FACULTY OF HEALTH AND MEDICAL SCIENCES
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PhD thesis

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Epidemiology of *Streptococcus pneumoniae* in Greenland

Colonization, Invasive Disease and Vaccine Impact

Department of Epidemiology Research
National Center of Health Data and Disease Control
Statens Serum Institut
Copenhagen, Denmark

STATENS SERUM INSTITUT

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The thesis is based on the following original papers:

I. Nasopharyngeal bacterial carriage among young children in Greenland: A population at high risk of respiratory infections.
-Submitted

II. Population-based study of incidence, risk factors and mortality from Invasive Pneumococcal Disease in Greenland.
-Draft

III. Effect of the 13–valent pneumococcal conjugate vaccine on nasopharyngeal carriage by respiratory pathogens among Greenlandic children.
-Draft

Indhold

Acknowledgements

I first encountered patients with severe invasive pneumococcal disease during my internship as newly graduated medical doctor in Greenland, and was stunned and quite affected by the often rapid and very aggressive nature of the disease, which sometimes within 24 hours after the patient presented at the hospital, resulted in multiple organ failure being refractory to treatment.

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ABBREVIATIONS

IPS: Invasive pneumococcal disease

AOM: Acute otitis media

CFR: Case fatality rate

CSF: Cerebrospinal fluid

DCC: Day-care center

IR: Incidence rate

PCV: Pneumococcal conjugate vaccine

PCV-7: The 7-valent pneumococcal conjugate vaccine

PCV-13: The 13-valent pneumococcal conjugate vaccine

PPV23: The 23-valent pneumococcal polysaccharide vaccine

VT: vaccine type pneumococci

NVT: non-vaccine type pneumococci

SSI: Statens Serum Institut

OR: Odds ratio

RR: Rate ratio

HR: Hazard ratio

CI: Confidence interval

STGG: Skimmed milk Tryptane Glycosis Glycerol medium

S. Pneumoniae: *Streptococcus pneumoniae*

NTHi: Non-typeable Haemophilus influenzae

M. Catarrhalis: *Moraxella catarrhalis*

S. Aureus: *Staphylococcus aureus*

H. Streptococci: Hemolytical streptococci

WHO: World Health Organization

SUMMARY

Background
Like the Inuit populations of Canada and Alaska, the Greenlandic Inuit have a high prevalence of infectious diseases caused by the bacterium *Streptococcus pneumoniae* (pneumococci), causing higher morbidity and mortality than among non-Inuit. Reasons for this ethnic health disparity are not clarified but are likely multifactorial. The first essential step in developing pneumococcal disease is the carriage of pneumococci in the nasopharynx, from which the bacterium may either be cleared by the immune system or may be transmitted to other individuals. Occasionally it causes local mucosal infections in the respiratory tract, such as otitis media or pneumonia, or it may penetrate the mucosa to cause serious diseases such as bacteremia and meningitis known as invasive pneumococcal diseases (IPD).

The most important recent advance against pneumococcal diseases is the introduction of the pneumococcal conjugate vaccines (PCV) first licensed in 2000. In contrast to previous pneumococcal vaccines, such as the 23-valent pneumococcal polysaccharide vaccine (PPV-23), the PCV's are able to mount a protective immune response in children < 2 years, the age-group together with elderly >65 years, having the highest morbidity and mortality of pneumococcal diseases. The vaccine however, only covers a subset of the 93 known pneumococcal serotypes (subtypes). The first generation of PCV's included 7 serotypes (PCV-7) and successfully reduced the burden of disease among vaccinated. However, while IPD caused by the serotypes included in the vaccine (VT) decreased, IPD caused by non-vaccine serotypes (NVT) increased. This replacement phenomenon has been observed in most countries post PCV-introduction and except for certain population, only to modest degrees. However, among Inuit and other natives of Alaska the benefits of vaccination have been counterbalanced by substantial increases of NVT-IPD with subsequent increased disparity in IPD-rates compared with the general US population.

Besides the impact on IPD, the PCV's have proved efficient in preventing nasopharyngeal pneumococcal carriage by VT both among vaccinated and non-vaccinated individuals, since the chain of nasopharyngeal pneumococcal transmission to other individuals in the community is interrupted, which results in a herd immunity effect. However, a substantial increase in NVT carriage has emerged with subsequently little or no net change in overall carriage-rates. Furthermore, recent studies have indicated that other co-colonizing bacteria may change in carriage prevalence post PCV-introduction, which subsequently may cause altered disease patterns after widespread use of PCV's.

In September 2010, Greenland introduced the latest expanded edition of the PCV covering 13 serotypes (PCV-13). The PCV-13 was chosen after recommendations from the Public Health Medical Officer of Greenland based on the existing knowledge on pneumococcal serotype distribution in Greenland. Although the existing PPV-23 has a broader protection, it is poorly immunogenic in infants and in Greenland it is recommended to adults with immune deficient conditions and after splenectomy. The PCV-13 is administered concomitantly with the other pediatric vaccines included in the Childhood Vaccination Program, at ages three- and five months and a booster at twelve months.

Objectives
The aims of this thesis were to describe the prevalence and risk factors of nasopharyngeal carriage by pneumococci and other important potential pathogenic bacteria in young Greenlandic children. Furthermore, we aimed to evaluate the effect of the recently introduced PCV-13 in Greenland, on bacterial colonization patterns. Finally, we intended to describe the epidemiology of IPD in Greenland (i.e. incidence, age-distribution, regional variation, serotype-distribution, risk factors and mortality) during the last four decades (1973-2013).

Methods
We conducted two cross-sectional population-based studies of the nasopharyngeal bacterial carriage in children living in West-Greenland (Sisimiut and settlements) and East-Greenland (Tasiilaq and settlements) in 2011 and again in 2013. Using a randomized register extraction from the Civil Registration System, we identified and invited children less than seven years of age. After parental consent, the parents completed a questionnaire regarding potential risk factors for carriage. Subsequently, we took a nasopharyngeal swab-sample for later analyses at the Statens Serum Institut.
To describe the natural history of IPD in Greenland, we conducted a matched case-control study nested in the Greenlandic population. In order to have as complete as possible identification of IPD-cases, we retrieved data from three different registries; the pneumococcal database at the Statens Serum Institut, the microbiology laboratory at Dronning Ingrids Hospital in Nuuk, Greenland and finally, since IPD has been a mandatory reportable disease in Greenland since 1995, we got access to all invasive bacterial cases reported to the public health medical officer in Greenland. Since IPD requires a microbiologic identification of *S. pneumoniae* and laboratory facilities are limited in Greenland, with only one microbiologic laboratory in Nuuk, some degree of underreporting particularly from the districts was anticipated. After identification of cases, they were individually matched 1:10 with a group of controls randomly selected from the Greenlandic population after matching on age and ethnicity. Information on potential risk factors for IPD was retrieved from national registries in Greenland and Denmark.

Results (study I and III)
The carriage-studies revealed that bacterial carriage begins at an early age (two weeks of age) in Greenlandic children, and that carriage rates peak during the second year of life and persist with relative high rates during childhood and up to pre-school age. In addition, the children were frequently colonized with multiple species at the same time. Risk factors for carriage included young age, gender, ethnicity, PCV-13 vaccination, living in Tasiilaq, having siblings attending a daycare and recent episodes of respiratory infections within the last three months. Some interesting positive and negative inter-bacterial associations were furthermore observed between *S. pneumoniae* and other colonizing bacteria. In 2013, three years after the PCV-13 introduction in Greenland, a noticeable serotype shift was observed both among vaccinated and unvaccinated children, with reductions in VT-pneumococci counterbalanced by increases in NVT. In addition, noticeable changes in non-pneumococcal bacteria were observed among vaccinated children, including increases in carriage rates of *M. catarrhalis* and reductions in rates of *S. aureus.*

Results (Study II)
The study regarding IPD in Greenland demonstrated that the incidence rate (IR) of IPD increased almost ten-fold during the study period. The overall IR of IPD was 22.6/100,000 person years,

highest among young children and the elderly. We confirmed a higher risk of IPD among Inuit compared to non-Inuit. Other risk factors for IPD included being male, having certain underlying comorbidities and living alone. Mortality from IPD was higher among Inuit than non-Inuit, particular among young children less than two years and adults aged 50 to 65 years and among those with meningitis, with a high degree of comorbidity (Charlson-score ≥2) and those living in the rural districts of Greenland. Finally, the first indications of PCV-13 vaccine impact on IPD in Greenland showed reductions in the overall incidence-rates of IPD, including reductions in IPD caused by VT and NVT. Thus, no indications of replacement IPD in Greenland was observed so far.

Discussion

Unexpectedly, in this population of Greenlandic children at high risk of respiratory infections, we found bacterial carriage rates to be comparable with other pediatric populations at low-risk of respiratory infections. In contrast, the early acquisition of bacterial carriage in Greenlandic children differed from western pediatric populations, where the median age is six months for first acquisition. This pattern of early age at first bacterial acquisition and ongoing polymicrobial infection pressure through infancy and childhood may facilitate a carriage-state characterized by persistent inflammation and mucosal damage as part of the explanation of the high disease burden in this Greenlandic Inuit population. The risk factors identified for carriage confirms previous studies in other settings, dominated by crowding-related factors and recent infections. However, the fact that ethnicity only marginally were associated with *M. catarrhalis* carriage, indicates that environmental exposures rather than a potential genetic susceptibility are significant for bacterial carriage in this population. The dramatic serotype shift observed among both vaccinated and non-vaccinated children indicate a herd-immunity effect facilitated by the PCV-13. Since the carriage of VT pneumococci when vaccinated is reduced, less exposure of VT pneumococci to non-vaccinated children occurs resulting in an indirect protection. However, the increasing rates of NVT are likely to reflect so-called serotype replacement and thus the overall vaccine effect on pneumococcal carriage is limited. Besides the serotype-shifts, carriage rates of other bacteria changed. This potentially will affect the prevalence of the corresponding infections related to these bacteria, but to what extent these alterations may occur requires further surveillance to clarify.

The incidence of IPD in Greenland was similar to observations from other Arctic countries prior to introductions of the PCV's, however; we believe the estimates are likely conservative due to presumable under-diagnosing in the rural districts of Greenland, where microbiological services are limited. Individuals of Inuit origin were at higher risk of IPD and had higher mortality from IPD than compared with Inuit from Alaska and Canada as well as Danish citizens. Among children < 2 years old, the mortality was up to five-times higher than Danish children with IPD and among adults aged 50 to 60 years mortality was up to two-times higher than in Denmark. Reasons for this disparity in health may be related to a genetic susceptibility for these infections and a high degree of premorbid comorbidity in the Greenlandic population. Since the carriage studies revealed increases in NVT carriage, one might expect increasing rates of IPD caused by NVT. However, the first three years of PCV-13 usage in Greenland has so far not resulted in replacement-IPD. This may be due to lower virulence of the NVT serotypes, since pneumococcal serotypes have been shown to be very heterogeneous in their disease-potential, with some more likely to colonize the nasopharynx, whereas others typically are involved in pediatric infections.

Overall, we believe our work has added to the existing knowledge on pneumococcus epidemiology in Greenland and hopefully this will aid the surveillance of the vaccine impact on pneumococcal infections and assist public-health planners as well as clinicians in appropriate risk stratification and prevention of pneumococcal diseases in Greenland.

DANSK SAMMENDRAG

Baggrund

I lighed med inuitter i Canada og Alaska har befolkningen i Grønland en meget høj forekomst af infektionssygdomme forårsaget af bakterien *Streptococcus pneumoniae* (pneumokokker), der er forbundet med højere sygelighed og dødelighed end hos individer med anden etnisk baggrund. Baggrunden for denne etnisk betingede ulighed i sygelighed er fortsat ikke afklaret, men er formentlig multifaktoriel. Det er imidlertid velkendt at det at bære bakterien i næsesvælget (kolonisering) er et nødvendigt første trin i udviklingen af pneumokoksygdomme. Herfra kan bakterien enten blive fjernet af immunforsvaret eller smitte andre individer. Endvidere kan bakterien forårsage lokale infektioner i luftvejene såvel mellemørebetændelse som lungebetændelse eller trænge ind i ellers sterile dele af kroppen og medføre alvorlig såkaldt invasiv pneumokoksygdom (IPS) såsom blodforgiftning eller meningitis.

Et vigtigt nyere fremskridt i kampen mod pneumokoksygdomme er den konjugerede pneumokokvaccine (PCV). I modsætning til tidligere vacciner er PCV'en i stand til at fremkalde et tilstrækkeligt immunrespons hos børn < 2 år, den aldersgruppe der sammen med ældre > 65 år har højest sygelighed og dødelighed af pneumokoksygdomme. Vaccinen dækker imidlertid kun et fåtal af de 93 serotyper (undertyper) der kendes til. Den første generation af PCV'en dækkede 7 serotyper (PCV-7) og reducerede effektivt IPS-tilfældene forårsaget af de 7 vaccinetyper (VT), men til gengæld steg IPS-tilfældene forårsaget af de andre serotyper, der ikke var inkluderet i vaccinen (såkaldte non-vaccine typer eller NVT). Dette erstatningsfænomen (eng. 'replacement') er observeret i de fleste lande efter vaccinen er taget i brug, men fraset visse befolkningsgrupper, oftest kun i beskeden grad. Dog særligt blandt Inuit og den øvrige oprindelige befolkning i Alaska, er effekten af vaccinen blevet markant udlignet pga. store stigninger i IPS-tilfælde forårsaget af NVT. På trods af et overordnet fald i IPS tilfældene, har den store 'replacement' effekt samlet set resulteret i en endnu større ulighed i IPS-forekomsten blandt Inuitter i Alaska sammenlignet med den generelle amerikanske befolkning.

Udover effekten på IPS, har tidligere studier vist, at vaccinen er effektiv til at forebygge næsesvælg koloniseringen af vaccine-type (VT) pneumokokker. Dette er observeret både hos vaccinerede og ikke-vaccinerede, idet smittekæden fra næsesvælget stoppes og dermed spredningen af bakterier i samfundet. Denne positive effekt kaldes for flok-immunitet (eng. 'herd immunity'). Imidlertid er der sket en tilsvarende stigning i koloniseringen af pneumokokker der ikke dækkes af vaccinen (non-vaccine typer - NVT) og dermed er netto effekten på den overordnede pneumokok-kolonisering meget lille eller ikke eksisterende. Nylige studier viser, at frekvensen af andre bakterier der koloniserer næsesvælget formentlig også påvirkes af vaccinen, hvilket har givet anledning til bekymring om hvorvidt sygdomsmønstret for andre bakterier efter længere tids vaccinebrug vil ændres.

Formål

Formålene med denne afhandling var dels at bestemme bærergraden og risikofaktorerne for at have pneumokokker og andre bakterier, der hyppigt er relateret til luftvejssygdomme, i næsesvælget (*S. aureus*, *M. catarrhalis* og non-typeable haemophilus influenzae - 'NTHi'). Derudover ville vi evaluere effekten af den nyligt indførte PCV vaccine i Grønland, der dækker 13 serotyper, på henholdsvis næsesvælg koloniseringen og forekomsten af IPD. Endelig var det målet at beskrive epidemiologien af IPS i Grønland (incidens, aldersfordeling, regional variation,

serotypefordeling, risikofaktorer og dødelighed) over en 40 årig periode i Grønland (1972-2013).

Metoder
Vi udførte to populationsbaserede tværsnitsstudier af unge børn bosat i Vest- og Øst-Grønland (Sisimiut og Tasiilaq) i henholdsvis 2011 og 2013. Via et randomiseret registerudtræk fra det centrale personregister, identificerede vi grønlandske børn under 7 år, og inviterede dem (via forældrene) til at deltage i undersøgelserne. Efter forældre samtykke, blev et spørgeskema udfyldt vedrørende potentielle risikofaktorer for at have bakterier i næsesvælget. Efterfølgende tog vi en næsesvælg podning til senere analyser på Statens Serum Institut.
For at kunne beskrive naturhistorien for invasive pneumokoksygdomme i Grønland, udførte vi et matchet case-kontrol studie i den grønlandske befolkning. For at få så komplet som mulig identifikation af alle IPS-tilfældene, indhentede vi oplysninger fra tre forskellige registre. Dels fra pneumokok databasen på Statens Serum Institut, dels fra det mikrobiologiske laboratorium på Dronning Ingrids Hospital i Nuuk og endelig fra Embedslægeinstitutionen i Nuuk. Efter at have identificeret alle IPS tilfælde, blev de individuelt matchet 1:10 med en kontrolgruppe tilfældigt udvalgt fra den grønlandske befolkning men matchet på fødselsdato og etnicitet. Informationer vedrørende potentielle risikofaktorer blev indhentet fra forskellige nationale registre i Grønland og Danmark.

Resultater (studie I og III)
Bærerstudierne viste at den bakterielle kolonisering starter i en tidlig alder (allerede fra 2-ugers alderen) og at bærerfrekvensen af bakterier topper i det andet leveår og forbliver relativt højt op gennem barndommen indtil førskolealderen. Desuden var børnene ofte koloniseret med flere bakterier på samme tid. Vi fandt følgende risikofaktorer for at være koloniseret med bakterier: ung alder, køn, etnicitet (dog kun en svag sammenhæng), PCV-13 vaccination, bopæl i Tasiilaq, at have søskende i en daginstitution samt at have haft en nylig luftvejsinfektion inden for de seneste tre måneder. Vi fandt endvidere nogle interessante positive og negative associationer mellem pneumokokkerne og de andre bakterier. I 2013 så vi en markant ændring i pneumokokserotyperne efter 3 års anvendelse af PCV-13 vaccinen i Grønland, både blandt vaccinerede og ikke-vaccinerede børn. Mere specifikt fandt vi en reduktion i koloniseringen med VT der blev udlignet af en stigning af NVT. Yderligere fandt vi påfaldende ændringer i raterne af de andre koloniserende bakterier blandt de vaccinerede børn i forhold til ikke vaccinerede, herunder en stigning i *M. catarrhalis* frekvensen og en reduktion i *S. aureus* frekvensen.

Resultater (studie II)
Studiet vedrørende IPS epidemiologien i Grønland demonstrerede at incidens raten af IPS steg med op til en faktor 10 under studieperioden. Den overordnede incidens rate af IPS var 22.6/100.000 person år, højest blandt små børn < 2 år og voksne mellem 50 og 65 år. Vi kunne bekræfte at Inuitter har en højere risiko for IPS i forhold til individer med anden etnisk baggrund. Yderligere risikofaktorer for IPS var at være mand, at have kroniske underliggende sygdomme og at bo alene. Dødeligheden af IPS var højere blandt Inuitter, særligt blandt unge børn < 2 år og voksne i alderen 50 til 65 år, blandt patienter med meningitis og dem med høj grad af konkurrerende sygdomme (Charlson score ≥2) samt hos individer bosat i yderdistrikterne. Endelig viste studiet de første tegn på vaccine effekten på forekomsten af IPS tilfælde, hvor en overordnet

reduktion kunne ses, både af de tilfælde forårsaget af VT og tilfældene forårsaget af NVT. Dermed er der foreløbigt ikke set tegn på 'replacement' sygdom i Grønland.

Diskussion

Noget uventet fandt vi i denne population af børn i høj risiko for luftvejsinfektioner, en koloniseringsgrad af bakterier der var sammenlignelig med befolkningsgrupper med lav risiko for luftvejsinfektioner i vestlige lande. Derimod adskiller den tidligere kolonisering vi så blandt grønlandske børn (fra 2 ugers alderen) sig fra vestlige børn, hvor median alderen for første kolonisering med bakterier typisk er 6 måneder. Dette mønster med en tidlig kolonisering, og multiple bakterier koloniserende på en gang, samt et vedvarende højt infektionstryk op gennem barndommen, kan medvirke til en bærertilstand, karakteriseret ved en kronisk inflammationstilstand af næsesvælgslimhinden og dermed en øget risiko for infektioner. Dette kan muligvis medvirke til at forklare den høje sygdomsbyrde af luftvejsinfektioner i Grønland. De risikofaktorer vi identificerede for kolonisering bekræfter tidligere studier, hvor crowding-relaterede faktorer dominerer samt nylige infektioner. Derimod indikerer den meget svage sammenhæng mellem etnicitet og kolonisering, at en eventuel genetisk følsomhed næppe er betydende for koloniseringen i Grønland, men nok snarere skal tillægges miljømæssige eksponeringer. Det tydelige skift i serotypefordelingen der kunne demonstreres både hos vaccinerede og ikke vaccinerede børn, peger på en flok-immunitets effekt, idet vaccinen direkte reducerer koloniseringen med VT hos vaccinerede børn som dermed i mindre grad indirekte vil smitte andre individer med VT. Derimod kan den observerede stigning i NVT pege i retningen af såkaldt 'serotype replacement', og dermed udlignes den overordnede effekt af vaccinen på den samlede pneumokok kolonisering. Ud over dette serotype skift, observerede vi ændringer i raterne af koloniseringen med andre bakterier blandt vaccinerede børn. I hvilket omfang dette vil påvirke forekomsten af infektioner relateret til disse bakterier er uvist og vil kræve yderligere monitorering at belyse.

Forekomsten af IPS i Grønland svarede til de observationer der er set i andre arktiske lande før indførelsen af PCV vacciner. Imidlertid er vores estimater formentlig noget konservative da det må formodes at en betydelig underdiagnosticering finder sted i distrikterne, hvor adgangen til mikrobiologiske faciliteter er meget begrænset. Individer af Inuit oprindelse havde en højere risiko for IPS samt højere dødelighed sammenlignet med Inuit fra Alaska og Canada såvel som danskere. For børn < 2 år var dødeligheden af IPS op til 5 gange højere end danske børn med IPS, og for voksne mellem 50 og 60 år dobbelt så høj. Baggrunden for denne etniske ulighed i sygelighed kan muligvis skyldes en genetisk betinget følsomhed for infektioner samt en højere grad af komorbiditeter blandt grønlandske voksne, men dette er fortsat uafklaret. Idet vi så en stigning i NVT bærertilstanden, kunne man forvente en tilsvarende stigning i forekomsten af IPS forårsaget af NVT. Imidlertid viste den foreløbige opgørelse af IPS forekomsten efter de første tre års brug af PCV-13 vaccinen et samlet i forekomst af IPS, både blandt tilfælde forårsaget af VT og NVT. Dermed er der ikke set tegn på 'replacement' sygdom i Grønland indtil videre. Dette kan muligvis forklares i den meget heterogene grad af sygdomspotentiale de enkelte serotyper har. Således har nogle mere tilbøjelighed til at kolonisere slimhinden, mens andre er mere virulente.
Overodnet kan disse studier bidrage til den eksisterende viden om pneumokoksygdomme i Grønland og vil kunne supplere overvågningen af vaccine effekten på pneumokoksygdomme.

Endelig kan resultaterne supplere sundhedsfagligt personale ved risikostratificeringen af patienter mistænkt for pneumokoksygdom, samt medvirke til den fremtidige planlægning af vaccinestrategier i Grønland.

JUSTIFICATION

Diseases caused by the bacterium *Streptococcus pneumoniae* (pneumococcus) constitute a major public health problem worldwide. Clinical manifestations ranges from asymptomatic carriage and common respiratory tract infections such as ear infections (otitis media) [1] and lung-infections (pneumonia), to severe life-threatening diseases such as blood poisoning (bacteremia) and meningitis, also known as invasive pneumococcal diseases (IPD) [2].

On a global scale, the pneumococcus is the leading cause of otitis media, bacterial respiratory infections, bacteremia and meningitis [3]. Mortality of pneumonia exceeds that of any other illness, even that of AIDS, malaria and measles combined [4]. The World Health Organization (WHO) estimated in 2007 that 14.5 million cases of severe pneumococcal disease occurs annually and that more than 1.6 million people, including up to 1 million children < 5 years of age, die of IPD each year [5].

However, the burden and mortality of pneumococcal diseases differs greatly worldwide. Otitis media is a leading cause of frequent health care visits in western countries, but complications and long-term sequelae are nevertheless most prominent in the developing part of the world [6] and among certain indigenous populations such as the Inuit of Greenland [7]. Likewise, with respect to IPD disease-burden , this is most prominent in the developing part of the world [8] but is also frequent among certain high-risk ethnic groups including the indigenous population of Australia [9], Bedouins of Israel [10], Maori from New Zealand [11], American Native Indians [12] and among the Inuit-populations of the arctic, i.e. Alaska, USA [13], northern Canada [14] and Greenland [15,16].

Surveillance of IPD in the Arctic has revealed a pattern of ethnic health disparity with Inuit suffering from markedly higher morbidity and mortality caused by IPD as compared with non-Inuit [16–18]. In Greenland, almost 90% of the population is of Inuit origin. Within the Arctic region, current data indicates that the Inuit in Greenland are among those with worst outcomes, i.e. with the highest morbidity and mortality [15]. Reasons for this disparity have not yet been clarified, but besides genetics, the populations across the circumpolar region share characteristics such as living conditions and socio-economic challenges, each of which may play a critical role.

In order to establish appropriate preventive measures it is essential to clarify the importance of risk factors for the development of IPD using retrospective data. There is, however, only limited data on the national epidemiology of IPD in Greenland. Currently primarily descriptive data have been published on risk factors and mortality of IPD in the Arctic [13,16,19,20].

In 2010 a new pneumococcal vaccine, directed against a subset of the pneumococcal subtypes was introduced in Greenland. The experience with the vaccine from other Arctic countries has overall been a success with reduction in overall IPD-rates. In Alaska the rates of IPD among Alaskan native children less than 2 years of age, were reduced from 440.6 to 177.5 and in Canadian native children from 229.3 to 92.6, all pr. 100,000 [15]. However, the vaccine (PCV-7 Prevenar© Pfizer) only covers seven of the more than 93 known pneumococcal serotypes (subtypes), and while the rate of IPD-cases among Alaskan natives caused by vaccine-types (VT) decreased by 80% (children less than two years of age 93%), an 122 % increase in cases related to non-vaccine-types (NVT) was observed (children less than two years of age) [18]. This phenomenon named 'serotype-replacement' (substitution) is one of the highest reported in the world.

The second generation of the vaccines is an expanded version of the PCV-7 targeting additionally six serotypes (PCV-13), and it was introduced in Greenland in September 2010. The impact on pneumococcal disease in Greenland is so far unknown.

This thesis addresses the above mentioned issues in various ways. First, we studied the very first step in pneumococcal disease, namely the carriage of the bacteria in the posterior part of the nose (nasopharynx). In 2010 we conducted a population-based cross-sectional study of young children living in two different regions of Greenland. Using nasopharyngeal samples and data on potential risk factors obtained from questionnaires we estimated the carriage rates of pneumococci and other clinically relevant bacteria, frequently causing respiratory infections in children, as well as risk factors for carriage and inter-bacterial associations. To evaluate the impact of the PCV-13 a second cross-sectional study was conducted in 2013 three years post PCV-13 introduction and data was compared with the study-results from 2011. Furthermore we studied IPD, the most serious outcome of pneumococcal disease, using a matched case-control study design nested in

the Greenlandic population, including all known cases of IPD in Greenland from 1973 to 2013. By linking these data to national registries new information regarding the regional distribution of IPD, risk factors, morbidity and mortality as well as the first signs of vaccine-impact on the serotype distribution was obtained.

The studies contribute to the evaluation of the pneumococcal vaccine impact and support future surveillance in Greenland by providing solid surveillance data and thus, form a better platform for future vaccine policies in Greenland. Also it may be of help for decision-makers when planning public health measures and future implementation of pneumococcal immunization programs in Greenland as well as in other settings.

INTRODUCTION

Streptococcus pneumoniae (The pneumococcus)

The bacteria was identified in 1881 by Sternberg, USA and Pasteur, France who independently isolated diplococci from rabbits that had been inoculated with human saliva [21]. The pneumococcus is a lancet shaped gram-positive diplococcus characterized by a surrounding polysaccharide capsule (fig. 1). Since it is alpha hemolytic, bile soluble and optochin sensitive, it can be identified by conventional microbiological tests, using morphological characteristics and biochemical tests [22]. Based on the structure of the capsule, the pneumococci can be sub-classified and more than 90 antigenically different serotypes (subtypes) have been identified. Furthermore, some non-encapsulated variants of pneumococci exist, also known as non-typeable pneumococci [23].

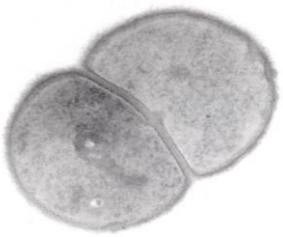


Figure 1. Electron microscope picture of *Streptococcus pneumoniae*

Serotyping

Traditionally, pneumococci are classified by the nomenclature developed at Statens Serum Institut, Copenhagen, Denmark, where the serotypes are grouped into serogroups, containing one to five serotypes with related antigens. The serogroups are numbered one to 48 and serotypes within each group are identified by an additional alphabetical letter [24].

The golden Standard of serotyping is performed on pneumococcal isolates identified by culturing on blood-agar. First, a suspension of pneumococci is mixed with serotype-specific antisera and observed under the microscope. If the capsule swells and appears large or agglutinated, the reaction is positive – known as the Neufeld test or Quellung reaction [22]. The method is still widely used but is labor-intensive and therefore not ideal when laboratory facilities are limited. Thus, other methods have been developed.

One approach is the simple latex agglutination test for grouping and serotyping of pneumococci which has been developed at Statens Serum Institut [25] and which consists of 14 different pooled pneumococcus antisera (pools A to I and pools P to T) applied to latex-particles. This method is sensitive and less expensive since it may be used as a screening tool to quickly identify the serotype, which subsequently can be confirmed by Quellung reaction. Second, molecular methods

exist which consists of polymerase chain reaction (PCR)-based amplification of serotype-specific genes encoding the capsule locus [26]. Third, the multiplex assays exist using mono- and polyclonal antibodies [27]. However, the three described methods have some limitations. First, they do not allow for antimicrobial susceptibility testing as the culture-step is left out. Second, serotyping based on genetics does not necessarily reflect capsule specificity and thus, the serotype actually expressed by a viable or agar grown pneumococcus. The sequence diversity of the DNA encoding the capsular locus can result in mismatches between results based on one of the three molecular methods and thus, problems in distinguishing the closely related serotypes from each other. Consequently, this may challenge the interpretation of the identified pneumococcal serotypes in for example nasopharyngeal carriage studies, giving rise to overestimating carriage-rates of co-colonizing serotypes. On the other hand the Quellung methods are also limited with regards to quantification of co-colonizing serotypes, since it includes a culture step which theoretically may disturb the original nasopharyngeal proportions of serotypes [28].

NASOPHARYNGEAL CARRIAGE

Carriage of the pneumococcus in the posterior part of the nasal cavity, so-called nasopharyngeal carriage, is considered an essential precursor for developing pneumococcal infections [29]. Although carriage as such, is asymptomatic, it may progress to local mucosal infections such as otitis media and sinusitis, or lower respiratory tract infections, such as pneumonia and occasionally the most severe manifestation may occur when the bacteria invades the mucosal surfaces of the body causing infections such as bacteremia or meningitis, so-called invasive pneumococcal disease (IPD) (fig.2) [29]. Besides *S. pneumoniae,* other potential pathogenic bacteria transiently colonize the mucosa of the nasopharynx, sharing the niche with numerous commensal microbes – also

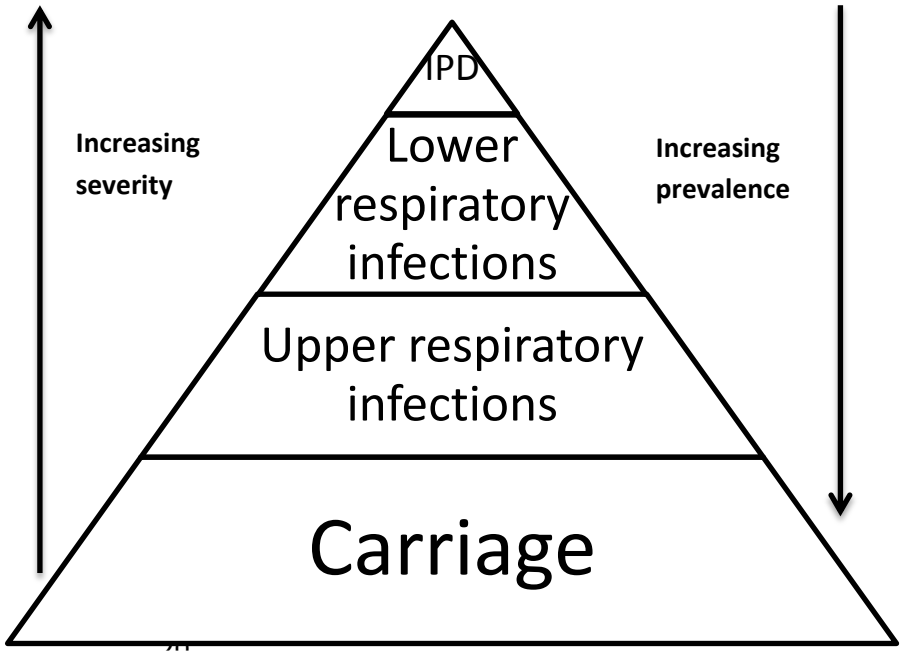


Figure 2. Relative proportions of *S. pneumoniae* involvement in carriage and diseases

known as the resident flora. Besides causing sporadic infections within the host, the colonization of the nasopharynx is the source of bacterial transmission to other individuals in the community [30].

Among the colonizing bacteria, some of the major contributors and thus most clinically relevant to the polymicrobial respiratory infections in childhood include *S. pneumoniae*, *non-typeable Haemophilus influenzae* (NTHi), *Moraxella catarrhalis* and S*taphylococcus aureus*[23,31,32]. The bacteria transiently colonize the nasal cavity, contributing to the existing flora and thus join a very complex and dynamic environment characterized by recurrent acquisition and elimination of a variety of microbes, interacting with each other and with the local immune system of the host. This microbial ecosystem (also known as the microbiome) is as such considered beneficial for the host when in a balanced state, providing a protective barrier against invading pathogens and stimulating the immune system. However, if the homeostatic balance is skewed, the bacteria may cause either local respiratory infections such as otitis media, sinusitis and bronchitis, or more severe conditions such as pneumonia, bacteremia, arthritis, osteomyelitis, peritonitis and meningitis – also known as invasive bacterial diseases [29,30].

Risk factors for pneumococcal carriage

Various risk factors have been identified for carriage of these potential pathogens, in particular young age. Carriage rates of *S. pneumoniae* increases during the first year after birth, peak in the second and third year, and decline gradually hereafter [2]. In the developing part of the world such as The Gambia but also among some indigenous populations such as Australian aboriginals [33,34], carriage tends to begin only few days after birth, to reach very high rates (approximately 90%) among infants less than two years[34] and frequently to present with co-colonization of other potential pathogens. In pediatric populations from Europe and USA, however, peak-carriage generally ranges from 40 to 60% in two-year-olds and decreases below 10% by the age of ten years [2,30]. The mean age of first acquisition in these populations is six months [30,35].
Other risk factors include: crowding (especially with other young children), ethnicity (children from low-income countries such as India, Gambia, New Guinea) , socio-economic status, passive smoking, lack of breast-feeding, recent use of antimicrobials and recent or current respiratory tract infections including viral infections [2,30].

Invasive pneumococcal disease (IPD) in selected Arctic regions

ALASKA, USA

IPD is highly prevalent in the circumpolar areas, with the highest rates among children less than two years and adults older than 65 years [20]. In 1986 the highest incidence rates of IPD was found among the Eskimo population of Alaska [13]. IPD-rates among Alaskan Inuit children younger than two years were 450 cases per 100.000, three and a half times higher than non-natives of same age. Similar ethnic health disparities have been reported among other Arctic populations including the Inuit population of Northern Canada [36] and Greenland [15,16]. For this reason the International Circumpolar Surveillance System for Invasive Pneumococcal Disease was established in 1999 using available laboratory as well as demographic and clinical data collected for each IPD-case by the participating countries (fig 3.) [20]. The surveillance network included Alaska and northern Canada in 1999, in 2000 Greenland was included followed by Norway, Iceland and Finland in 2001 and northern Sweden in 2003, whereas Russia is not participating.

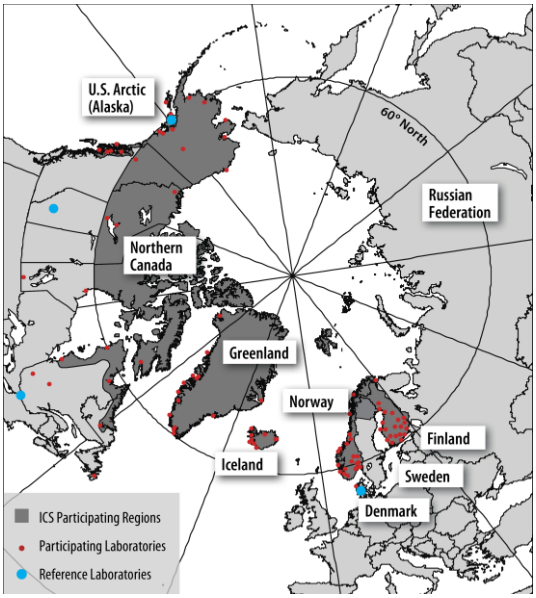


Figure 3. Participating regions of The International Circumpolar Surveillance System for Invasive Pneumococcal Disease, reprinted

CANADA

In Northern Canada, in the prevaccine (PCV-7) period, the IPD rate among indigenous children less than two years of age was 229/100.000 [14]. Nunavik is the most northern region in Quebec in Canada and with 90% of the population being of Inuit origin. The reported average rate of IPD from 1997 through 2010 in the Nunavik region was 56/100.000, which is five times higher than the rate reported in the whole Quebec population in the same time period (11/100.000) [17]. These estimates from the Arctic part of Canada, are likely to be underestimated, since they are based on laboratory surveillance which in the Arctic is particular challenging due to limited infrastructure, sparse laboratory facilities, lack of physicians in all the communities and thus a pragmatic

approach to patients presenting with fever and signs of acute infections, where antibiotics are prescribed without necessarily performing blood cultures for diagnostics.

GREENLAND

According to a small retrospective study of Greenlandic IPD cases between 1998 and 2002, the annual incidence rate of IPD was 54/100.000 for Inuit and 17/100.000 for non-Inuit [16]. These rates were calculated based on laboratory-data from the capital Nuuk since underreporting from the districts in Greenland is most likely to occur due to similar logistic challenges as described for the Arctic part of Canada. Besides being three to three and a half times higher than incidences among non-Inuit and populations in Scandinavian countries [37,38] the age-distribution revealed the highest rates among children less than two years of age but also a sudden increase among adults older than 35 years.

Risk factors for IPD

Well established risk factors for IPD include: age (children less than two years and adults older than 65 years [39]), male sex [3], ethnicity (indigenous population of arctic Canada [36] and Alaska [13], American native Indians [40], Australian aboriginals [9], Maori from New Zealand [11], Bedouins from Israel [10], and Afro-Americans[41]), and socio-economic status [42]. Specific factors for children associated with increased risk for IPD are preterm birth, low birth weight [43], co-morbidity [44], daycare institution-attendance and crowding, and for adults, alcohol abuse [45], cigarette-smoking [46], congestive heart disease, recent influenza infection, diabetes, and immune-compromising diseases [47].

Only few studies have investigated specific risk factors for IPD among Inuit-populations and primarily descriptive data have been published. In Alaska and Northern Canada, cigarette smoking, alcohol abuse, chronic lung disease (including asthma), diabetes mellitus, immunosuppressive therapy, injection drug use and asplenia have been associated with an increased risk of IPD, however, the analyses did not include a control group [38]. Among 315 Native Alaskan IPD-patients during 1986-1990, the most frequent preexisting conditions were: alcohol abuse, anemia, chronic lung disease, major congenital defects, heart diseases, low birth weight or prematurity,

unspecific seizure disorders and malignancy [13].

In Greenland, Meyer et al [48] found that among Inuit, pneumococci dominated among invasive bacterial diseases and were associated with high morbidity and mortality. In a retrospective study of IPD in Nuuk, the capital of Greenland, between 1996 and 2002, 51 cases were identified, among which 42 were adults [16]. Of these 40% had pre-existing comorbidity, 33% were unemployed and 33% suffered from alcohol abuse. Risk factors for children were not assessed in this study. Thus, nationwide epidemiological data is sparse with respect to regional incidences of IPD, specific risk factors for IPD compared to an age-matched control-group, and with respect to mortality from IPD in Greenland compared with other Arctic regions.

PREVENTION

Despite the existence of different pneumococcal vaccines, the pneumococcus remains the leading cause of vaccine-preventable deaths in children less than 5 years (fig 4) [49].

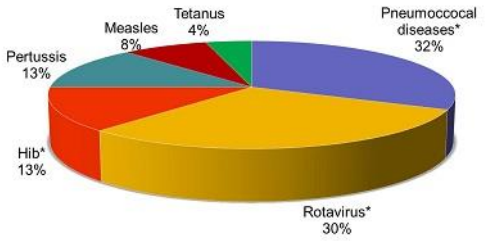


Figure 4. Proportions of deaths among infants, due to vaccine preventable diseases [114]

Two licensed vaccines against invasive pneumococcal disease exist, both of which are based on antibodies against capsular polysaccharides. In the 1980's the first generation vaccine was licensed, composed of 23 of the most prevalent serotypes at that time in Europe and USA (Pneumovax® 23 Merck or PPV-23) [50]. The vaccine is also available in Greenland and is recommended for immunocompromised individuals and elderly older than 65 years. However, the immunologic response to this vaccine is T-cell independent and thus with poor immunologic memory. Furthermore, the use of the vaccine in children less than two years of age, the age-group most susceptible to IPD, is not indicated due to low immunogenicity [50]. Results on the efficacy of the PPV-23 are heterogeneous and in the United Kingdom the vaccine is no longer recommended due to the lack of evidence on reducing IPD, despite widespread use in the UK since 2003 [51]. The second generations of pneumococcal vaccines have been conjugated with a carrier protein which results in a T-cell dependent response to the cell surface polysaccharides. These conjugated vaccines (PCV's) are approved for infant use and induce immunologic memory, and have recently also been approved for use in adults and elderly [52].

The first PCV was licensed in 2000 and is 7-valent (Prevenar®; Wyeth/Pfizer) (PCV-7). It contains polysaccharides from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F conjugated to an inactivated non-toxic diphtheria toxin CRM197 [53]. The selection of serotypes was based on the existing data on serotype distribution of IPD among children at the time. Due to complex manufacturing processes, the number of included serotype-specific polysaccharides is limited. Moreover, the potential coverage at the time of licensure was varying across regions, ranging from less than 50% in Asia, 60-70% in Europe to 80% in North-America and Australia [54]. Since then, a high number of countries have introduced the PCV-vaccines (fig. 5) [55] in the country specific children vaccination programs and in September 2010, the latest edition of the PCV's, the 13-valent vaccine PCV-13 (Prevnar13®, Pfizer) was introduced. The PCV-13 is an expanded version of the PCV-7 containing

additionally six serotypes (1, 3, 5, 6A, 7F and 19A) [56]. The vaccine was integrated in 2010 in the Childhood Vaccination Program in Greenland and was administered at ages three and five months and with a booster at twelve months.

Figure 5. Countries having introduced the pneumococcal conjugate vaccine.
(International Vaccine Access Center (IVAC), Johns Hopkins Bloomberg School of Public Health. Vaccine Information Management System (VIMS) Global Vaccine Introduction Report, March 2014.)

PCV-7 impact

The impact of the PCV-7 (Prevnar® Pfizer) on IPD has been evaluated in randomized controlled trials [57–60] showing reductions in rates of IPD both among vaccinated and non-vaccinated children and adults. Furthermore, a decline in carriage-rates of vaccine-type (VT) pneumococci has been observed in the intervention groups compared with controls [40,57,58]. The reduction of colonization by VT pneumococci has led to diminished circulation of the bacteria in the community and thus to reduction in IPD and colonization, resulting in protection of non-vaccinated groups or so-called 'herd-immunity'. However, after widespread use of the PCV-7, colonization by non-vaccine type pneumococci (NVT) has increased resulting in no overall effect on pneumococcal carriage-rates a phenomenon known as serotype-replacement [61]. Furthermore, some of the NVT have also been shown to be capable of causing diseases in various degrees [62]. For instance, in the US the PCV-7 has led to sustained decreases in IPD in all age-groups [63], but new non-vaccine types have emerged to become major causes of IPD such as 3, 7F and 19A [64]. To avoid

this serotype replacement, new vaccines with higher valences have been developed as previously described. In 2009 the Synflorix™ (Glaxo Smith Kline) was licensed in Europe containing 10 serotypes and conjugated to protein D from non-typeable Haemophilus influenzae and in 2010 the PCV-13 was licensed (Prevnar13® Pfizer)[53].

The PCV-experience from the Arctic

After the introduction of the PCV-7 in the Children Immunization Program in Alaska and Canada in 2000 and 2001, rates of IPD caused by PCV-7 serotypes have decreased, both among vaccinated and non-vaccinated individuals due to herd-immunity [36,45]. Among native Alaskan children less than two years of age, rates of IPD caused by the seven vaccine serotypes (VT) declined from 275 to 10/100.000. However, rates of IPD caused by non-PCV-7 serotypes increased with 129% among children less than two years from 95 to 228/100.000, which is among the highest reported degrees of replacement globally (fig. 6).

Among native Alaskans the overall incidence rates of IPD declined from 49 to 46/100.000, whereas the rates among non-natives declined from 16.7 to 11.1/100.000. Thus, a four-fold increased risk of IPD has remained among native Alaskan adults and children less than two 2 years of age compared with the US white population of similar ages due to IPD caused by non-vaccine serotypes (replacement). The overall IPD-rates have thus decreased but the disparity in IPD-rates between Alaskan natives and the general US population has in fact increased in the post-PCV-7 era [65].

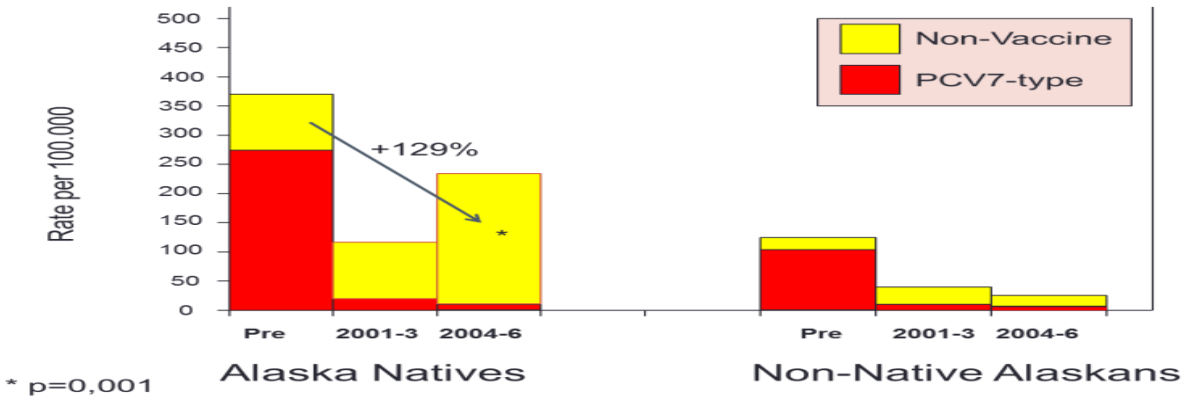


Figure 6. Rates of invasive pneumococcal disease among children < 2 years, by time period (pre-and post PCV-7 introduction) in Alaska. Reproduced from [115]

PCV-13 impact

In 2010, after ten years of PCV-7 usage in Alaska, the vaccine was replaced by the successor, the PCV-13 [66]. A recent carriage-study of pneumococcal colonization among Alaskans found no overall change in carriage-rates post-PCV-13 introduction compared with the PCV-7 period. However, among vaccinated and unvaccinated children, a significant reduction was found in carriage of the six additional serotypes included in the PCV-13 compared with the prior PCV-7 period, as well as increasing rates of NVT [67]. Regarding PCV-13 impact on IPD in Alaska, surveillance has observed reducing rates of IPD among vaccinated caused by both VT and NVT [68] and thus, no indications of replacement-disease so far.

PCV-7 impact on other co-colonizing bacteria

Besides the vaccine-related impact on the pneumococcal serotypes, studies have indicated changes among vaccinated in carriage-rate of other bacterial species co-colonizing the nasopharynx, as compared to a group of non-vaccinated controls [69]. These changes may be temporary and without any clinical consequences, but recent studies show long-term changes post-PCV-7 vaccination [70]. In Holland, Spijkerman et al. [70] found persistently higher prevalence rates of *NTHi* carriage and pneumococcal non-PCV-7 serotypes among young asymptomatic children three and four and a half years after PCV-7 vaccination as well as an almost complete eradication of PCV-7 type pneumococci compared with the pre-PCV-7 period. Besides the changes in carriage rates of colonizing bacteria, disease-rates may also be affected by these alterations. Bogaert et al. observed increasing proportions of *S. aureus* colonization as well as otitis media caused by *S. aureus* in PCV-7 vaccinated children, compared to a group of controls [71]. Moreover, the Finnish Otitis Media trials revealed increases in the proportion of acute otitis media caused by *M.catarrhalis* and *NTHi* among PCV-7 vaccinated as compared to non PCV-7 vaccinated [72].

Based on the observed serotype-shifts in pneumococcal carriage and pneumococcal diseases as well as the indications of changes in carriage-rates of other co-colonizing bacteria post PCV-introduction in a number of countries, and in particular among the Inuit populations of Alaska and Canada, this PhD.-project aims to clarify the effects of the PCV-13 on carriage and IPD among the population of Greenland where the vaccine was recently introduced.

OBJECTIVES

The main purpose of the thesis is to describe the epidemiology of pneumococcal carriage in Greenland by determining the changes in nasopharyngeal pneumococcal carriage during a three-year period after the introduction of the PCV-13. Furthermore, based on all known cases of IPD, we aimed to describe risk factors, mortality and the changes in incidence of IPD in Greenland during the period from 1973 to 2013.

The specific aims were to:

1. Determine the frequency of nasopharyngeal bacterial carriage, antimicrobial susceptibility patterns, risk factors and inter-bacterial associations between four key bacteria, frequently involved in respiratory and invasive infections in Greenlandic children less than seven years of age. (PAPER I)
2. Estimate the impact of the recently introduced PCV-13 on nasopharyngeal carriage-rates of *Streptococcus pneumoniae*, *non-typeable Haemophilus influenzae* (NTHi), *Moraxella catarrhalis* and *Staphylococcus aureus* among children less than 7 years of age. (PAPER III)
3. Describe the incidence, age distribution, season- and regional- variation, clinical manifestation and mortality from IPD in Greenland. (PAPER II)
4. Identify risk factors for IPD among children less than two years of age and adults older than 18-years.
   (PAPER II)
5. Determine the serotype-specific frequency of IPD in the period between 2010 and 2013 (the post-vaccine-era) and assess the degree of replacement by comparing with the period between 2000 and 2009 (pre-vaccine period). (PAPER II)

MATERIAL AND METHODS

The thesis consists of three studies. Two of them are based on bacterial carriage-data obtained by conducting two sequential cross-sectional studies of the nasopharyngeal bacterial colonization among young children living in East- and West-Greenland. The work conducted as part of the study included field-work as well as laboratory work. For the laboratory work, I was temporarily associated with the Department and Laboratory of Microbiology and Infection Control, including The Neisseria and Streptococcus Reference Center, Statens Serum Institut, where I performed the initial plating and culturing of nasopharyngeal swab samples, as well as the pneumococcal serotyping under the supervision of the laboratory technicians. The third study on IPD is purely register-based and based on register-data collected from different Greenlandic and Danish registers, with detailed information on clinical samples, hospitalizations, demographic and socio-economic data.

DATA-SOURCES

The Danish/Greenlandic Civil Registration System (CRS)

This registry was established in 1968 in Denmark, and in 1972 in Greenland and contains daily updated demographic information on all residents in the two countries [73]. At birth, all persons are assigned a unique personal identification number ('CPR-number') allowing for accurate linkage of individual level data across various health registers. We used information from the CRS on date- and place of birth, gender, history of residence, emigration, vital status, and birth order as well as individual level kinship information (parents, siblings and children).

Since a specific register for IPD-cases in Greenland does not exist, the identification of IPD-cases was obtained from different data-sources in order to get as complete as possible registration of all verified IPD cases in Greenland during 1973-2013.

Identification of IPD-cases in Greenland

(Please see table 1. below for an overview of available registries.)

1) IPD-cases from the microbiology laboratory at Dronning Ingrids Hospital, Nuuk, Greenland

The 16 rural health districts scattered around the coast line in Greenland, submit clinical samples for microbiological analysis to Dronning Ingrids Hospital (DIH), Nuuk, where the only microbiological laboratory in Greenland is located. If pneumococci are isolated, they are routinely shipped to the National Neisseria and Streptococcus Reference Center (NSR) at Statens Serum Institut for serotyping. We retrieved a list of all available pneumococcal isolates from invasive samples, registered by the laboratory staff. Data was available from 1990 and onwards.

2) Reports of IPD from the Public Health Medical Officer of Greenland

In Greenland it is mandatory to report all clinical or microbiologic verified cases of meningitis or invasive pneumococcal diseases to the Public Health Medical Officer of Greenland. We thus examined all available reports of IPD in the period from 1990 to 2013.

3) The Danish pneumococcus database

This database has previously been described in details elsewhere [74,75]. In brief, it was constructed at the national Neisseria and Streptococcus Reference (NSR) laboratory at the Statens Serum Institut, by linking laboratory data with information from the CRS. The NSR receives isolates from all the microbiologic laboratories in Denmark as well as from Greenland from which isolates for serotyping are routinely shipped, since the Statens Serum Institut functions as a tertiary laboratory for Greenland, and is the only laboratory in Denmark performing pneumococcal serotyping. We retrieved all IPD-cases related to Greenland from this database by linking the unique personal identification number from all persons alive in 1972 and onwards with residence in Greenland from the Civil Registrations System to the NSR.

Identifying risk factors for IPD

The Greenlandic National In-patient Register

This register used in paper II, was established in 1987 and contains data on admittance, treatment- and discharge diagnosis of all inpatients in Greenlandic hospitals. Outpatients are not recorded as well as emergency room contacts not leading to admittance. Discharge codes are based on WHO International Classification System ICD-8 up to 1993 and ICD-10 from 1994. We used these data to categorize organ-specific comorbidity, including all admissions within the last three years and up to one month prior to IPD-diagnosis or sampling of control subjects (sampling of control-subjects described later in method section). Furthermore data from this registry was used for the Charlson co-morbidity score index [76] in the survival analysis (paper II).

The Greenlandic Medical Birth Registry

This registry contains data on all live and stillbirths by women with residence in Greenland for the purposes of health surveillance and research. Reporting to the registry has been mandatory for midwifes since 1990, and the registry contains a variety of birth-related variables such as gestational age, birth weight, Apgar-score at 5 minutes, mode of delivery, BCG-vaccination etc.

Greenland Statistics

Greenland statistics keeps annually updated information on a variety of demographic and socio-economic variables and covers the entire Greenlandic population. For the study (paper II) in the present thesis, this register supplied information on household income and level of education. Data was available from 2003-2013.

Table 1. Overview of available registries and study period used.

Register	Available period	Included studyperiod	Study
The civil registration system (Greenland)	1972 – 2013	1973 - 2013	I, II & III
The Danish Pneumococcus database	1938 – 2013	1973 – 2013	II
The Greenlandic National In-patient Registry	1987 – 2013	1990 - 2013	II
The Greenlandic Medical Birth Registry	1990 – 2013	1990 - 2013	II
Greenland Statistics	2003 – 2013	2003 - 2013	II
The Public Health Medical Officer of Greenland	1990 – 2013	1990 - 2013	II
The microbiology laboratory at Dronning Ingrids Hospital, Nuuk, Greenland	1990 - 2013	1990 - 2013	II

STUDY DESIGNS:

Study Population

Greenland is the world's largest island with more than three quarter covered by ice and a population of 56,370 (2013) persons living in towns and settlements scattered along the coastline, which also makes it the least densely populated country in the world. Approximately 90% of the population are Inuit and the rest mainly Caucasians (Danes) [77].

Ethnicity was in these studies defined on the basis of parental birthplace i.e. both parents born in Greenland (Inuit), one parent born in Greenland (mixed) and if parents were born outside Greenland (other) i.e. mainly Danish or Scandinavian.

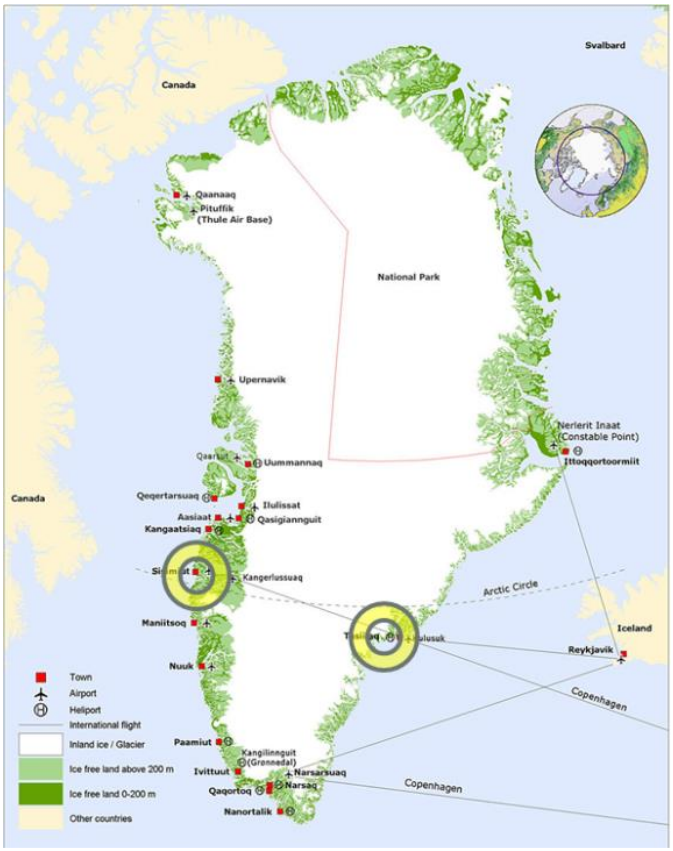


Figure 7. Greenland.
Yellow circles represents the study sites: Tasiilaq & Sisimiut.

Study I

To assess the nasopharyngeal carriage-rates of four clinically relevant pathogenic bacteria among children less than seven years, we conducted a population-based cross-sectional study.

Two towns and nearby settlements were chosen as the study area. The towns represents the two largest towns in Greenland on the west- and east-coast, except for Nuuk, and were chosen partly due to logistic reasons, availability, and partly to evaluate potential regional difference in carriage. Tasiilaq, one of two towns on the East coast with 1,800 inhabitants and 800 persons living in three settlements, and Sisimiut on the West coast, the second-largest town of Greenland with 5,460 inhabitants and 350 persons in one settlement (fig. 7).

First, we identified all children aged 0 to 6 years and their parents living in the study area in October 2011 by use of the CRS. Second, we did a randomized register-extraction of 500 children and invited them to participate. After written- and oral informed consents were obtained from

parents or caretakers, a questionnaire, written in both Danish and Greenlandic, was completed. The questionnaires had been tested in pilot-study during the summer of 2010 in Sisimiut to evaluate its usage. Based on the pilot-study it was decided that during the cross-sectional studies, if necessary, the form was completed under the supervision of an interpreter. It included questions regarding potential confounders such as; the number of siblings, day-care institution attendance, breastfeeding, recent antibiotic use, domestic tobacco exposure, recent respiratory tract infections, hospitalizations, self-experienced housing standard, number of rooms, number of people sleeping in the same room, in-house water supply and heating source. Data on PCV-13 vaccination status was obtained through local medical files.

Nasopharyngeal sampling

The standard procedure recommended by WHO in 2003 was chosen [78]. A nasopharyngeal swab sample was taken inserted via the nasal cavity to the posterior wall of the nasopharynx. Johan Navne undertook the nasopharyngeal sampling, which he has been trained to during his work as a physician at pediatric departments. The samples were stored at -20˚C for a maximum of three weeks before transported by air at - 20 ˚C to Statens Serum Institut, Copenhagen, Denmark, for storage at -80˚C. We used the STGG medium (Skim milk-Tryptone-Glucose-Glycerin) which has proven useful for the study of respiratory pathogens including *S. pneumonia*, NTHi and *M. catarrhalis* [79] for storage.

Study III

The evaluation of the PCV-13 impact on nasopharyngeal carriage of potential pathogenic bacteria was based on another cross-sectional carriage study conducted in 2013 in October - November. We chose the same study-site as in study I, Tasiilaq and Sisimiut, invited the same age-group of children less than seven years and used the same sampling-method and laboratory analyses as described in study I. We determined the carriage rates of *S. pneumonia*, NTHi, *M. catarrhalis* and *S.aureus* and grouped the pneumococci by serotypes included or not in the PCV-13. Information regarding potential confounders was obtained through questionnaires as previously described. The results were subsequently compared with the data from study I representing a proxy of baseline-data.

Study II

To assess the natural history of IPD in Greenland, we conducted a case-control study nested in the Greenlandic population. IPD-cases were matched randomly with Greenlandic inhabitants. The study-population was defined as individuals with residence in Greenland (at the time of sampling of control subjects and IPD-cases respectively), during the study-period (1973 to 2013). Potential risk factors for IPD were identified a priori on the basis of the literature [43,80–83].

A Greenlandic cohort was created consisting of all individuals alive between 1st of October 1973 and 1st of October 2013 registered in the CRS and, with residence in Greenland prior to the date of IPD-diagnosis for the corresponding index-case. After creating this cohort, we were able to link the individuals with other registries using the unique personal identification number. In order to have as complete as possible identification of cases, we retrieved data from three different registries as previously described; the pneumococcal database at the Statens Serum Institut, the microbiology laboratory at Dronning Ingrids Hospital in Nuuk, Greenland and finally we got access to all invasive bacterial cases reported to the public health medical officer in Greenland. After identification of cases, they were individually matched 1:10 with a group of controls randomly selected from the Greenlandic cohort after matching on age and ethnicity.

Laboratory analyses (Study I & III)

At the Statens Serum Institut, the collected nasopharyngeal swab samples were plated on 5% horse blood agar, a chocolate agar and an antibiotic chocolate agar plate. To increase the likelihood of detecting low-density carriage and multiple pneumococcal serotype carriage, we also added 50µl of the swab-samples to a 1 ml. serum-ox broth and incubated in CO_2, 37˚C for 24 hours, before plating again as described above. This method has previously proved efficient in detecting multiple and low-density pneumococcal carriage [84].

Bacterial identification was based on colony morphology by conventional microbiologic procedures and verified by MALDI/TOF mass spectrometry [85]. All isolates were tested for antimicrobial susceptibility using the disk diffusion test and EUCAST breakpoints [86].

Pneumococci were identified based on α-hemolysis, optochin sensitivity and capsular reaction (Quellung). Non-typeable pneumococci were identified using bile solubility-test.

Pneumococcal group-determination was performed directly on the serum-broth enriched NP-

samples by Pneumotest latex ® agglutination and serotypes identified with *Quellung* [87] reaction by the use of type-specific antisera from the Statens Serum Institut [25,84].

STATISTICS

In study I

In this study, risk factor analyses and tests for inter-bacterial associations were performed using logistic regression models (PROC LOGISTIC, SAS V.9.3). Each exposure variable was tested by separate inclusion in a univariable model and only when significant at a 5% level included in the final multivariable model. Furthermore, test of inter-bacterial associations was performed by logistic regression adjusting for potential confounders, such as recent antimicrobial use or PCV-13 vaccination.

Study III

The study was designed to demonstrate potential changes in pneumococcal carriage after the introduction of the PCV-13 as the primary outcome, and changes in NTHi*, M. catarrhalis* and *S. aureus* as secondary outcomes. Based on a previous study [88] carriage rate prevalence were estimated to be 67%, 42% and 8% for NTHi*, M. catarrhalis* and *S. aureus,* respectively. Given these estimates a sample size of 350 in each cross-sectional study, would be sufficient to detect a minimal difference in carriage of 10%, 11% and 7%, respectively with 80% power and a significance-level of 5%. Differences in prevalence rates were tested by chi-square tests, with a p-value < 0.05 considered significant. Associations between nasopharyngeal carriage by the four bacteria and PCV-13 vaccination were estimated by multivariable logistic regression analyses. Possible confounders were tested by separate inclusion in basic adjusted models with adjustments for age (one-year intervals), sex, PCV-13 vaccination and sampling-year. Only when significant in a basic model at a 5%-level, it was included in the fully adjusted models. The fully adjusted models included adjustments for age, sex, ethnicity, region (East/West), current day-care attendance, having siblings attending a day-care, recent respiratory infections (otitis media, ear-discharge, tonsillitis or pneumonia within the last three months or rhinitis within the last week). For analytical purposes we grouped pneumococcal serotypes in vaccine-types (VT), i.e. serotypes included in the PCV-13 and non-vaccine types (NVT), not included in the PCV-13. In case of

potential repeated measurements from individuals appearing in both of the two cross-sectional studies, we did a robustness analyses using a general estimation equation (GEE) [89] to account for correlated data. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina)

Study II

In this study, each IPD-case was individually matched 1:10 by age and ethnicity, using the risk-set sampling technique [90] i.e. controls were selected from subjects at risk in the Greenlandic population at the time of disease-occurrence of the case. This gives the possibility of expressing the association between a risk factor and IPD by rate-ratios (RR). Risk factor analysis was done separately for children less than two years of age and adults older 18 years using conditional logistic regression. Exposure variables were retrieved from various registers as described previously. Data on comorbidity, retrieved from the Greenlandic inpatient register, was based on ICD-codes and was grouped by organ-system for analytical purposes in the risk factor analyses. Only ICD-codes registered within the last three years and up to one month prior to IPD-admission were included. Only in the mortality analyses we accounted for comorbidity by using the Charlson comorbidity index score which has been validated for this purpose and described previously [76]. In short, the Charlson score is a validated method accounting for comorbidity as a competing risk for mortality in prognostic studies (table 2). It takes each individual's age and comorbid conditions into account, including both the number and seriousness of comorbid diseases. The scoring system does not include alcohol-related conditions, which may be of great importance for the prognosis of IPD. We therefore created a group of alcohol-related conditions, based on ICD-codes related to heavy alcohol consumption (29109, 29129, 29199, 30309, 30319, 30320, 30328, 30329, 30390, 30399, 98099, DF10, DF100, DF1000, DF101, DF102, DF1024, DF1025, DF103, DF1030, DF104, DF105, DF106, DF108, DF109, DT51, DT510, DT519, DZ721 and DE512).

Table 2. Charlson Comorbidity Index Scoring System (reproduced from [76]**).**

Score	Condition
1	Myocardial infarction
	Congestive heart failure
	Preipheral vascular disease
	Cerebrovascular disease: CVA with mild or no sequelae or TIA
	Dementia
	Chronic pulmonary disease
	Connective tissue disease
	Peptic ulcer disease
	Mild liver disease (including chronic hepatitis)
	Diabetes without end-organ damage
2	Hemiplegia
	Moderate or severe renal disease
	Diabetes with end-organ disease (retinopathy, neuropathy, nephropathy)
	Tumor without metastasis
	Leukemia (acute or chronic)
	Lymphoma
3	Moderate or severe liver disease
6	Metastatic solid tumor
	AIDS (not just HIV-positive)

Abbreviations: CVA, cerebrovascular accident; TIA, transient ischemic accident; AIDS, Acquired immunodeficiency syndrome

HIV: human immunodeficiency virus

For each decade > 40 years of age, a score of 1 is added to the above score.

ETHICAL APPROVAL

The studies fulfilled the Helsinki II Declaration and were scientific ethically approved by the Greenlandic Scientific Commission (Journal no. 2011 – 056257, doc. no. 738293, Journal no. 2012-060783, doc.no. 821618) and were approved by the Danish Data Protection Agency (2008-54-0427).

RESULTS

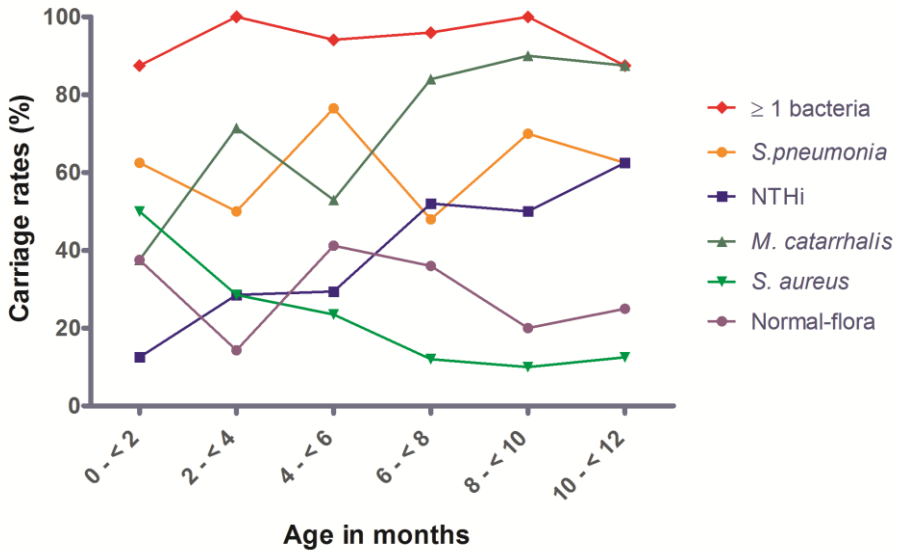
PAPER I

The first cross-sectional study aimed to describe nasopharyngeal carriage patterns, risk factors for carriage and bacterial interactions for four important clinically relevant bacteria frequently associated with infections in young children. We found nasopharyngeal pneumococcal carriage in healthy Greenlandic children to occur very early in life (beginning at two weeks of age) compared with a median age of six months for first acquisition in healthy pediatric low-risk populations. The overall carriage-rate of ≥ 1 bacteria (*S. pneumoniae*, *M. catarrhalis*, NTHi or *S. aureus* as grouped) was 83%. Pneumococcal carriage-rates reached levels up to 60%, peaking among 2-year-olds (fig. 8). Furthermore, we found that the Greenlandic children were often colonized with multiple bacterial species (52%) and that carriage rates in this population tended to remain relatively high in pre-school age with only moderate reductions after peak-age, as otherwise observed in low-risk populations. Among risk factors for carriage, young age and crowding-related circumstances dominated whereas ethnicity had no influence on carriage. The study further indicated various associations between the colonizing bacteria (table 3). NTHi was positively associated with non-vaccine type pneumococci – (NVT) as well as with *M. catarrhalis*, but negatively associated with *S. aureus. M.catarrhalis* on the other hand, was positively associated with both VT and NVT, but negatively associated with *S. aureus.* Overall our results indicate that colonization does not occur at random but that there are important inter-bacterial associations. These bacterial interactions may in turn affect the new balance of the nasopharyngeal flora anticipated after PCV-13 implementation in Greenland, and thus, potentially affect future prevalence of respiratory infections after wide spread use of PCV-13 in Greenland.

Figure 8.

Nasopharyngeal carriage of *S. pneumoniae*, NTHi, *M. catarrhalis*, *S. aureus*, ≥1 bacteria (all four bacteria grouped) and the presence of normal flora, according to age.

(Top graph: age 0 to < 1 year, Bottom graph: age 0 to <6 years).



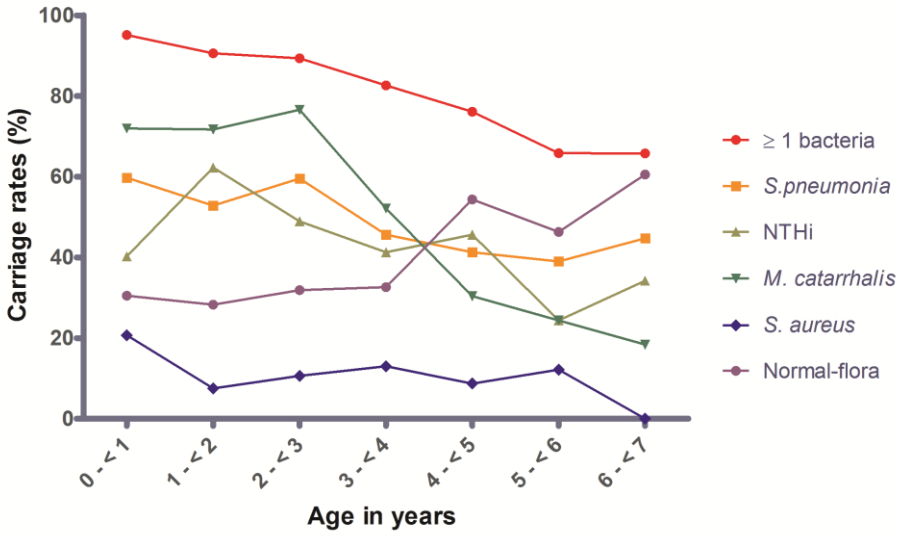


Table 3. Odds of co-colonization between pneumococcal vaccine (VT) or non-vaccine (NVT) serotypes, *non-typeable H. influenzae (NTHi)*, *M. catarrhalis* and *S. aureus* among 353 Greenlandic children aged less than 7 years. Analyses are based on isolates from original swab-samples without serum-broth enrichment and with no restrictions regarding other potential co-colonizing bacteria. OR are adjusted for age, sex, ethnicity and PCV-13 status.

Bacterium		**VT[b]**			**NTHi**			***M.catarrhalis*[d]**			***S. aureus*[e]**			**Normal flora[f]**	
	n	**aOR**	**p**	**n**	**aOR**	**p**	**n**	**aOR**	**p**	**n**	**aOR**	**p**	**n**	**aOR**	**p**
NVT[a]															
No	113	1 (ref)		34	1 (ref)		85	1 (ref)		105			105	1 (ref)	
Yes	4	**0.15 (0.1-0.3)**	<0.01	41	**2.3 (1.4-3.7)**	<0.01	32	1.6 (0.95-2.5)	0.08	12	0.75 (0.35-1.4)	0.44	12	**0.2 (0.1-0.8)**	0.03
VT[b]															
Yes				42	1 (ref)		42	1 (ref)		50	1 (ref)		55	1 (ref)	
No				18	0.7 (0.4-1.5)	0.40	18	**3.4 (1.7-6.5)**	<0.01	10	1.2 (0.4-3.9)	0.68	5	0.7 (0.3-1.3)	0.22
NTHI[c]															
No							46	1 (ref)		46	1 (ref)		108	1 (ref)	
Yes							37	**4.5 (2.6-7.8)**	<0.01	37	**0.2 (0.1-0.9)**	0.04	34	**0.3 (0.2-0.6)**	<0.01
M.catarr[d]															
No										64	1 (ref)		76	1 (ref)	
Yes										6	**0.1 (0.02-0.4)**	<0.01	15	**0.4 (0.2-0.6)**	<0.01
S. aureus[e]															
No													39	1 (ref)	
Yes													2	0.6 (0.2-1.8)	0.38

Abbreviations: aOR (adjusted Odds Ratios); PCV-13 (the 13-valent pneumococcal conjugate vaccine); p: p-value

a. NVT: pneumococcal serotypes not included in the 13-valent pneumococcal conjugate vaccine
b. VT: pneumococcal serotypes included in the 13-valent pneumococcal conjugate vaccine
c. NTHi: non-typeable Haemophilus influenzae
d. M.catarr: *Moraxella catarrhalis*
e. S.aureus: *Staphylococcus aureus*
f. Normal flora: A group of commensal bacteria, primarily Moraxella species (*non liquefaciens*), non-hemolytic streptococci and coagulase negative staphylococci.

Significant findings in bold ($p < 0.05$)

PAPER II

This study aimed to describe the natural history of IPD in Greenland, including incidence rates (IR), risk factors and mortality. A total of 230 cases of IPD were identified during 1973-2013. The majorities of patients were Inuit (91.3%), were men (59.6%) lived in Nuuk (51.7%) and had a very low Charlson comorbidity-score of 0 (84.6%). Short education (i.e. primary school) and low or very-low income dominated. Overall the IPD incidence rate (IR) in Greenland was 22.6/100.000 person-years (PY). The highest age-specific IR (fig. 9) was observed among children < 2 years and adults aged 50 – 60 years (59 and 51.7/100.000 PY), respectively, during 2000-2009. Regional variation in IR was evident, with the highest IR in Nuuk 51.1 (95% CI 39.6-66.0), followed by an IR in Tasiilaq of 46.2 (95% CI 26.6-79.5) and an IR in Narsaq of 21.52 (95% CI 8.08-57.34) and the lowest IR in Ilulissat of 6.7 (95% CI 2.2-20.7.) The frequency of bacteremia varied throughout the season with highest frequencies during the winter-months, whereas the variation throughout the year was virtually non-existent for meningitis (fig. 10).

Figure 9: Age-specific incidence rates (IR) per 100.000 person years of invasive pneumococcal disease in Greenland 1973-2013.

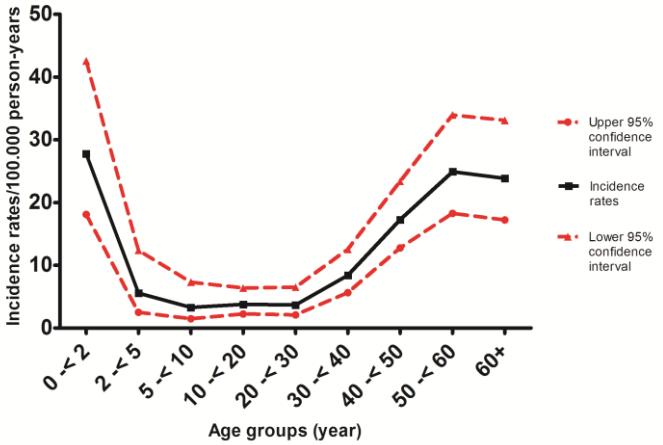
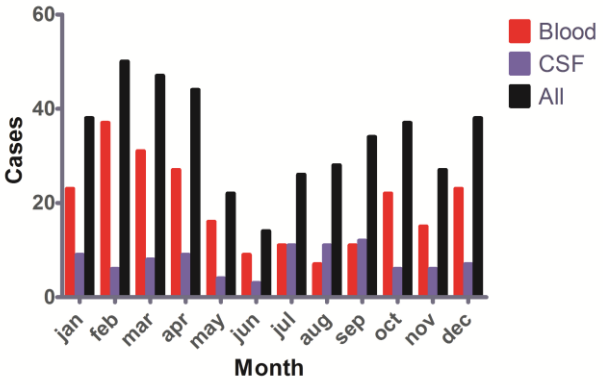


Figure 10: Prevalence of invasive pneumococcal disease accoridng to season and type. Greenland 1973-2013



Risk factors for infants included crowding related factors and neonatal complications such as prematurity, asphyxia and respiratory distress syndrome. Furthermore, previous hospitalizations with infections not related to the IPD-diagnosis increased the risk. Neither gender nor ethnicity was associated with IPD in infants.

Among adults > 18 years, being Inuit increased the risk of IPD up to 4-times (aOR 4.68; 95% CI 1.92-11.62) compared to non-Inuit, despite adjustments for sex, region, family size and underlying comorbidities. Furthermore, being male, living alone or having a chronic medical condition

(oncological, previous infections not related to the IPD-episode or ophthalmological conditions) increased the risk of IPD. Crowding-related factors were not associated with IPD, but in contrast living alone increased the risk of IPD despite adjustments. The level of education was not related with IPD, however, when including income as a continuous variable in the model, increasing income seemed to reduce the risk of IPD, although the results did not reach statistical significance. The total case-fatality risk during the period 1973-2013 was 23.5%. Males had higher mortality than females aOR 1.4 (95% CI 0.99-2.00).

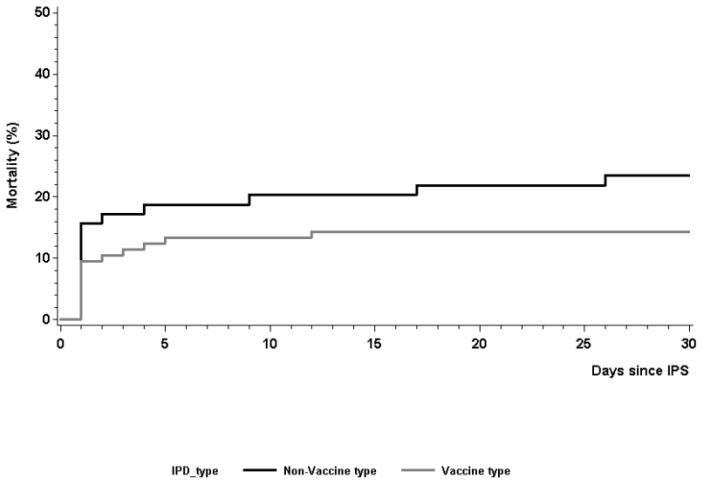
The 30-day mortality (figures 11 and 12)

The 30-day mortality-rate was defined as rates of mortality during the first 30 days after admission to hospital due to IPD. The 30-day mortality-rates decreased over the study-period, with highest rates in 1990-<2000 (36%) and lowest rates occurring between 2010 and 2013 (15%). Overall, Inuit had higher 30-day mortality (25%) than individuals with mixed ethnicity (9%) and non-Inuit (0%). IPD patients living in the districts had a higher mortality rate than Nuuk patients, with a hazard ratios (HR) of 2.34 (95% CI 1.07-5.13) in the southern region of Greenland compared to Nuuk. According to age-groups, adults (aged 50-<65 years) had the highest mortality (almost 35%) whereas school-children and young adults had the lowest (9%). Moreover, the mortality rate of cases of meningitis was twice the mortality-rate of cases with bacteremia. When adjusting the analyses for Charlson score the increased mortality among Inuit-cases compared to individuals with a mixed ethnicity was attenuated and no longer significant (aHR 0.55; 95% CI 0.07-4.12). However, 30-day mortality among patients with meningitis remained higher than among patients with bacteremia (aHR 2.92; 95% CI 1.29-6.62). Also living in the districts, in particular South-Greenland were associated with increased mortality compared to Nuuk-citizens although statistically insignificant (aHR 2.07; 0.95-4.53). The first signs of PCV-13 impact on IPD incidence rates in Greenland were studied by comparing the pre-vaccine period (2000-2009) with the post-vaccine period (2010-2013) (fig.13). Overall incidence rates of IPD declined in all age-groups except a minor increase among elderly aged > 60 years. The reductions were observed both for IPD caused by VT and NVT.

Figure 11. Mortality from invasive pneumococcal disease (IPD) in Greenland during 1973 to 2013, according to the number of days after admission to the hospital.

Top: IPD-cases are grouped according to those caused by serotypes included in the 13-valent pneumococcal conjugate vaccine (Vaccine-type) or not included in the vaccine (Non-vaccine type).

Bottom: Mortality according to the clinical manifestation of IPD (only bacteremia and meningitis included).



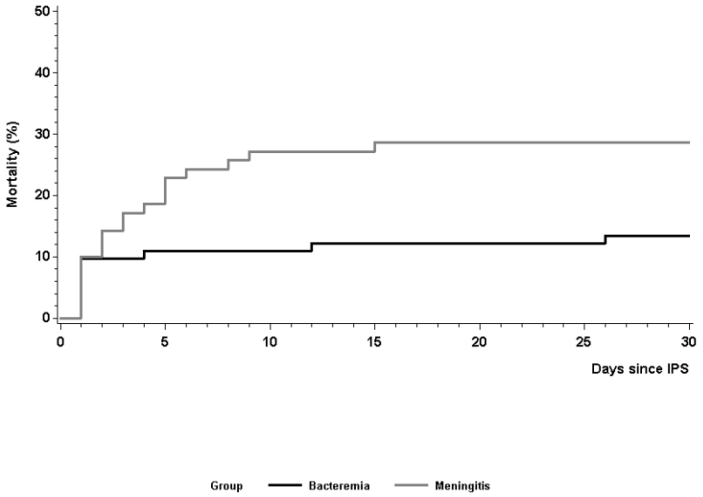
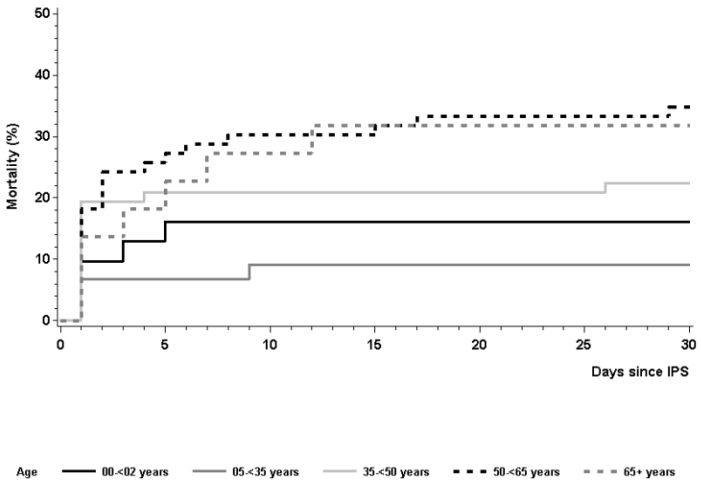


Figure 12. Mortality from invasive pneumococcal disease (IPD) in Greenland during 1973 to 2013, according to the number of days after admission to the hospital.

Top: Mortality according to age group

Last: Mortality according to calendar-period.



IPS - Mortality

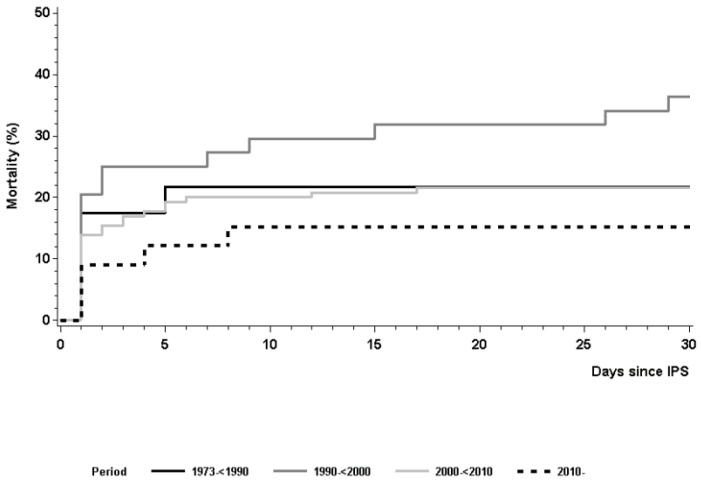
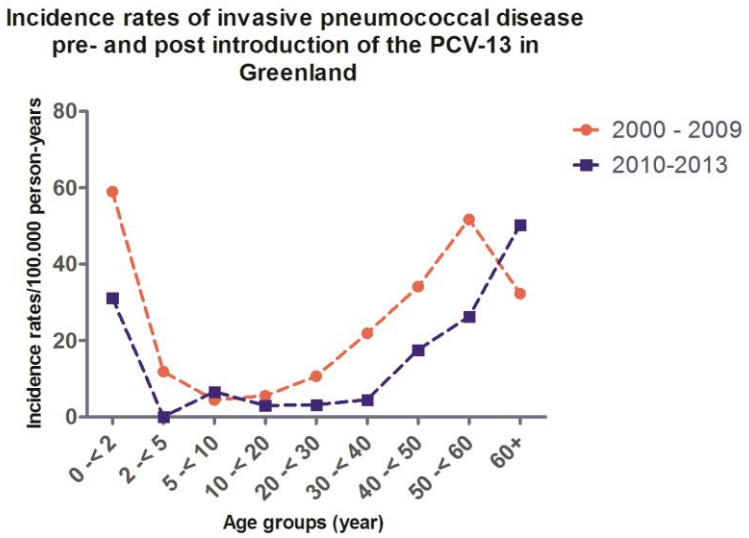
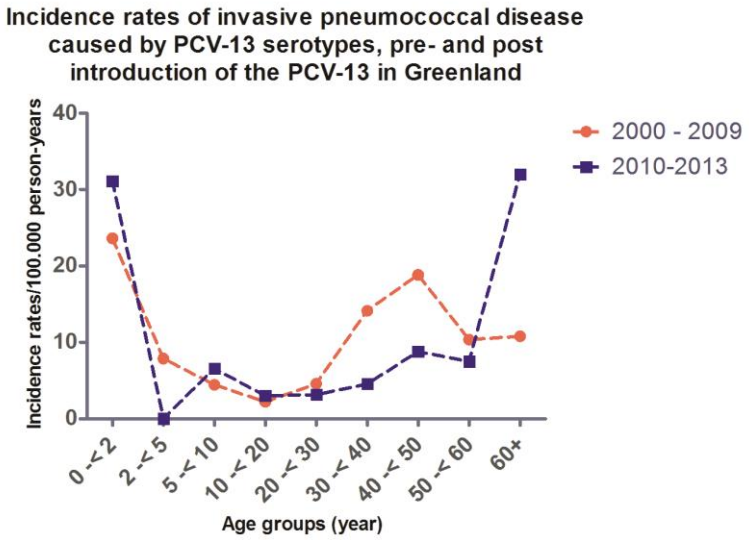
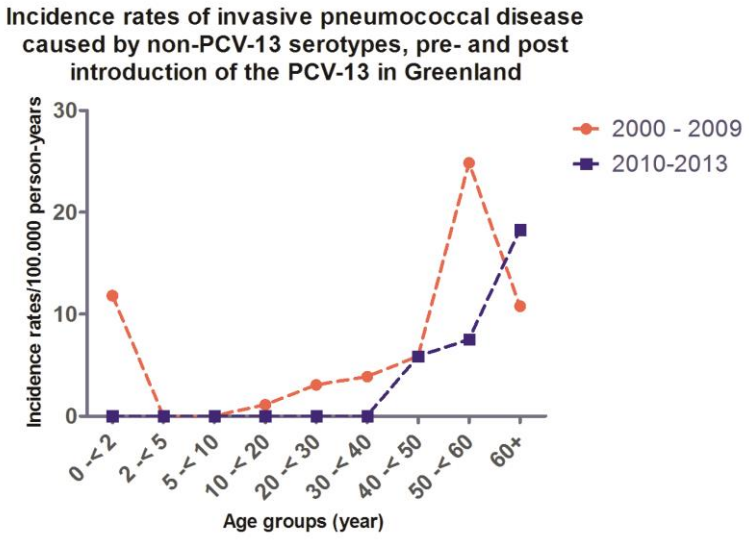


Figure 13. Age-specific incidence rates of invasive pneumococcal disease (IPD) in Greenland, pre- (2000-2009) and post (2010-2013) introduction of the 13-valent pneumococcal conjugate vaccine (PCV-13).

Top: Overall pneumococcal IPD. Middle and Bottom: According to serotype.





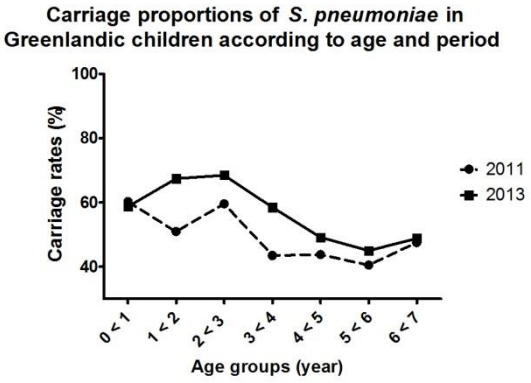


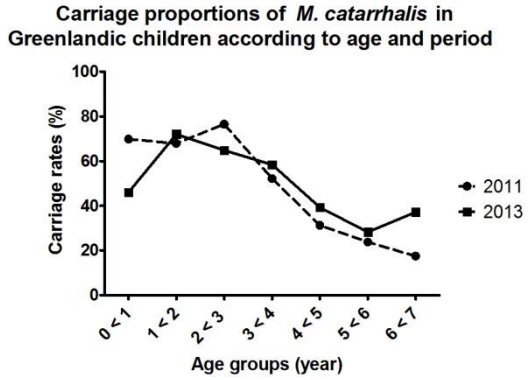
PAPER III

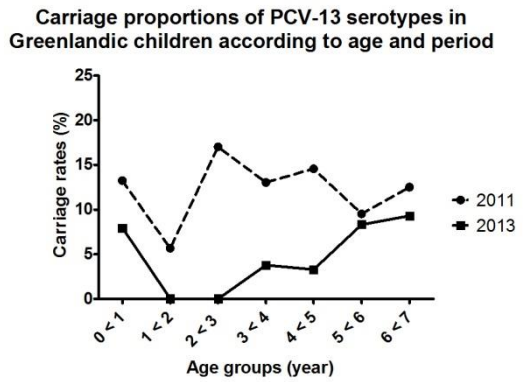
The aim was to evaluate the impact of the 13-valent pneumococcal vaccine (PCV-13) on nasopharyngeal carriage according to four important and clinical relevant bacteria. A total of 377 children aged 0 to 6 years participated in the cross-sectional study in 2013. Data was compared with main findings from the cross-sectional study conducted in 2011 (table 4). In order to account for potential confounders affecting the observed estimates of changes in carriage-prevalence, we performed risk factor analyses using logistic regression models. The following factors were associated with bacterial carriage: age, sex, ethnicity, period (year of sampling), PCV-13 vaccination, region of Greenland, day-care attendance, having siblings attending a daycare and episodes of recent respiratory infections (otitis media, ear-discharge, rhinitis, tonsillitis or pneumonia within the last three months).

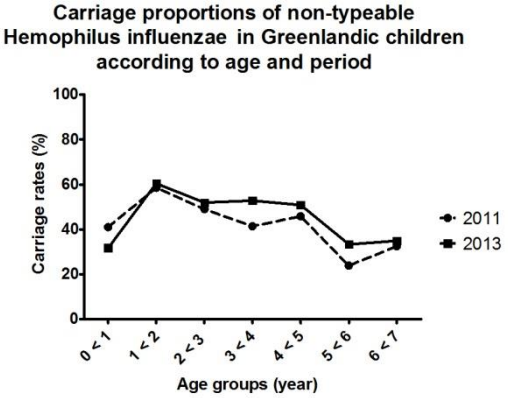
Three years post PCV-13 introduction, pneumococcal serotype distribution had changed (figure 15). A non-significant change occurred in overall pneumococcal carriage from 51% (in 2011) to 56% (in 2013), whereas carriage-rates of VT-serotypes decreased significantly from 12% to 5% counterbalanced by increases in NVT carriage-rates from 38% to 49% (figure 14). In multivariate regression analyses a significant reduction in VT-carriage was seen among vaccinated (aOR 0.38, 95% CI 0.18-0.82) (table 5) controlling for age-group, year- and geographic region of sampling, breastfeeding, day-care attendance, exposure to tobacco smoke, recent respiratory infections and having siblings attending a day-care center (table 4). Likewise the NVT-carriage increased significantly (aOR 1.64, 95% CI 1.07-2.52) both among vaccinated, but also independently of PCV-13 status (OR 1.87, 95% CI 1.08-3.24).

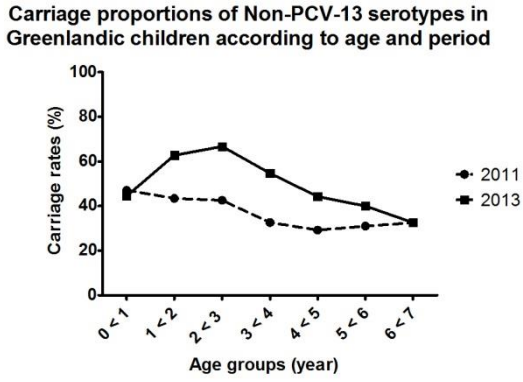
Figure 14: Carriage patterns of *S. pneumoniae*, PCV-13 serotypes, Non-PCV-13 serotypes, Non-typeable Hemophilus influenzae, *M. catarrhalis* and *S. aureus*, according to age-groups and period. Dotted lines represents carriage rates from a previous study conducted in 2011 [Paper I].











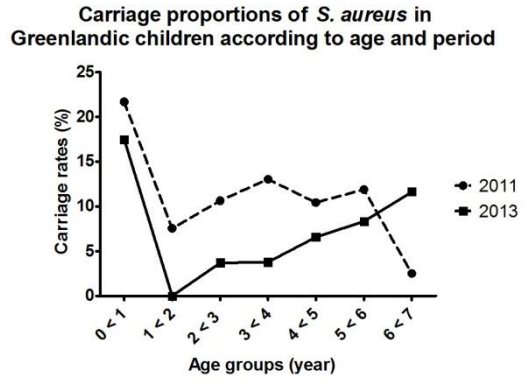


Figure 15. Proportions of nasopharyngeal pneumococcal serotype distribution among Greenlandic children aged 0 – 6 years in 2013 and compared with serotype distribution in 2011 [Paper I]. Arrows indicates serotypes included in the 13-valent pneumococcal conjugate vaccine.

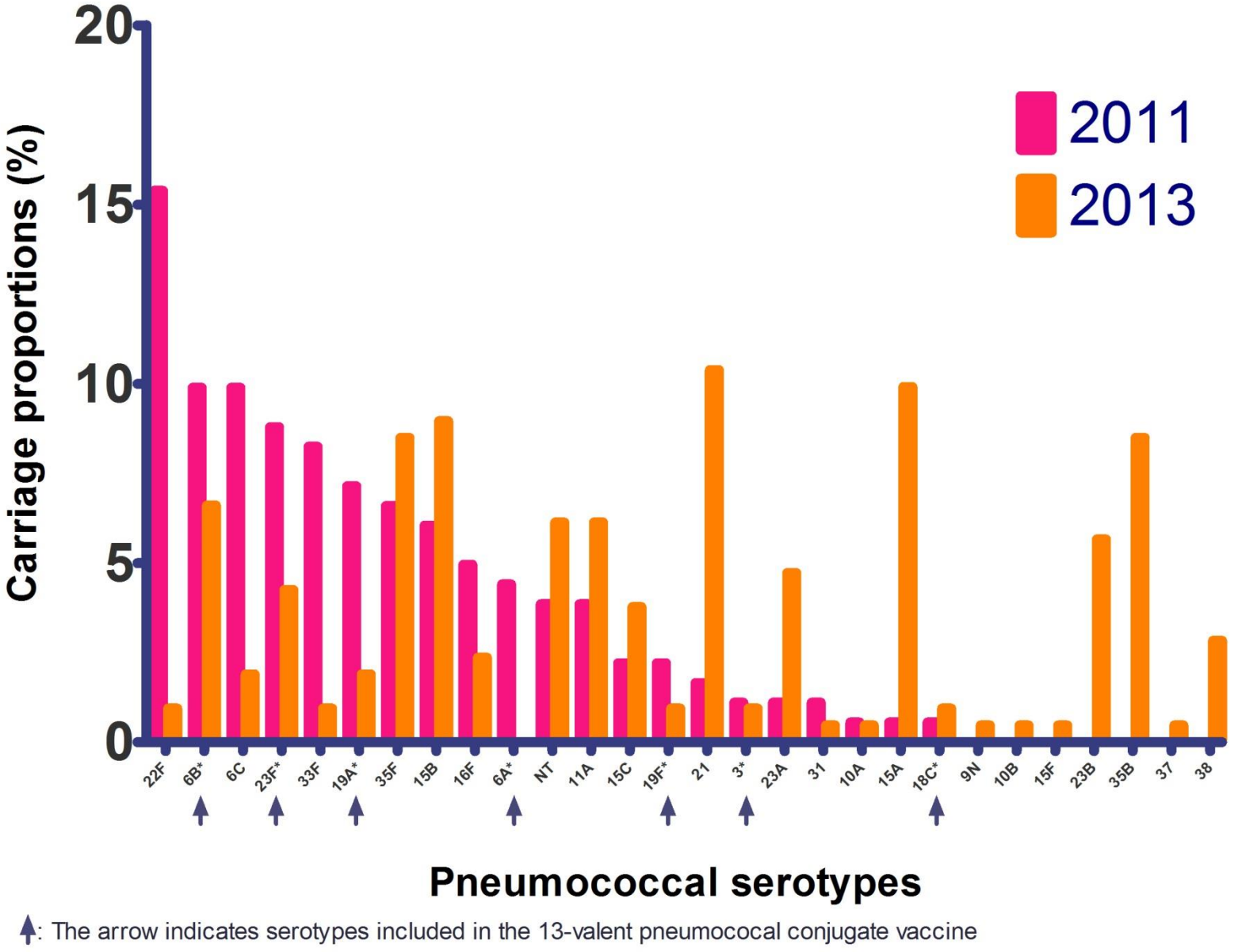


Table 4: Demographic characteristics of study population 2013 (n=377 children) and comparison population 2011 (n=352) (Paper I). Unselected children aged 0-6 years living in two regions of Greenland.

Variable	Level	Year 2013 n = 377 (%)	Year 2011 n = 352 (%)	p[a]
Gender	Male	192 (51)	179 (51)	0.98
Age, years	Median (Q1;Q3)	3.50 (1.68; 5.12)	2.85 (1.08; 4.84)	0.34
Ethnicity	Inuit	343 (91)	320 (91)	0.35
Region of Greenland[b]	East-coast	186 (49)	128 (36)	**<0.001**
	West-coast	191 (51)	224 (64)	
Daycare attendance (current)	yes	232 (62)	260 (74)	**<0.001**
Having siblings in DC[c]	yes	134 (36)	139 (39)	0.29
Inhouse smoking[d]	yes	39 (10)	67 (19)	**0.002**
Sharing bedroom with children < 5 years of age	yes	210 (56)	210 (58)	0.45
Number of persons per room[e]	0	23 (6)	14 (4)	0.41
	1	181 (48)	167 (47)	
	2	167 (44)	163 (47)	
Resp. infection < 3 months[f]	yes	229 (61)	206 (58)	0.43
Antibiotics < 3 months[g]	yes	53 (14)	58 (16)	0.39
PCV-13 vaccinated[h]	yes	212 (56)	127 (36)	**<0.0001**
***S. pneumoniae* detected**	yes	212 (56)	178 (51)	0.13
VT-pneumococci detected	yes	18 (5)	44 (12)	**<0.001**
NVT-pneumococci detected	yes	185 (49)	137 (38)	**0.003**
***S. aureus* detected**	yes	29 (8)	38 (11)	**0.04**
NTHi detected	yes	168 (45)	152 (42)	0.54
***M. catarrhalis* detected**	yes	183 (49)	186 (53)	0.38
Any of the bacteria detected[i]	yes	317 (84)	290 (82)	0.55

Abbreviations: DC: Day-care center, AOM: Acute otitis media, PCV-13: the 13-valent pneumococcal conjugate vaccine, S. pneumoniae: *Streptococcus pneumoniae*, VT-pneumococci: Pneumococcal serotypes included in the PCV-13, NVT-pneumococci: Pneumococcal serotypes not included in the PCV-13, S. aureus: *Staphylococcus aureus*, NTHi: non-typeable Haemophilus influenzae, M.catarrhalis: *Moraxella catarrhalis*.

a. P-value based on chi-square test for difference
b. Region: East (Tasiilaq, Kuummiut, Sermiligaaq, Kulusuk), West (Sisimiut, Sarfannguaq)
c. Having siblings attending a day-care institution
d. Tobacco smoke inside the house
e. Number of persons per room: (0= less than 1 person per room in household), (1= 1 to <2 persons per room), (2= ≥2 persons per room)
f. Respiratory infections: Any episode of rhinitis, acute otitis media, ear-discharge, tonsillitis or pneumonia within the last three months prior to nasopharyngeal sampling
g. Having received treatment with antimicrobial drugs within the last three months
h. PCV-13 vaccinated: Vaccinated with ≥1 dose of the 13-valent pneumococcal conjugate vaccine
i. Any bacteria: The detection of either *S. pneumoniae*, *M. catarrhalis*, NTHi or *S. aureus* in the nasopharyngeal sample

Table 5.

Crude- and adjusted odds ratios for bacterial nasopharyngeal carriage by *Streptococcus pneumoniae*, non-typeable Haemophilus influenzae, *Moraxella catarrhalis* or *Staphylococcus aureus*, among Greenlandic children aged 0 to 6 years in 2013 compared with data from a cross-sectional study in 2011 (Paper I).

	PCV-13				Year			
	No	Yes			2011	2013		
Bacterium	**1 (ref.)**	**OR[a] (95% CI)**	**aOR[b] (95% CI)**	**p**	**1 (ref.)**	**OR[a] (95% CI)**	**aOR[b] (95% CI)**	**p**
S. pneumoniae	1 (ref.)	1.31 (0.88-1.95)	1.19 (0.78-1.82)	0.41	1 (ref.)	1.23 (0.90-1.68)	1.18 (0.84-1.65)	0.33
VT	1 (ref.)	**0.44 (0.22-0.92)**	**0.43 (0.20-0.90)**	0.02	1 (ref.)	**0.42 (0.23-0.75)**	**0.44 (0.24-0.82)**	0.01
NVT	1 (ref.)	**1.65 (1.11-2.46)**	**1.63 (1.07-2.48)**	0.02	1 (ref.)	**1.45 (1.06-1.99)**	1.36 (0.97-1.90)	0.07
NTHi	1 (ref.)	1.17 (0.78-1.75)	1.29 (0.84-1.98)	0.24	1 (ref.)	1.07 (0.78-1.48)	1.10 (0.79-1.54)	0.58
M. catarrhalis	1 (ref.)	**1.72 (1.14-2.58)**	1.52 (0.99-2.33)	0.06	1 (ref.)	0.83 (0.60-1.16)	0.82 (0.58-1.16)	0.27
S. aureus	1 (ref.)	**0.51 (0.27-0.94)**	**0.48 (0.25-0.91)**	0.03	1 (ref.)	0.70 (0.42-1.18)	0.62 (0.35-1.07)	0.09
Any bacterium	1 (ref.)	1.58 (0.88-2.84)	1.43 (0.76-2.68)	0.27	1 (ref.)	1.13 (0.74-1.74)	1.04 (0.65-1.66)	0.87

Abbreviations: PCV-13: the 13-valent pneumococcal conjugate vaccine, OR: Odds-ratio, aOR: adjusted Odds-ratio, p: p-value for adjusted OR, CI: Confidence interval, S. pneumoniae: *Streptococcus pneumoniae*, VT: pneumococcal serotypes included in the 13-valent pneumococcal conjugate vaccine. NVT: pneumococcal serotypes not included in the 13-valent pneumococcal conjugate vaccine. NTHi: non-typeable Haemophilus influenzae, M. catarrhalis: *Moraxella catarrhalis,* S. aureus: *Staphylococcus aureus*. Any bacterium: Odds of carrying either *S. pneumoniae*, NTHi, *M. catarrhalis* or *S. aureus* (grouped).

a. Odds ratios mutually adjusted for year of sampling and PCV-13 vaccination, as well as age groups (1 year intervals) and sex.
b. Odds ratios mutually adjusted for year of sampling and PCV-13 vaccination, as well as age groups (1 year intervals) and sex. Additional adjustments: region (East-/West-Greenland), recent respiratory infection (otitis media, ear-discharge, nasopharyngitis, tonsillitis or pneumonia within the last three months), current day-care attendance (yes/no), having siblings in a day-care (yes/no) and ethnicity ('Inuit', 'mixed Inuit/other ethnicity' or 'non-Inuit').

DISCUSSION

Nasopharyngeal bacterial carriage (study I)

The first cross-sectional study aimed to describe carriage patterns and risk factors for the four most frequently bacteria involved in pediatric respiratory infections. We were expecting high rates of carriage, since high colonization-rates are often observed in populations with a high burden of respiratory diseases such as in Greenland [33,91]. Surprisingly, this was not the case, and instead we found carriage-rates comparable to other healthy pediatric populations with low-risk of infections. However, as opposed to these populations, we observed a pattern of very early and persisting colonization by a heterogeneous group of bacteria, since the Inuit children were colonized shortly after birth, often by multiple species and with relatively high carriage rates persisting into pre-school age. This pattern may result in a carriage-state with a high degree of inflammation and mucosal damage leading to chronic mucosal disease and consequently a high risk of clinical infections as proposed by John et al. [92]. Based on that hypothesis they suggest probiotics as beneficial to homogenize the bacterial flora of the nasopharynx and thus prevent entering a state of chronic inflammation. Another way of breaking the vicious circle of multiple species carriage may be to inhibit the species in producing biofilm, since biofilm production seems to be enhanced when multiple species co-colonize the nasopharynx [93]. Biofilm is known to protect or hamper the immunological elimination of the bacteria [94]. Xylitol, a sweetening substitute for sucrose often added to chewing-gum, has in clinical trials shown to reduce the rates of otitis media in children compared with a control group [95]. The hypothesis that xylitol inhibits the bacterial biofilm production was tested by a Finnish research group who confirmed an inhibitory effect on biofilm production by *S. pneumoniae* [96], however, the effect was not observed when other carbon sources were present.

With respect to risk factors for carriage, we identified primarily crowding and young age as having a significant impact, and these associations have in part been attributed to close contacts and high transmission rates between young children combined with an immature immunity towards these bacteria in infancy. Ethnicity, as such, did not affect the risk of carriage, indicating that potential genetic variants related to immunologic deficiency most likely is of less importance rather than exposure to environmental factors. This is further supported by previous studies of mannose-binding-lectin insufficiency or cytokine responses in relation to respiratory infections among Greenlandic Inuit, which did not indicate any impaired immune reactivity to infections being able

to explain the high burden of infectious diseases among Inuit [97,98]. We observed some important bacterial associations in this study. More specifically pneumococcal serotypes seemed to differ in their associations to other co-colonizing pathogens, depending on whether they belonged to vaccine-type (i.e. included in the 13-valent pneumococcal vaccine) or non-vaccine type pneumococci. NTHi and *M. catarrhalis* were positively associated to pneumococci, whereas *S. aureus* was competitive with *S. pneumoniae*. Lijek et al. found in a study on bacterial co-colonization in mice, that specific proteins related to pneumococcal colonization stimulates an immune response which elicits antibodies capable of cross-reacting and hence inhibits *S. aureus* acquisition and colonization [99]. Furthermore, Margolis et al. have shown that density and rates of nasopharyngeal carriage by NTHi and *S.pneumoniae* are higher when they co-colonize the nasopharynx as opposed to if one of the species is absent, which indicates a synergistic interaction [100]. Overall, these findings indicate that the bacterial composition of the nasopharynx may be skewed after widespread use of the PCV-13 in Greenland, where selective elimination of vaccine-type pneumococci is expected. However, the exact mechanisms of the complex inter-microbial interactions and how the nasopharyngeal microbiome interacts with the local host-immune system and environmental exposures have not been fully elucidated, but these factors are likely to play a critical role in the composition of the colonizing bacteria [100].

Serotype-shifts and changes in carriage-proportions (study III)

In study III we examined the possible consequences of PCV-13 introduction in Greenland on nasopharyngeal bacterial carriage. As expected, we found substantial serotype-shifts with reductions in vaccine-types (VT) and increase in non-vaccine types (NVT). This indicates a direct vaccine effect, which is likely facilitated by high concentrations of serotype-specific antibodies elicited by the PCV immunization, migrating from the serum to the nasopharyngeal mucosa to prevent VT colonization. Furthermore, as observed in other studies and as indicated in the present study, reductions in VT-carriage occurs independently of PCV-13 status, which points to an indirect effect of the vaccine (herd immunity), since the nasopharyngeal transmission of VT pneumococci from vaccinated children to other individuals are interrupted. In fact, calculations of the indirect vaccine effect has shown that the PCV globally has prevented far more IPD-cases among unvaccinated than among vaccinated due to herd-immunity [101].

However, in some settings such as among native Alaskans, this beneficial effect it offset by dramatic increasing rates of NVT-IPD. The possible explanations to the observed increasing rates of NVT may include several mechanisms [62]. It may be caused by natural fluctuations in serotypes circulating in a community, which may not be identified if the surveillance-period is short and the increases in NVT may thus be unrelated to the vaccine-introduction. On the other hand, in a situation with multiple pneumococcal serotype colonization, the reduction of vaccine-type (VT) pneumococci may lead to so-called 'unmasking' of previously unidentified non-vaccine-type (NVT) pneumococci already residing in the flora but in such low density that they are undetectable by traditional culture-methods. Moreover, another theory of the mechanism behind increasing NVT is the so-called 'True replacement'-theory, where an actual increase in the frequency of NVT occurs, which may be explained by different scenarios. First, it may be an expansion of existing NVT with previous low frequency of carriage, or second, new clones of NVT being introduced in the community or third, it may be a result of so-called 'capsular switch'. Capsular switch may be explained by the unique plasticity of the pneumococcus, which makes it capable of responding to selective vaccine- or antimicrobial pressure by expressing another locus of the pneumococcal genome encoding the capsular structure. This may subsequently result in a new capsule structure and thus serotype. The pneumococcus is also capable of exchanging DNA horizontally from other co-colonizing pneumococci in the nasopharynx or even from related streptococcus species, which then results in capsular switch, hence the capsular structure is modified and thus, the serotype changed [62].

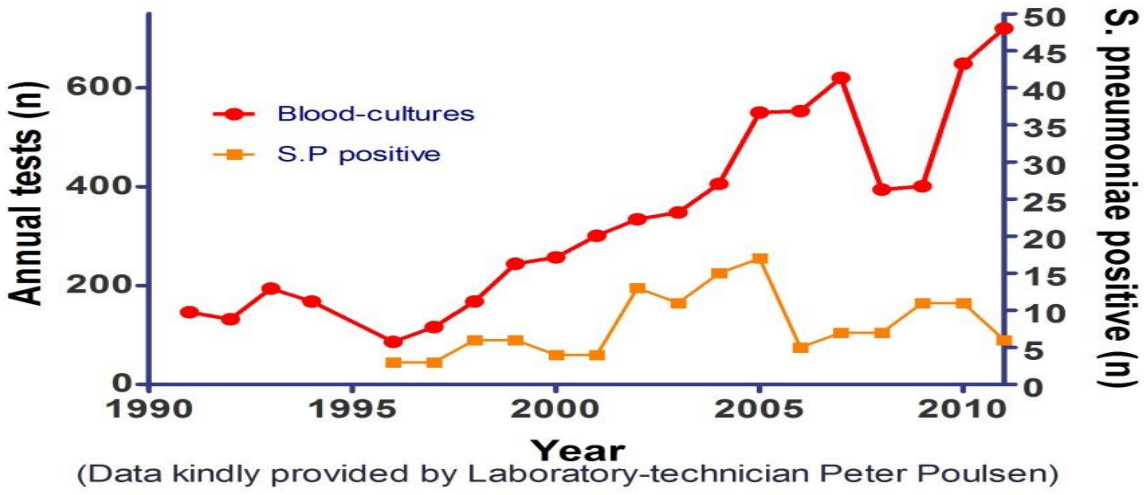
Regarding non-pneumococcal bacteria, we observed two important findings. First, the data demonstrated an increase in carriage-rates of *M. catarrhalis* among PCV-13 vaccinated children. This pathogen is frequently involved in acute otitis media (AOM), which is a major public health problem in Greenland, with early onset and recurrent episodes progressing to chronic otitis with subsequent hearing disabilities and language impairment [7,102–104]. Whether the increases in carriage-rates of *M. catarrhalis* observed in this study are temporary or persistent, may be clarified by continued surveillance, but recent studies have indicated that PCV may skew the balance not only in colonization-composition, but also in disease-prevalence. Studies of PCV-7 impact on acute otitis media (AOM) have shown increases in proportions of children with *M.catarrhalis* and NTHi in middle ear fluid among those vaccinated with the PCV-7, compared to

non-vaccinated age-matched controls with AOM indicating an association [72,105–107].

IPD in Greenland (Study II)

The study of IPD in Greenland during 1973 to 2013 demonstrated dramatic increases in incidence rates from the 1970s to the 2000s. This change may partly be caused by so-called surveillance bias. Changes in clinical practice or case reporting over time may have occurred. For example the proportions of febrile patients having a blood-culture taken when presenting at the doctor in the districts as compared to the hospital of Nuuk is likely to differ. Moreover, the awareness of a potential increased susceptibility to bacterial infections among Inuit may have changed over the period and thus influenced clinical practice. During the 1990s and 2000s a substantial overall increase in the number of annual blood-cultures performed at the laboratory of DIH, Nuuk have been observed (fig.16).

Figure 16. Annual numbers of blood-cultures performed at the Laboratory of Dronning Ingrids Hospital, Nuuk Greenland, during 1990 to 2011



(Data kindly provided by Laboratory-technician Peter Poulsen)

However, despite the increase in annual number of blood cultures performed, a true increase in IPD is likely to have occurred, since the incidence of meningitis has also increased during the same calendar period (Navne unpublished data). Though we did not have data on annual number of cerebrospinal fluid-analyses, it is less likely that indications for performing lumbar puncture during

the period have changed largely, given the invasive nature of the procedure and the observations may thus represent a true increase in IPD in Greenland.

IPD was found to be most prevalent among young children less than two years of age and adults aged 50 to 65 years. This pattern is comparable to other countries, where age-specific IPD-incidences typically form a U-shape. In Greenland though, we noticed a modification of the U-shape, since increases were observed already from age 35 and peaked among middle-aged from 50 to 65 years and the leveled out. The reason for this early peak in incidence rate of IPD may be a relatively higher degree of comorbidity in this population as compared with other, with almost 50% of the IPD-patients in this age group having a high Charlson score (≥2). The study furthermore confirmed that Inuit are at high-risk of IPD with up to four-times increased risk compared with non-Inuit despite adjustments for age, sex, environmental factors and comorbidity as observed in Alaska and Canada[13,14]. Thus, a genetic susceptibility among Inuit cannot be ruled out. Other risk factors included being male, living alone and having certain underlying conditions especially malignancies and previous infections requiring hospitalizations. The overall 30-day mortality among Inuit with IPD was higher than among the Inuit populations in Canada and Alaska, particular among young children and middle-aged adults, and among those with meningitis and a high Charlson score. Furthermore, significant differences in regional mortality-rates were observed within Greenland, with highest rates in the rural districts compared with Nuuk, where mortality-rates were comparable to rates observed in Denmark. Overall, this may indicate several things; first, that the premorbid comorbidity levels of IPD-patients in Greenland may be higher than in other regions (i.e. a high proportion of patients with high Charlson score), second, that only those with the worst prognosis are diagnosed in Greenland (resulting in higher mortality rates, since milder cases are more unlikely to be identified) and third, that outcome of IPD is highly dependent on fast access to specialized treatment, with respect to the variations in mortality-rates between the rural districts and the capital Nuuk.

STRENGTHS, LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

Carriage studies (study I and III)

Due to the great variety of available methods for pharyngeal sampling, identification and serotyping of pneumococci, a working-group under the WHO published a protocol for conducting pneumococcal nasopharyngeal carriage studies by standardized methods in order to better compare studies across regions and avoid bias related to the chosen methods [108]. The protocol describes all the necessary steps for conducting carriage studies, from choosing the right utensils, the correct sampling method, transport-medium, temporary and long-term storage conditions etc. Since studies from the developing part of the world are sparse and are still to some extent lacking, a simple and inexpensive transport medium was also proposed by the WHO. This medium is made of Skimmed milk, Tryptane, Glycosis and Glycerol (STGG). The protocol was updated in 2013 [28] with minor modifications. The carriage-studies presented in this thesis have been based on the WHO protocol (2003) with exactly the same methodology in both of the studies, which minimizes potential technical bias. The first study was conducted soon after vaccine-introduction in a setting where most of the children were unvaccinated and thus with minimal potential confounding by vaccine induced herd immunity.

Furthermore, the carriage-studies presented in this thesis, are the first to include non-pneumococcal bacteria in an Inuit-population, when evaluating the impact of the PCV, which is of great importance in order to describe the overall effect on nasopharyngeal bacterial carriage. We have used systematic confounder control by including data from Greenlandic translated questionnaires completed by the parents often under the assistance of an interpreter if the questions gave rise to any uncertainties. However, we did not have regular baseline carriage data prior to the PCV-introduction and thus, we cannot rule out potential natural fluctuations causing the observed changes or at least to some degree to have influenced the results. In addition, we have only sampled a subset of the Greenlandic pediatric population and the results may not be generalizable to the entire population.

Serum-broth enrichment.

Despite the use of the pneumococcus-specific transport medium STGG, asymptomatic carriage of *S. pneumoniae* in the nasopharynx may be a challenge to estimate under circumstances where traditional culture methods are used and direct inoculation of the samples is not possible. The colonies of pneumococci may be present in very low numbers and thus less likely to survive temporary storage (up to three weeks) and transportation (from Greenland to Denmark) to a laboratory with microbiologic facilities (Statens Serum Institut). A traditional culture method may not be able to detect low-density growth and may thus underestimate the actual carriage rates. Thus, to increase the detection-level of pneumococci we added 50 µL of the original swab samples to an enrichment broth (1 ml of serum-ox broth - Statens Serum Institut) which previously has shown efficient in increasing the growth of pneumococci and to detect co-colonization by multiple serotypes [84].

Thus, in our carriage-studies, the proportion of *S. pneumoniae* positive samples increased by up to 78% when using the serum-broth enrichment (table 5). The increased detection-level was not reserved to pneumococci, but also other bacteria; however, the increased growth was not equally distributed among the different bacterial species.

Table 5. Overall nasopharyngeal carriage rates among 353 healthy Greenlandic children aged 0 to < 7 years. Results listed according to positive culture from either the original nasopharyngeal samples, the serum-broth enriched samples, positive in samples either with or without serum-broth enrichment and finally positive in both types of samples.

	S.pneumoniae	NTHi[a]	*M.catarrhalis*	*S. aureus*	Normal flora[b]	Others[c]	Sterile
Type of sample	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Original swab	137 (39)	108 (30)	177 (50)	22 (6.8)	103 (29)	6 (1.5)	27 (7.5)
Swab with serum-broth enrichment	175 (50)	141 (40)	88 (25)	39 (11)	90 (25)	11 (3)	8 (2)
Positive in either original or serum-broth	178 (50)	152 (43)	188 (53)	41 (11.6)	137 (39)	14 (4)	32 (9)
Positive in both original & serum-broth	136 (38)	97 (27)	77 (22)	20 (5.6)	56 (16)	3 (1)	3 (1)

a) NTHi: non-typeable Haemophilus influenzae
b) Normal flora: Primarily Moraxella species in particular nonliquefaciens, but also coagulase negative staphylococci and non-hemolytic streptococci.
c) Others: Include Haemophilus influenzae type B, E and F, n=five (1%) and Hemolytic streptococci group A, B and G, n=nine (2.5 %).

These selective growth advantages observed after the addition of serum-broth enrichment may be due to bacterial interactions occurring within the samples with both synergistic and competitive associations. Based on these observations we chose to calculate carriage-rates from the proportions of positive samples in either the original or the serum-broth enriched samples. The rationale behind was that if an original sample went from negative to positive after enrichment, then the bacteria must have been present from the beginning in the original swab sample but in such low numbers or density that traditional culture method would not detect it.
However, when analyzing for inter-bacterial associations we only used the original swab samples to avoid potential bias by the selective growth induced by the serum-broth enrichment.

Risk factor analyses

In the risk factor analyses for IPD, we used validated nationwide registers with detailed information on clinical samples, hospitalizations and demographic data. Thus, demographic and risk factor information for identified IPD cases and controls was almost complete and reliable with a fixed pre-IPD observational period for each person. However, not all register information may be reliable such as e.g. information on income. Economy in Greenland, in particular in more remote

towns and settlements, involves some element of non-official income such as that from hunting for one's own use, the degree of which may not be reflected in official registers. We did not have access to direct information on important factors such as breast feeding, smoking and alcohol, as this is not contained in national registers. Instead, we had to rely on an indirect measure for alcohol use, namely alcohol associated co-morbidity, which may not be an accurate measure of alcohol use. Finally, only comorbidities resulting in hospitalizations were addressed, as outpatient contacts are so far not registered in nation-wide registers. This is important, as e.g. recurrent acute otitis media, which is highly prevalent in Greenland [102,104], may be a risk factor for IPD [109].

Furthermore, some misclassification of comorbidity and Charlson-score may be present for those individuals diagnosed with chronic medical conditions in Denmark and who after immigration to Greenland are hospitalized with IPD or selected as a control subject, since they may have missing history of comorbidity in the Greenlandic Inpatient Register. However, we only included ICD-codes from a three-year period prior to IPD-diagnosis to limit the potential influence of this type of bias. Furthermore, we include only individuals having lived in Greenland for the last three years prior to IPD-diagnosis in the analyses to minimize bias introduced by this type of misclassification.

Study period

Regarding nasopharyngeal carriage, some studies indicate that there is a transient flux in serotype prevalence in the first few years after vaccine-introduction but that a steady state is reached after some years [62]. Likewise, natural fluctuations in overall IPD incidence may occur, and these are not detected if only short time-periods pre- and post PCV-introduction are used as the basis of analysis [110]. Thus, to clarify if the observed changes in bacterial carriage-rates persist, we recommend continued surveillance of nasopharyngeal colonization in Greenland.

CONTRIBUTION AND PERSPECTIVES

In this PhD-project, we have contributed with high-quality IPD surveillance analyses over a three-year period based on multiple sources prior to the introduction of pneumococcal conjugate vaccines. We have confirmed that the incidence of IPD among Inuit of Greenland are three to four times higher than among non-Inuit, and that incidence rates are comparable to other arctic countries in the pre-PCV era. However, the estimates are most likely conservative due to presumable under-diagnosing in the rural districts. Risk factors for IPD in Greenland have been identified, based on a matched case-control design, adding to the existing knowledge and highlighting important high-risk groups: young children < 2 years of age and middle-aged adults who have the highest incidences of IPD and also a higher mortality from IPD than what is observed in other arctic countries, especially among patients presenting with pneumococcal meningitis. The mortality from IPD among Greenlandic children is up to five-times the rates observed in other Arctic countries. Also middle-aged adults, primarily men, with underlying comorbidities and those living alone, form a special group at high risk of IPD and with high mortality-rates from IPD. Overall, a genetic importance contributing to IPD risk among Inuit cannot be excluded since the estimates did not change despite adjustments for comorbidity and environmental factors.

We have demonstrated dramatic serotype-shifts among colonizing pneumococci in both vaccinated and unvaccinated children after the introduction of the PCV-13 in Greenland, with reductions in vaccine-type (VT) pneumococci. However, the effect was counterbalanced by increasing rates of non-vaccine-serotype (NVT) pneumococci, and the question is to what extent the NVT will cause disease. Based on the experience from other countries having introduced the PCV's, some degree of replacement disease is likely to occur in Greenland, however, current data does so not indicate that. Furthermore, we have documented changes in other colonizing bacteria, associated with frequent infections in children, which likely makes the net effect of the vaccine on the overall disease-burden from respiratory and invasive infections limited to some extent. As in other countries the demographic pattern in Greenland includes an aging population with an increasing proportion of people > 60 years [77], the age-group with the highest IPD-associated mortality rate. To what extent the PCV-13, which recently has been licensed to all age groups including adults [52] might be beneficial to high-risk groups such as elderly with certain comorbidities, may rely on several factors. First, continued surveillance of IPD may clarify the

degree of indirect herd-immunity from the PCV-13 and thus to what extent reductions in IPD-rates among unvaccinated occur. Second, the disease potential of colonizing NVT may differ among children and adults, since some so-called pediatric serotypes are more associated with infections in children, causing local mucosal infections such as otitis media [111] whereas other serotypes may be more associated with asymptomatic carriage or even be more virulent and cause IPD [112]. In the long term the pneumococci are likely to adapt to the vaccine pressure and thus only vaccines with higher valency or vaccines which are serotype-independent may proove efficient in preventing pneumococcal diseases.

FUTURE STUDIES

An important question to answer is if the observed changes in bacterial carriage rates post PCV-13 introduction in Greenland persist in the long-term or only represent a transient state of reorganization within the nasopharyngeal flora. A suggestion could be to implement biannual carriage studies as a surveillance tool including both pneumococci and other potential pathogenic bacteria. To minimize logistic challenges, local health-care facilities could be included and selected staff members trained in performing nasopharyngeal swab samples. Carriage studies are relatively inexpensive and more feasible as compared with nation-wide monitoration of IPD. Since carriage is the essential first step in developing the corresponding infectious diseases, carriage studies may serve as proxies for vaccine impact on infectious diseases[113]. Whether the increases of NVT carriage may result in increased pneumococcal infections, including NVT-IPD is an important question for future studies, especially since data from the present study indicates that IPD caused by NVT is associated with a higher mortality than IPD caused by VT. However, as previously described results so far on PCV-13 impact on IPD in Greenland shows reductions in overall IPD rates and no indications of substantial replacement IPD. Yet, a longer follow-up time is needed to clarify if these changes are temporary or persist as the coverage of the PCV-13 is widespread in Greenland.

To further clarify a potential genetic susceptibility of IPD among Inuit, we propose conducting a study comparing the risk and mortality from IPD among Inuit living in Greenland and Inuit having immigrated to Denmark, including the necessary Danish register data for confounder control.

In addition, to further elucidate the background of the increased risk of IPD among adult Greenlanders studying the nasopharyngeal bacterial carriage among both children and adult Greenlanders might provide the necessary data to determine which specific bacterial transmission routes exist in this population at increased risk of respiratory and invasive infections.
We have studied the PCV-13 impact on selected bacteria residing in the nasopharynx. However, the entire microbiome may be even more interesting to study, since microbial interactions are far more complicated than what may be observed between selected bacteria. However, these analyses are expensive and the enormous data generated from the numerous species in the microbiome may be a challenge to interpret not least in the context of other environmental confounders.

SUMMARY OF MAIN-FINDINGS:

- Greenlandic children are colonized with *S. pneumonia*, NTHi, *M. catarrhalis* and *S. aureus* in rates that are comparable to other low-risk pediatric populations (PAPER I)
- However, carriage begins at very young age (two weeks) and frequently multiple bacteria are carried simultaneously. (PAPER I)
- Risk factors for bacterial carriage includes: young age, gender (females and *M.catarrhalis*), Ethnicity (Inuit and *M.catarrhalis*), PCV-13 vaccination (increased NVT and *M.catarrhalis*, but reductions in *S. aureus* carriage), living in Tasiilaq (increased S. pneumoniae and M. catarrhalis carriage), having siblings attending a daycare (increased NTHi) and having experienced episodes of respiratory infections within the last three months (otitis media, pneumonia, tonsillitis or rhinitis) (PAPER III)
- Nasopharyngeal bacteria exhibit important synergistic- and antagonistic associations, which may indirectly cause alterations in the nasopharyngeal bacterial composition after widespread use of the PCV-13. (PAPER I)
- After the introduction of the PCV-13 in Greenland, substantial changes in serotype-distribution have occurred both among vaccinated and unvaccinated, with marked reduction in VT carriage counterbalanced by increased rates of NVT carriage. Consequently, the PCV-13 seems to cause a herd-immunity effect, with no or little overall effect on the pneumococcal colonization rates. (PAPER III)
- Carriage rates of *M.catarrhalis* have increased among vaccinated children post-PCV-13 introduction, whereas rates of *S. aureus* have declined. This may have implications for the prevalence of respiratory- and other *S.aureus*-related infections in the post PCV-13 era. (PAPER III)
- The overall incidence rate of Invasive pneumococcal disease in Greenland is comparable to other Arctic countries prior to the introduction of pneumococcal conjugate vaccines, however, the estimate is likely conservative due to presumable under-diagnosing in the rural districts. (PAPER II)
- Risk factors for IPD in Greenland include ethnicity (Inuit), being male, living alone and having certain underlying comorbidities, whereas crowding and socio-economic factors do not seem to influence the risk. (PAPER II)

- Overall mortality from IPD is higher among Inuit compared with non-Inuit, particular among young children (up to five-times higher than Danish children with IPD) and adults (two-times higher than Danish IPD-patients), among patients with comorbidity (high Charlson score ≥2), among patients with pneumococcal meningitis and among those living in the districts of Greenland. (PAPER II)

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APPENDIX

PAPER I
PAPER II
PAPER III

NASOPHARYNGEAL BACTERIAL CARRIAGE AMONG YOUNG CHILDREN IN GREENLAND: A POPULATION AT HIGH RISK OF RESPIRATORY INFECTIONS

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Abstract

Background: The incidence of childhood respiratory infections in Greenland is among the highest globally. Nasopharyngeal bacterial carriage precedes disease. We aimed to describe rates and risk factors for carriage of four key bacteria associated with respiratory infections, their antimicrobial susceptibility and inter-bacterial associations.

Methods: Population-based study of children in Greenland. Nasopharyngeal swab tested for *Streptococcus pneumoniae (S. pneumoniae)*, non-typeable *H. influenzae* (NTHi), *Staphylococcus aureus* and *Moraxella catarrhalis* and the presence of *normal flora (Moraxella non liquefaciens,* non-hemolytic streptococci and coagulase negative staphylococci). *S. pneumoniae* was grouped by serotypes included (VT) or not (NVT) in the 13-valent pneumococcal conjugate vaccine. Risk factor information was obtained through questionnaires. Statistical analyses included logistic regression.

Results: 352 children aged 0 - 6 years participated. Overall co-colonization with two or more of the studied bacteria was 52%. *S. pneumoniae* was detected from two weeks of age with a peak carriage rate of 59.5% in 2-year-olds. Increasing age (aOR 0.8; 95% CI 0.68-0.9) and having siblings in a day-care (aOR 1.6; 95% CI 1.1-2.2) but not ethnicity (aOR 0.7; 95% CI 0.3-1.5) was associated with pneumococcal carriage. The carriage of normal flora and NTHi (aOR 0.3; 95% CI 0.2-0.6), *M. catarrhalis* (aOR 0.4; 95% CI 0.4-0.6) and NVT (aOR 0.2; 95% CI 0.1-0.8) were inversely related. NTHi and NVT were positively associated (aOR 2.3; 95% CI 1.4-3.7) whereas *S. aureus* were negatively associated with NTHi (aOR 0.2; 95% CI 0.1-0.9) and *M.catarrhalis* (aOR 0.1; 95% CI 0.01-0.4).

Conclusion: Significant respiratory carriage is present already early in life and with frequent co-colonization. Domestic crowding increased odds of carriage whereas ethnicity did not. Due to important bacterial interactions we suggest future surveillance of pneumococcal conjugate vaccine impact on carriage in Greenland to also include non-pneumococci, since emergence of NVT may secondary increase carriage-rates of the important oto-pathogen NTHi.

Keywords: risk factors, nasopharyngeal carriage, pneumococci, children, Greenland, Inuit, PCV-13

Running title: Bacterial Carriage Among Inuit Children

INTRODUCTION

Respiratory infections are a major healthcare problem in the Inuit population of the Arctic. In Greenland [1], where almost 90 % of the population is of Inuit origin, the incidence of clinically verified upper- and lower respiratory infections in children aged 0 to 2 years is among the highest in the world. In particular, otitis media is highly prevalent, characterized by early onset, recurrent episodes progressing to chronic otitis media with long-term consequences such as hearing loss and impaired language acquisition [2,3]. The burden of disease is almost exclusively carried by the Inuit population, whereas non-Inuit in Greenland have lower risks of respiratory infections [1]. In addition, the incidence of invasive pneumococcal disease (IPD) is markedly high in native populations of the Arctic including Greenlandic Inuit with significantly higher mortality than among non-Inuit [4]. However, reasons for this ethnic health disparity and the high incidence of respiratory tract infections are basically unknown. In other populations, genetic as well as environmental factors including socio-economic conditions have been shown to influence the risk of respiratory infections [4]. Except for use of child care centers, passive smoking, and a parental history of recurrent infections, [1,5] particular risk factors for respiratory tract infections have not been identified in Inuit.

Worldwide, some of the most clinically relevant bacteria involved in childhood respiratory and invasive bacterial diseases include *Streptococcus pneumoniae, non-typeable Haemophilus influenzae, Moraxella catarrhalis*, and S*taphylococcus aureus*[6–8]. The bacteria colonize the nasopharynx except for *S. aureus* which resides in the anterior nasal cavity [9]. Nasopharyngeal carriage, which is highly frequent among young children, is considered the essential first step in the development of respiratory and invasive bacterial infections. Although asymptomatic in themselves, the bacteria may migrate to cause either local infections such as otitis media, sinusitis and pneumonia or systemic potentially life-threatening invasive diseases such as bacteremia and meningitis [10]. Nasopharyngeal carriage may thus be considered an infectious reservoir for bacterial auto-infections as well as transmission to other individuals in the community [11,12].

Factors known to influence bacterial carriage rates are besides age: bacterial and viral intra- and interspecies interactions (commensal and pathogenic), function of the immune system, and environmental factors [12].

In 2010 the 13-valent pneumococcal conjugate vaccine Prevenar 13® (PCV-13) was introduced in the Greenlandic childhood vaccination program at 3 and 5 months of age and with a booster at 12 months. The predecessor the 7-valent pneumococcal conjugate vaccine (PCV-7), that has not been used in Greenland, has in other settings been shown not only to reduce incidence of IPD caused by pneumococcal serotypes included in the vaccine (also called vaccine serotypes - VT), but also to prevent nasopharyngeal carriage by VT [13,14]. However, invasive disease caused by non-vaccine pneumococcal serotypes (NVT) and other bacteria (so-called 'replacement disease') may have emerged as a consequence of the vacant nasopharyngeal niche being refilled by non-vaccine serotype pneumococci or other respiratory pathogens [13–20] or it may be caused by temporal changes in serotype prevalence unrelated to serotype replacement. One of the first countries to introduce the PCV-7 was the US in Alaska in 2000. They observed significant pneumococcal serotype shifts, particularly among the Native Alaskan population, with reduced prevalence of VT-IPD but also one of the highest reported degrees of IPD caused by NVT and a total incidence of IPD reaching the pre-PCV7 vaccination level [15]. The impact of PCV-13 introduction in Greenland is unknown.

In this cross-sectional population-based study of Greenlandic children aged 0 – 6 years of age, we aimed to determine prevalence and risk factors for nasopharyngeal carriage by *S. pneumoniae, non-typeable H influenzae* (NTHi)*, M. catarrhalis* and *S. aureus*, including antibiotic susceptibility patterns and bacterial interactions, as well as to provide surveillance data during the introduction of the PCV-13 in Greenland.

METHODS AND MATERIALS

Population/Study design

Greenland is the world's largest island with more than three quarters covered by ice and a population of 56,370 (2013) persons living in towns and settlements scattered along the coastline. Approximately 88% of the people are Inuit and the rest mainly Caucasians (Danes) [21].

Two towns and nearby settlements were chosen as the study area. Tasiilaq, one of two towns on the East coast with 1,800 inhabitants and 800 persons living in three settlements, and Sisimiut on the West coast, the second-largest town of Greenland with 5,460 inhabitants and 350 persons in one settlement.
Since 1972 all citizens of Greenland have been given a unique identification number registered in the Civil Registration System (CRS). The daily updated CRS contains vital information on place and date of birth, gender, birth order, siblings, parents, current and earlier addresses [22], and the unique personal identification number allows for accurate linkage between other national registers. We identified all children aged 0 to <7 years in October 2011 in the CRS and their parents living in the study area and invited them to participate. After written- and oral informed consents were obtained from parents or caretakers, a questionnaire was completed regarding number of siblings, day-care institution attendance, breastfeeding, recent antibiotic use, domestic tobacco exposure, recent respiratory tract infections, hospitalizations, self-experienced housing standard, number of rooms, number of people sleeping in the same room, in-house water supply and heating source. Data on PCV-13 vaccination status was obtained through nationwide medical files.

Nasopharyngeal sampling

The standard procedure for nasopharyngeal sampling recommended by World Health Organization in 2003 was chosen [23]. The Skim milk-Tryptone-Glucose-Glycerin medium (STGG) has proven useful for the study of respiratory pathogens including *S. pneumonia*, NTHi and *M. catarrhalis* [24]. A nasopharyngeal swab sample was taken using Minitip Flocked nylon Swabs (FLOQSwabs™) inserted via the nasal cavity to the

posterior wall of the nasopharynx, rotated 180 degrees and then placed in 1 ml of STGG medium. After cutting the excess wire with a sterilized scissor, the cap was tightened and the sample stored at -20˚C for a maximum of three weeks before being transported by air at - 20 ˚C to Statens Serum Institut, Copenhagen, Denmark, for storage at -80˚C.

Laboratory analysis

After thawing, the specimens were vortexed and 50µl of each sample was spread with a sterile plastic loop into three streaks on a 5% horse blood agar, a chocolate agar and an antibiotic chocolate agar plate (Statens Serum Institut). The blood agar was subsequently cross-streaked with a strain of *S. aureus.* To increase the likelihood of detecting low-density carriage and carriage of multiple pneumococcal serotypes, we also added 50µl of the nasopharyngeal-sample to a 2 ml serum-ox broth and incubated in CO_2, 37˚C for 24 hours, before plating again as described above. This method has proven efficient in increasing the detection-level of nasopharyngeal pneumococci [25].

Bacterial identification was based on colony morphology as ascertained by conventional microbiologic procedures and verified by MALDI/TOF mass spectrometry[26]. All isolates were tested for antimicrobial susceptibility using the disk diffusion test and EUCAST breakpoints [27]. Pneumococci were identified based on α-hemolysis, optochin sensitivity and capsular reaction (known as 'Quellung'). Non-typeable pneumococci were identified using bile solubility-test.

Pneumococcal group-determination was performed directly on the serum-broth enriched nasopharyngeal-samples by Pneumotest latex ® agglutination. Serotypes were identified with *Quellung* [28] reaction by the use of type-specific antisera from the Statens Serum Institut [25,29]. For analytical purposes, a group of 'normal flora' was defined which consisted primarily of Moraxella species in particular *M. non liquefaciens* but also non-hemolytic streptococci and coagulase negative staphylococci.

Statistical analyses

The use of serum-broth enrichment increased the detection of *S. pneumoniae,* NTHi and *S. aureus*, whereas detection of *M. catarrhalis* and the normal flora were reduced when using this enrichment. Due to these selective growth advantages, we decided to base the analysis of carriage rates on either a positive original or serum-broth enriched nasopharyngeal sample, whereas the analyses of risk factor- and bacterial interaction were based solely on results from bacterial growth on the 5% horse blood agar, chocolate agar and antibiotic chocolate agar plates.

Risk factor analyses and tests for inter-bacterial associations were done by logistic regression analysis (PROC LOGISTIC, SAS. V.9.3). Each exposure variable was first tested separately in a univariable model and if significant at a 5 % level included in a multivariable model. Based on this, the final model was adjusted for age, sex, ethnicity and PCV-13 status (i.e. having received ≥ 1 dose of PCV-13 or not). Pneumococcal serotypes were grouped in vaccine-types (VT), i.e. serotypes included in the PCV-13 and non-vaccine types (NVT), not included in the PCV-13, and treated as separate groups of bacteria in the analyses.

Ethics

The study fulfilled the Helsinki II Declaration and was scientific ethically approved by the Greenlandic Scientific Commission (Journal no. 2011 – 056257, doc. no. 738293) and the Danish Data Protection Agency (2008-54-0427).

RESULTS

A total of 450 children were invited and 352 children aged 0 – 6 years (median 2.8 years, 25% and 75% quartiles 1.1 and 4.8 years) consented and were enrolled. The majority (92%) was of Inuit origin and 52% were males (table 1). Approximately one third of the sample had received one or more doses of PCV-13 vaccination (12% one dose, 16% two doses and 8% three doses).

The serum-broth enrichment increased the detection of low density growth, in particular *S. pneumoniae,* NTHi and *S. aureus*, whereas the detection of *M. catarrhalis* and the normal flora was reduced (table 2).

Carriage of potentially pathogenic bacteria

Overall, 293 children (83%) carried one or more potentially pathogenic bacteria (fig.1a). Carriage was established at an early age with 85% of infants less than 2 months of age (n=17) being colonized, and 100% in 2 to 4 months old children (n=16). Carriage rates fluctuated between 85 and 100% during the first year (fig 1a). Hereafter a steady decline to reach an overall carriage rate of 70% in 6-year-olds (n=25) was observed (fig 1.b). In contrast, the lowest rates of carriage of normal flora were observed among 0 – 3 year-olds (30%, n=55) increasing up to 60% among 6-year-olds (n=23).

M. catarrhalis

The most frequent colonizing bacteria overall was *M. catarrhalis* with an overall carriage rate of 53% (188 isolates). Rates increased during the first year and peaked among 1 – 2 year-olds (n=36, 70%), then decreased to 20 % in preschool children (n=17). All isolates were resistant to penicillin and ampicillin, intermediate resistant to cephalosporins (cefuroxime) and fully susceptible to macrolides.

S. pneumoniae

A total of 185 *S. pneumoniae* isolates were detected in 177 children, resulting in an overall carriage rate of 50%, with the highest carriage-rate around 60% observed in the years before 3 years of age levelling off to 45% in subsequent years (fig 1b). Carriage was detected as early as 2 weeks of age, and by 2 months of age 50% of children carried pneumococci (fig. 1a). The most frequently carried serotypes were 6B, 6C, 15B, 16F,

19A, 22F, 23F, 33F and 35F accounting for 78% of all isolates. Among these only serotypes 6B and 19A are included in the PCV-13. Co-colonization with multiple serotypes occurred in 4% of participants. All *S. pneumoniae* isolates were fully susceptible to penicillin (oxacillin) except one isolate of type 6C that also showed resistance to erythromycin and clindamycin.

NTHi

Overall carriage was 40% (142 isolates), with increasing rates during the first two years from 10% to 60%. After peaking in 2 year-olds rates dropped to 35% among preschool children. We only identified three capsular *Haemophilus influenzae* isolates (type B and E). Among NTHi isolates 36% were penicillin-resistant and β-lactamase-producing and thus resistant to ampicillin, amoxicillin and piperacillin according to EUCAST breakpoints v.3.1 [27]. Five percent of NTHi isolates were Trimethoprim-sulfamethoxazole resistant.

S. aureus

A different carriage pattern was observed for *S. aureus* with highest rates among the 0 to 1 month-old children (50%), followed by a decline to around 10% among 1 year-olds where it stabilized until preschool age. A total of 41 isolates (11.5%) were identified. The majority (90%) of *S. aureus* isolates were penicillin-resistant but susceptible to dicloxacillin.

Risk factors for carriage

For all pathogens young age was significantly associated with increased bacterial carriage (Table 3). Having normal flora in the nasopharynx was, however, associated with increasing age. The group of 4 to 6 year-olds were more likely to carry normal flora (OR 2.4, 95% CI 1.1-5.4) compared to children aged 0 – 1 years. Crowding related factors such as attending a day-care institution, having a sibling in day-care or living 2 or more persons per room, increased the odds of bacterial carriage (except for *S. aureus),* whereas ethnicity did not show a clear association with carriage. Furthermore, we found significantly higher carriage rates of NTHi and *M. catarrhalis* in children from Sisimiut compared with children from Tasiilaq even when adjusting for day-care attendance. Having received ≥1 dose of PCV-13 was not associated with an increase

in overall carriage on non-pneumococcal bacteria.

Bacterial interaction

The results of Co-colonization analyses are based on multivariable logistic regression adjusting for age, sex, ethnicity and PCV-13 status (i.e. having received ≥ 1 dose of PCV-13 or not). Co-colonization between any two or more of the studied bacteria appeared in 52% of children, primarily among young infants, and most frequently between *S. pneumoniae* and *M. catarrhalis* (33%). NTHi showed synergistic interactions with NVT pneumococci and *M. catarrhalis, respectively*, but a competitive interaction with *S. aureus* and the normal flora (Table 4). *M. catarrhalis* was positively associated with *S. pneumoniae*, particular vaccine-type pneumococci, and negatively associated with S. aureus and the normal flora. When restricting the pairwise interaction analysis to individuals with no concurrent colonization by any other of the studied bacteria none of the estimates changed in direction, but some interactions were less pronounced. The association between NTHi and *M. catarrhalis*, however, remained synergistic (OR 4.0, 95% CI 1.3-11.7) and the competitive association between NVT pneumococci and the normal flora was robust (OR 0.1, 95% CI 0.01-0.9).

DISCUSSION

Overall we found nasopharyngeal bacterial colonization in Greenlandic children to occur very early in life, with frequent bacterial co-colonization and continuing high carriage rates in older children. Furthermore we found crowding-related risk factors for bacterial carriage and indications of clinically important associations between the normal flora and four key pathogens frequently related to respiratory and invasive infections in young children.

Pneumococcal carriage was acquired within the first two weeks of living and followed by an increasing prevalence during the next two years until reaching a peak-carriage rate of almost 60% in two year-olds. This is comparable to Navajo and White Mountain Apache (63% in children < 6 years)[30] and Danish

children (53% aged 12 to 72 months)[31] but less than Australian aboriginals (67% children aged 2-15 years) and Gambian infants (78% aged 0 to 12 months). In a systematic review of nasopharyngeal carriage in children, Adegbola et al. found that pneumococcal carriage rates in children from low and lower-middle income countries generally were higher (up to 93%) as compared to middle- and high income countries were highest reported carriage-rates were 58%[32]. High carriage rates of pneumococci have been associated with a high prevalence of respiratory infections [12] . Greenland has one of the highest incidences of respiratory tract infections [33]. Yet, unlike most other high risk populations with very high rates of bacterial carriage [34–36], this population-based study shows carriage-rates comparable to those of pediatric populations from Europe and the USA with much lower incidences of respiratory tract infections [12,31]. In other words, the carriage rate in itself, although in the upper end, does not seem to be the most significant feature for the high disease burden among Greenlandic children. In contrast, we observed that carriage in our population occurred very early, already at two weeks of age, as compared with a mean age of six months for first acquisition in pediatric populations from Europe and the US [10]. Furthermore, we saw a high rate of co-colonization in our population, which is likely to have implications for the polymicrobial respiratory infections. These findings correspond to findings in other high-risk groups, although relative few studies have described the combined carriage of respiratory pathogens. In a carriage study among Australian aboriginals aged 0 to 2 years, who share the same very high disease-burden from otitis media as the Greenlanders, they found higher carriage rates of respiratory pathogens in aboriginal children compared with non-Aboriginals. Furthermore carriage by multiple pathogens was observed more frequently in Aboriginal children and colonization began at an earlier age. Overall carriage rates of NTHi and *M.catarrhalis* were 50% and 41% respectively[34]. In comparison we found overall rates of 48% (NTHi) and 71% (*M.catarrhalis)* in children less than 2 years. Moreover, overall carriage rates, which after peak incidence among 2 year-olds often decrease gradually in low-risk populations [11], were in our study found only to be moderately reduced with age, indicating that the relatively high carriage rates in the youngest children remain in later childhood.

This pattern of early age at first acquisition and ongoing polymicrobial infection pressure through infancy and childhood may facilitate a carriage-state characterized by persistent inflammation and mucosal damage as part of the explanation of the high disease burden in this Inuit population. Furthermore, other unknown factors increasing infectious pressure including environmental and bacterial risk factors may play a role. Whether the Inuit have particular immune deficiencies associated with impaired clearance or poor immune response mechanisms after the bacterial invasion of the nasopharyngeal mucosa is unknown, but previous studies of mannose-binding-lectin insufficiency or cytokine responses in relation to respiratory infections among Inuit do not indicate an impaired immune reactivity to infections [37,38].

To our knowledge previous studies on bacterial nasopharyngeal carriage among the Inuit population, primarily Alaskan Natives, have almost exclusively focused on pneumococcal carriage without including other potential pathogenic bacteria [39,40]. Only a single Greenlandic study from 1993 has described nasopharyngeal bacterial and viral co-carriage among young children with acute otitis media (AOM) and healthy controls. However in that study, few risk factors for carriage of specific bacteria were identified, except for young age and current AOM that were associated with increased *S. pneumoniae* carriage-rates [41].

We consistently found that young age increased the odds of bacterial carriage regardless of the studied species. This association has partly been attributed to close contacts and high transmission rates between young children combined with poorly developed immunity to these agents. In line with other studies[42] breastfeeding did not seem to protect against bacterial carriage. Breastfeeding has, however, been shown to lower the risk of otitis media and invasive pneumococcal disease [12,43]. This could indicate, that higher levels of serotype specific serum IgG than what is achievable from breastfeeding may be required to prevent or clear colonization. As suspected, crowding-related factors increased the risk of carriage. Attending a day-care institution is quite common in Greenland with 73% of the children in this study doing so. Furthermore, the average number of persons per household is higher in Greenland (3.4 in towns and

4.4 in settlements) as compared to e.g. Denmark (overall 2.1) [44,45]. Ethnicity in itself did, however, not show any association with carriage indicating that environmental factors, rather than genetics play the most critical role for the risk of bacterial carriage in this population.

The normal flora of the nasopharynx is generally considered a beneficial environment for the host, stimulating and maturing the host-immune system and protecting it from pathogen invasion by so-called 'colonization resistance' [46]. The flora is gradually established during the first year of life and bacterial antagonism is known to be of importance in maintaining a balance between components of the normal flora and transient invaders [12]. In Greenlandic children, first acquisition of the transient invaders seems to be very early in life, occurring few weeks after birth. We found that our definition of normal flora followed an inverse carriage pattern compared with the pathogenic bacteria, more specifically NTHi*, S. pneumoniae* and *M. catarrhalis*, whereas *S. aureus*, which resides in the anterior part of the nares did not affect the normal flora (Table 4). It is possible that the so-called 'colonization resistance' of the flora during the first weeks of life, may be more susceptible to disturbances from transient invaders, and thus affect the balance during the establishment of the flora towards a state dominated by potential pathogens. Early acquisition of NTHi has been associated with increased risk of recurrent otitis media, and thus time of first acquisition rather than the carriage-rate as such, may be the determining risk factor for respiratory infections.

Odds for NTHi carriage were substantially increased if NVT pneumococci or *M. catarrhalis* were also detected. Diverging results have been reported on co-colonization with these pathogens. [47]. Our findings are in line with Pettigrew et al., although these findings were among children with current upper respiratory infections [48], who found NTHi to be competitively associated with *S. pneumoniae* and *M. catarrhalis* respectively. Xu et al., who studied both healthy and symptomatic children at onset of an upper respiratory infection, found similar results and noted that bacterial interactions differed among healthy and symptomatic children. This may indicate that the inter-bacterial dynamics is influenced by infections [49].

The synergistic association between NTHi and NVT-pneumococci, but not VT-pneumococci, observed in our study may indicate that this population of children although asymptomatic, may have a more or less constant pre-infectious nasopharyngeal carriage pattern. It may also point towards a serotype-specific competitive effect between NTHi and pneumococcal serotypes. Spijkerman et al. [18] found persistently higher prevalence rates of NTHi carriage and pneumococcal non-PCV-7 types among young asymptomatic children 3 and 4½ years after PCV-7 vaccination as well as an almost complete eradication of PCV-7 type pneumococci compared to pre-PCV-7 vaccination period. The Finnish Otitis Media trials found increases in the proportion of otitis media caused by NTHi following PCV-7 vaccination as compared to the Pre-PCV-7 era [50]. This raises the question if increased NTHi-carriage rates can be expected after widespread use of PCV-13 in Greenland due to the inter-bacterial associations and the anticipated serotype shift from VT to NVT colonizing pneumococci. Theoretically, that may put PCV-13 immunized children at a higher risk of otitis media caused by NTHi. Among the identified NTHi isolates in our study 36% were β-lactamase producing in contrast to 4% in a similar pediatric population in Greenland twenty years ago [41]. NTHi is primarily involved in otitis media infections which in Greenland are most frequently treated with agents such as ampicillin and amoxicillin [51]. However, β-lactamase-production causes antibiotic resistance against these agents. A gradual eradication of susceptible strains may explain the increasing carriage rates and spread of the resistant strains to ampicillin and amoxicillin in Greenland, which is worrisome since this may lead to a higher degree of treatment failure [52].

This study has some limitations. Since we only recruited children from two areas of Greenland we cannot be certain that they are representative of the whole country. Also, the study was conducted within the first year after the introduction of the PCV-13 and thus the observed carriage rates may to some extent be confounded by this vaccine introduction. However, this effect is likely to be limited for various reasons. First, only 36% of the children had received one or more PCV-13 vaccinations, second only 8% of the children were fully vaccinated with three doses, and finally vaccination only occurred in the youngest children. The inter-bacterial associations we have observed may be temporary during the PCV-13

introduction. Whether these associations persist may be clarified by a follow-up study that is at present being carried out in the same study area. Finally, the association models between two species may be simplified and in fact not reflecting the complexity of events occurring within the nasopharyngeal flora, and thus only revealed in studies using more complex methods, such as microbiome studies.

In conclusion, we found nasopharyngeal colonization occurring very early in life in healthy children in Greenland, frequent bacterial co-colonization, and ongoing high carriage rates of four clinically relevant bacteria. Carriage seems primarily to be influenced by environmental factors such as crowding, whereas ethnicity as an expression of genetic factors did not show any association with carriage. Our results indicate that colonization does not occur at random but that there are important inter-bacterial associations. The balance of these interactions may be skewed by the PCV-13 vaccine, and introduction of this vaccine may subsequently affect both carriage of respiratory pathogens and respiratory infections in the future in Greenland. For these reasons we recommend further surveillance of the nasopharyngeal bacterial carriage in Greenland to monitor the impact of the PCV-13 after wide-spread use.

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Figure 1a. Nasopharyngeal bacterial carriage in healthy Greenlandic children less than one year of age

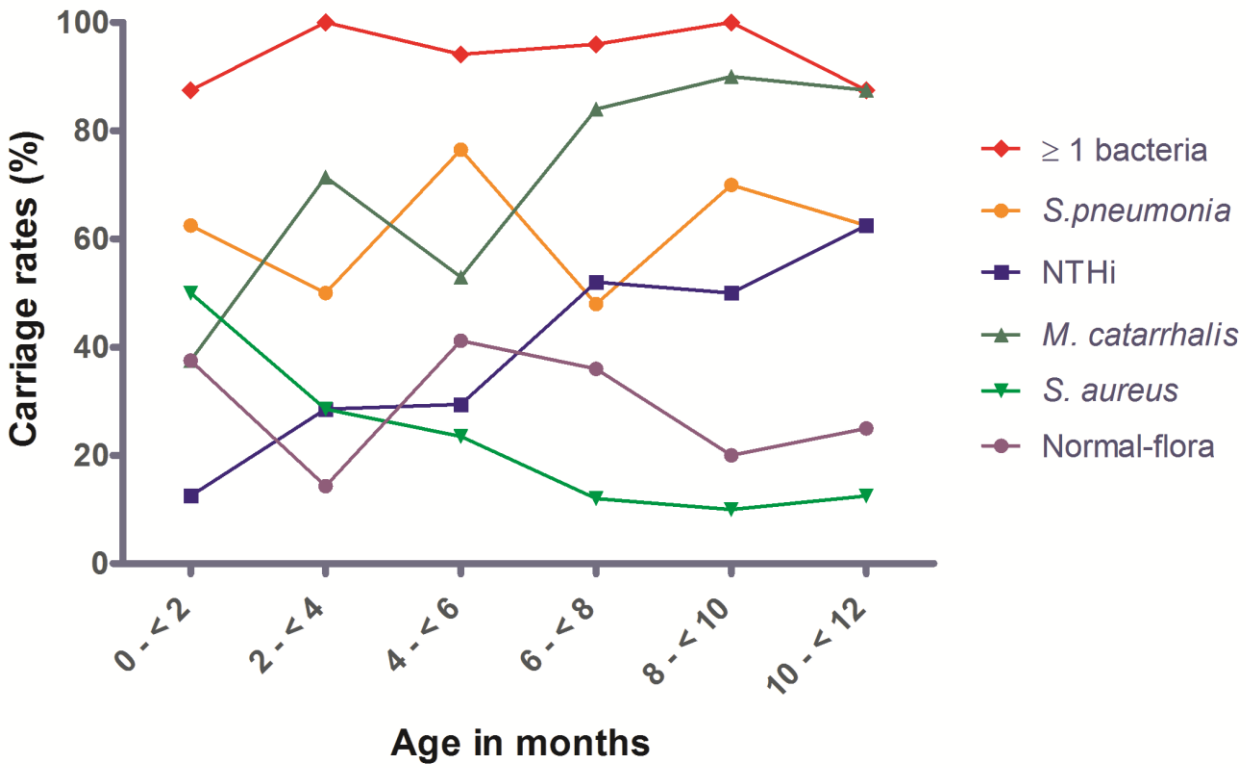

Figure 1b. Nasopharyngeal bacterial carriage in healthy Greenlandic children aged o – 6 years.

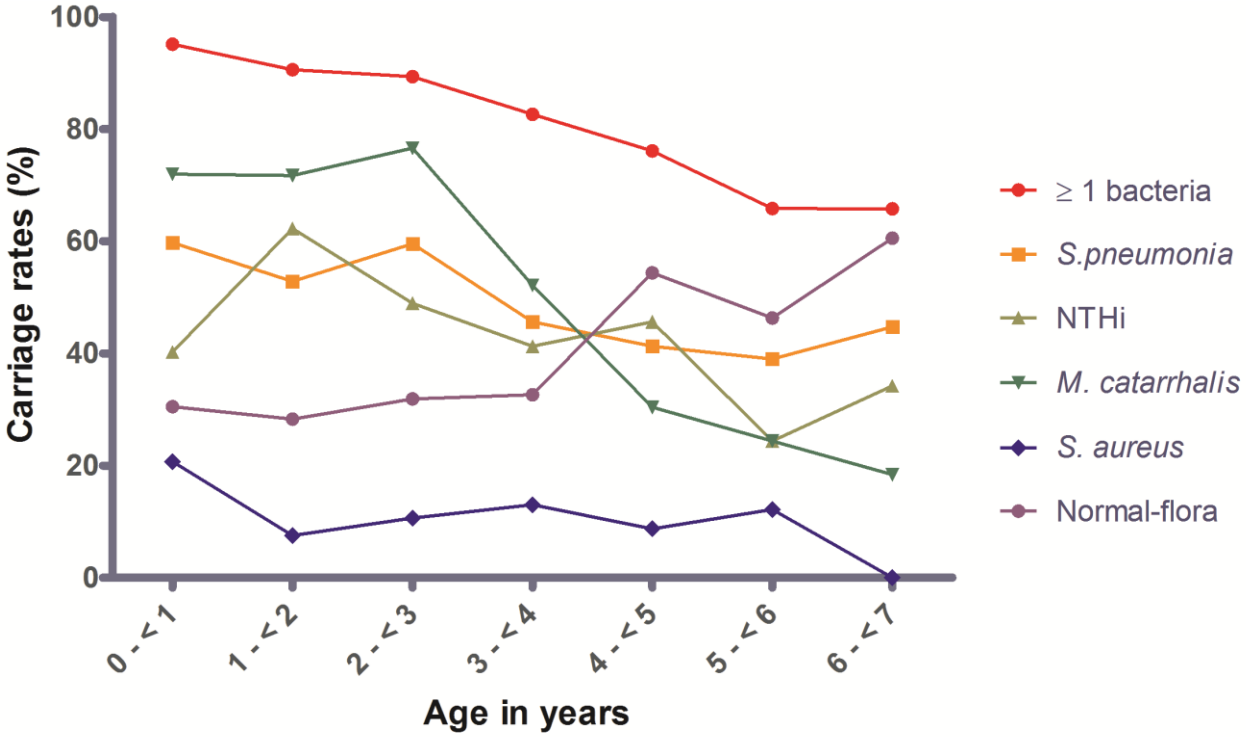

Table 1. Demographic characteristics of 352 healthy Greenlandic children aged 0 - 6 years, 2011, Tasiilaq and Sisimiut, Greenland

Category	Total n = 352 (%)
Age (years)	
0 < 2	134 (38)
2 < 4	93 (26)
4 < 7	125 (36)
Sex	
Female	169 (48)
Male	181 (52)
Ethnicity	
Inuit	324 (92)
Mixed	11 (3)
Other	17 (5)
PCV-13 vaccinated	
Yes	130 (37)
No	222 (63)
Geographic region in Greenland	
East-coast	122 (35)
West-coast	230 (65)
Day-care center attendance	
Yes	258 (73)
No	84 (24)
Missing	10 (3)
Current breast-feeding	
Yes	131 (37)
No	210 (60)
Missing	11 (3)
Ever breast-fed	
Yes	220 (63)
No	98 (28)
Missing	34 (9)
Having siblings attending a day-care center	
Yes	137 (39)
No	201 (57)
The number of persons per room in household	
0 < 2	251 (71)
2+	79 (22)
Missing	22 (7)
Exposure to tobacco-smoke	
Yes	83 (24)
No	269 (76)

a) PCV-13: Having received one or more doses of the 13-valent pneumococcal conjugate vaccine or not.
b) Geographical site: East-Greenland (Tasiilaq) and West-Greenland (Sisimiut).
c) Day-care attendance: Current attendance at a day-care center. Missing 10 (3%).
d) Sibling in day-care: Having siblings attending a day-care institution. Missing 14 (4%).
e) Person per room: Number of persons per household divided by number of rooms exclusive of kitchen, entrance-hall and bathroom.
f) Tobacco exposure: When one or both parents/caretakers smoke. Missing 6 (2%).

Table 2:

Overall nasopharyngeal carriage rates among 352 healthy Greenlandic children aged 0 - 6 years. Results listed according to positive culture from either the original nasopharyngeal samples, the serum-broth enriched samples, positive in samples either with or without serum-broth enrichment and finally positive in both types of samples.

	S.pneumoniae	**NTHi[a]**	***M.catarrhalis***	***S. aureus***	**Normal-flora[b]**	**Others[c]**	**Sterile**
Type of swab-sample	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Original	137 (39)	108 (30)	177 (50)	22 (6.8)	103 (29)	6 (1.5)	27 (7.5)
Serum-broth enriched	175 (50)	141 (40)	88 (25)	39 (11)	90 (25)	11 (3)	8 (2)
Original or serum-broth	178 (50)	152 (43)	188 (53)	41 (11.6)	137 (39)	14 (4)	32 (9)
Original and serum-broth	136 (38)	97 (27)	77 (22)	20 (5.6)	56 (16)	3 (1)	3 (1)

a) NTHi: non-typeable Haemophilus influenzae
b) N-flora: Normal flora, primarily Moraxella species in particular *nonliquefaciens*, but also coagulase negative staphylococci and non-hemolytic streptococci.
c) Others: Include Haemophilus influenzae type B, E and F, n=5 (1%) and Hemolytic streptococci group A, B and G, n=9 (2.5 %).

Table 3

Risk factors for carriage of *S. pneumoniae,* non-typeable Haemophilus influenzae*, M. catarrhalis, S. aureus,* ≥1 of either bacteria or normal flora among Greenlandic children aged 0 to <7 years in Tasiilaq and Sisimiut 2011, adjusted for age, sex, ethnicity and PCV-13 status.

Variable	N (%)	*S. pneumoniae*		NTHi		*M.catarrhalis*		*S. aureus*		≥ 1 bacteria		Normal flora	
		OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Age (years)	352 (100)	**0.8 (0.69-0.9)**	<0.01	**0.83 (0.7-0.9)**	<0.01	**0.65 (0.5-0.7)**	<0.01	**0.7 (0.6-0.9)**	0.04	**0.6 (0.5-0.7)**	<0.01	1.2 (0.9-1.3)	0.06
Sex													
Female	169 (49)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Male	183 (51)	1.3 (0.8-2.0)	0.26	1.0 (0.6-1.7)	0.78	1.5 (0.9-2.4)	0.06	0.9 (0.2-5.2)	0.97	1.4 (0.9-2.4)	0.14	0.9 (0.5-1.4)	0.51
Ethnicicty													
Inuit	324 (92)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Mixed/other	28 (8)	0.7 (0.3-1.5)	0.32	1.6 (0.7-3.5)	0.29	0.5 (0.2-1.2)	0.12	1.1 (0.2-5.2)	0.88	0.6 (0.2-1.5)	0.30	1.5 (0.7-3.5)	0.31
PCV-13[a]													
No doses	222 (63)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
≥ 1 dose	130 (37)	0.5 (0.3-1.1)	0.07	1.0 (0.6-2.0)	0.77	1.1 (0.6-2.1)	0.73	**0.2 (0.1-0.7)**	0.01	0.7 (0.3-1.5)	0.37	0.7 (0.3-1.3)	0.22
Region[b]													
West	230 (65)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
East	122 (35)	0.9 (0.6-1.6)	0.94	**0.4 (0.2-0.7)**	<0.01	**0.4 (0.3-0.8)**	<0.01	1.1 (0.4-3.1)	0.83	**0.6 (0.3-0.9)**	0.04	0.6 (0.3-1.1)	0.07
Day-care[c]													
No	84 (24)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Yes	258 (73)	1.0 (0.5-1.9)	0.96	**4.7 (2.3-9.6)**	<0.01	**2.4 (1.3-4.7)**	0.02	0.4 (0.1-1.6)	0.50	1.5 (0.7-3.1)	0.31	0.9 (0.5-1.9)	0.84
Siblings in DC[d]													
No	201 (57)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Yes	137 (39)	**1.6 (1.1-2.2)**	0.04	**1.7 (1.1-2.4)**	0.04	1.1 (0.7-1.9)	0.68	0.6 (0.2-1.6)	0.28	1.1 (0.6-1.9)	0.62	0.7 (0.4-1.1)	0.15
Persons/room[e]													
< 2	251 (72)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
2+	79 (28)	**1.7 (1.0-2.9)**	0.05	1.2 (0.7-2.1)	0.55	0.9 (0.5-1.7)	0.95	0.6 (0.2-2.3)	0.49	0.9 (0.5-1.8)	0.95	1.3 (0.7-2.3)	0.36
Tobacco exposure[f]													
No	83 (24)	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Yes	269 (76)	1.3 (0.7-2.2)	0.39	1.6 (0.8-2.9)	0.15	**1.9 (1.1-3.5)**	0.03	**0.3 (0.1-0.8)**	0.01	1.1 (0.6-2.0)	0.79	1.6 (0.8-3.0)	0.34

Abbreviations: OR (Odds ratios); NTHi (non-typeable Haemophilus influenzae); PCV-13 status (The 13-valent pneumococcal conjugate vaccine) ; DC (Child day-care institution); CI (Confidence interval)

a) PCV-13: having received one or more doses of the 13-valent pneumococcal conjugate vaccine or not.
b) Region: East-Greenland (Tasiilaq) and West-Greenland (Sisimiut).
c) DC-attendance: current attendance at a day-care center. Missing 10 (3%)
d) Sibling in DC, Having siblings attending a day-care institution Missing 14 (4%)
e) Person pr. room: number of persons per household divided by number of room exclusive kitchen, entrance-hall and bath. Missing 22 (7%)
f) Tobacco exposure: One or both parents/caretakers smoke. Missing 6 (2%)
Significant findings ($p < 0.05$) in bold.

Table 4

Odds of co-colonization between pneumococcal vaccine (VT) or non-vaccine (NVT) serotypes, *non-typeable H. influenzae (NTHi)*, *M. catarrhalis* and *S. aureus* among 352 Greenlandic children aged less than 7 years. Analyses are based on isolates from original swab-samples without serum-broth enrichment and with no restrictions regarding other potential co-colonizing bacteria. OR are adjusted for age, sex, ethnicity and PCV-13 status.

Bacterium	VT[b]			NTHi			*M. catarrhalis*[d]			*S. aureus*[e]			Normal flora[f]		
	n	aOR	p	n	aOR	p	n	aOR	p	n	aOR	p	n	aOR	p
NVT[a]															
No	113	1 (ref)		34	1 (ref)		85	1 (ref)		105			105	1 (ref)	
Yes	4	**0.15 (0.1-0.3)**	<0.01	41	**2.3 (1.4-3.7)**	<0.01	32	1.6 (0.95-2.5)	0.08	12	0.75 (0.35-1.4)	0.44	12	**0.2 (0.1-0.8)**	0.03
VT[b]															
Yes				42	1 (ref)		42	1 (ref)		50	1 (ref)		55	1 (ref)	
No				18	0.7 (0.4-1.5)	0.40	18	**3.4 (1.7-6.5)**	<0.01	10	1.2 (0.4-3.9)	0.68	5	0.7 (0.3-1.3)	0.22
NTHi[c]															
No							46	1 (ref)		46	1 (ref)		108	1 (ref)	
Yes							37	**4.5 (2.6-7.8)**	<0.01	37	**0.2 (0.1-0.9)**	0.04	34	**0.3 (0.2-0.6)**	<0.01
***M.catarrhalis*[d]**															
No										64	1 (ref)		76	1 (ref)	
Yes										6	**0.1 (0.02-0.4)**	<0.01	15	**0.4 (0.2-0.6)**	<0.01
***S. aureus*[e]**															
No													39	1 (ref)	
Yes													2	0.6 (0.2-1.8)	0.38

Abbreviations: aOR (adjusted Odds Ratios); PCV-13 (the 13-valent pneumococcal conjugate vaccine); p: p-value

a. NVT: pneumococcal serotypes not included in the 13-valent pneumococcal conjugate vaccine
b. VT: pneumococcal serotypes included in the 13-valent pneumococcal conjugate vaccine
c. NTHi: non-typeable Haemophilus influenzae
d. M.catarrhalis: *Moraxella catarrhalis*
e. S.aureus: *Staphylococcus aureus*
f. Normal flora: A group of commensal bacteria, primarily Moraxella species (*non liquefaciens*), non-hemolytic streptococci and coagulase negative staphylococci.

Significant findings in bold ($p < 0.05$)

POPULATION-BASED STUDY OF INCIDENCE, RISK FACTORS AND MORTALITY FOR INVASIVE PNEUMOCOCCAL DISEASE IN GREENLAND

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Background

The Inuit population of the Arctic suffers from high rates of invasive pneumococcal disease (IPD). However, data is scarce on risk factors and mortality of IPD in this high-risk population. The aim of the present study was to estimate incidence rates (IR), risk factors, and mortality from IPD among Greenlanders over a 40-year period.

Methods

A matched, case-control study was nested in the Greenlandic population during 1973 - 2013. Cases were identified from the Danish pneumococcus database at Statens Serum Institut and matched 1:10 to controls by age and ethnicity. Statistical analyses included socio-economic status, comorbidity, perinatal- and demographic characteristics using conditional logistic- and Cox-regression.

Results

A total of 230 IPD-cases were identified. Overall IR were 22.6/100.000 person-years (PY), highest among infants < 2 year (59.0/100.000 PY) and among adults aged 50 to <60 years (51.7/100.000 PY). Risk factors among infants included previous infections (aRR 4.86; 95% CI 1.03-23.05) and neurologic conditions (aRR 15.82 95% CI 1.46-170.5). Among adults significant risk factors were; ethnicity (non-Inuit aRR 0.22; 95% CI 0.09-0.53 compared to Inuit), being male (aRR 1.41; 95% CI 0.99-2.00), region (aRR 0.23; 95% CI 0.16-0.33 living in districts vs Nuuk), living alone (aRR 1.71; 95% CI 1.16-2.51), having cancer (aRR 5.06; 95% CI 2.06-12.43) and glaucoma or iridocyclitis (aRR 3.46; 95% CI 1.26-9.50). Overall 30-day mortality from IPD-admission was relatively high (25.2 per cent; 95% CI 19.4-30.6), among children <2 years (16.1 per cent; 95% CI 2.6-27.7) and adults aged 50 to <60 years (34.8 per cent 95% CI 22.9-44.9); among patients living in the southern district (40.5 per cent 95% CI 21.0-55.5); among patients with meningitis (28.6 per cent 95% CI 17.5-38.1) and in patients with comorbidities.

Conclusion

Overall IR of IPD in Greenland was comparable to other Arctic countries in the pre-vaccine era; however, the estimates are likely conservative due to under-diagnosing in the districts. Age-specific IR differed from low-risk population since increasing rates were observed already from age 50 years. The risk factor pattern mainly reflects Inuit ethnicity and comorbidity. IPD-mortality among infants and middle-aged is higher in Greenland than other arctic countries.

Introduction

Streptococcus pneumoniae (pneumococcus) remains the leading cause of vaccine-preventable deaths globally [1], with estimated 14.5 million cases of severe disease and more than 800.000 deaths annually among children less than 5 years of age [2]. Extensive geographical differences exist with regards to incidence rate (IR) of invasive pneumococcal disease (IPD) with the highest disease burden primarily in low-income countries and among certain high-risk ethnic groups [3].
Pneumococcal diseases originate from nasopharyngeal carriage of pneumococci [4]. Carriage in itself is asymptomatic but it may lead to respiratory infections such as otitis media and pneumonia and occasionally it may invade otherwise sterile body sites such as the blood and cerebrospinal fluid to cause IPD [5].

The Inuits in Arctic Alaska, Canada and Greenland share genetic and environmental characteristics such as living conditions and socio-economic challenges [6]. These populations also suffer from high incidences of IPD. In 1986-1990 the Inuits of Alaska had some of the highest reported IR of IPD [7] which led to the formation of circumpolar surveillance of IPD, confirming high rates of IPD in other Inuit populations. Reasons for the high IPD incidence among Inuit populations are mostly unknown, as only few and primarily descriptive studies have been published [6,8–10]. A retrospective small study of IPD in Greenland, found that 40% had pre-existing comorbidity, 33% were unemployed and 33 % suffered from alcohol abuse. However, risk factors among children were not assessed and there was no control group [9]. In an Alaskan study with no control group, cigarette smoking, alcohol abuse, chronic lung diseases, diabetes, immunosuppressive therapy, injection drug use and asplenia were found as risk factors among IPD-cases [11]. A high nasopharyngeal carriage rate has been associated with relatively higher risk of IPD [12]. However, we have recently shown that pneumococcal carriage-rates among Greenlandic Inuit children are comparable with pediatric populations at low risk of IPD (Navne et al, unpublished data).
Currently well-established risk factors for IPD include age (infants and elderly) [8,13], ethnicity (Aboriginals of Australia, Bedouin of Israel, Maoris of New Zealand and Native Americans including Inuit) [7,14–16], HIV infection and other chronic medical conditions, alcohol- and tobacco use [13,17], socioeconomic factors [18] crowding related factors [8] and respiratory viral infections such as influenza [19]. In children, low birth weight, exposure to other young children and chronic medical diseases are further known risk factors, whereas breastfeeding has shown to be protective [8,20,21]. To what extent these factors are responsible for the high incidence of IPD in Inuit populations is unknown.

Despite the availability of effective antibiotics and free medical health-care centers in every district of Greenland, IPD among Greenlandic Inuit may be associated with markedly higher morbidity and mortality

as compared to non-Inuit with case-fatality rates up to 33% as demonstrated by a previous minor study of IPD in Greenland [6,9,10]. In addition, mortality among Greenlandic Inuits seems to be higher than among Alaskan and Canadian Inuits [11].

The purpose of this study was to describe the risk factor pattern and estimate 30-day mortality from IPD in Greenland using nationwide registers with detailed information on clinical samples, comorbidity, perinatal factors, socio-economic characteristics and demographic data over the 40-year period 1973 – 2013.

Methods

We conducted a nation-wide matched, case-control study nested in the Greenlandic population. The Greenlandic population numbered 48,581 individuals in 1973 and 56,370 in 2013, about 90% of Inuit origin [22]. Information on IPD-cases, exposures and confounder variables on an individual level, were obtained from national registries in Greenland and Denmark using a unique 10-digit personal identification number (central person-register number or 'CPR-number') assigned to all citizens in Greenland since 1972 [23]. The study-population was defined as individuals with residence in Greenland at the time of sampling of control subjects and IPD-cases respectively, during the study-period 1973-2013. For risk factor analyses we restricted the cohort to only include individuals with a minimum of three years of residence in Greenland prior to IPD-admission in order to minimize bias by misclassification in registries among those individuals with recent immigration to Greenland. The term 'towns' was used to describe the major cities were the health-care centers are placed, whereas the smaller settlements which are staffed by a nurse or healthcare assistant were defined as 'settlements'. The term 'districts', refers to all the towns and settlements as a group excluding the capital Nuuk. Definitions of ethnicity were based on parental place of birth. If both parents were born in Greenland, ethnicity was defined as Inuit; one parent born in Greenland was defined as a 'mixed Inuit' ethnicity, and both parents born outside Greenland or of unknown origin were defined as 'other'. These were mainly of Caucasian ethnicity (Scandinavians). A case of invasive pneumococcal disease (IPD) was defined as a positive culture of *S. pneumoniae,* or verified pneumococcal antigen from a normally sterile clinical sample site such as blood, cerebrospinal- or peritoneal fluid. If cerebrospinal fluid isolates were present the case was defined as meningitis. IPD-cases where retrieved from registries at the microbiological laboratories at Dronning Ingrids Hospital in Nuuk, Greenland, and Statens Serum Institut, Copenhagen, and at the Public Health Medical Officer of Greenland given IPD being a reportable disease.

Registries

The Civil Registration System (CRS)

The CRS is updated on a daily basis and contains vital information on place and date of birth, gender, birth order, siblings, parents, current and previous addresses [23]. The unique CPR-number allows for accurate linkage between all national registers.

The Streptococcus pneumoniae registry of the Laboratory at Dronning Ingrids Hospital (DIH), Nuuk, Greenland

The 16 hospitals in the districts of Greenland outside the capital Nuuk, submit clinical samples for culture and microbiological analyses to DIH, in Nuuk, the only microbiological laboratory in Greenland. Pneumococcal isolates are subsequently submitted to the pneumococcus reference laboratory at Statens Serum Institut (SSI), Copenhagen Denmark, for serotyping and for surveillance purposes. All positive isolates registered at the laboratory at DIH since 1990 were identified and validated against the Danish pneumococcus database at SSI.

The Danish pneumococcus database

This unique registry has previously been described in details [24,25]. It is located at the national Neisseria and Streptococcus Reference (NSR) laboratory at Statens Serum Institut, Copenhagen, Denmark and contains nationwide detailed laboratory data from IPD cases in Denmark and Greenland including cases since 1938.

The Public Health Medical Officer of Greenland

IPD is a mandatory notifiable disease in Greenland reported to The National Board of Health. From this register additional IPD-cases were identified.

Selection of control subjects for risk factor analyses

For risk factor analyses we only included the period of 1990 to 2013, where data on co-variables from other registries were available. For each identified IPD-case we selected ten controls matched on age (individuals aged less than 3 years: +/- 15 days and individuals ≥ 3 years of age: +/- 6 months) and ethnicity by randomization from the CRS [23]. We did a second randomized register extraction matched on age but not ethnicity only to estimate the effect of ethnicity on IPD-risk. The risk-set sampling technique was used [27],

i.e. controls were selected from subjects at risk in the Greenlandic population at the time of disease-occurrence of the case. This gives the possibility of expressing the association between risk factors and IPD as rate ratios (RRs).

Risk factors

Data on possible risk factors were collected from national registries. Demographic data was obtained from the CRS from which we also created two crowding variables: 1) the number of household members at the time of IPD-diagnosis for the case/control person, and 2) number of children less than 6 years of age registered at the same address of the case or control, respectively, at time of IPD diagnosis. Information regarding hospitalizations, clinical manifestation, comorbidity and outcome of IPD was obtained from the Greenlandic National In-patient Register (GNIR).

The Greenlandic National In-patient Register

This register was established in 1987 and contains data on admittance, treatment- and discharge diagnosis of all patients in Greenlandic hospitals. Outpatients are not recorded as well as emergency room contacts not leading to admittance. Discharge codes are based on WHO International Classification System ICD-8 up to 1993 and ICD-10 from 1994.

Comorbidity was grouped by organ-systems for risk factor analysis. All ICD-codes within the last three years and up to one month prior to IPD admission was included in risk factor analysis for adults. As for infants we excluded comorbidity one month prior to IPD-diagnosis except the first week of living. For mortality analyses the Charlson comorbidity score-index was used [28] by means of ICD codes from all available discharge diagnosis registered in GNIR from 1990 and onwards. We defined three Charlson groups of comorbidity prior to IPD diagnosis, very low (score=0), low (score 1) and high (score ≥2). Since we did not have direct information on alcohol consumption, we created a new variable based on ICD-codes related to alcohol-consumption (See supplementary table 1). Information on socio-economic factors such as educational level, and household income was obtained from Statistics Greenland [22]. Information on perinatal factors for infant cases and controls was obtained from the Greenlandic Birth Registry with available data from 1990 and onwards.

Statistical analysis

Separate risk factor analyses for children less than 2 years of age and adults > 18 years of age were done using conditional logistic regression, estimated by odds ratios and expressed as rate ratios (RR) due to the study design. For infants RR was adjusted for sex, ethnicity and comorbidity and for adults for sex, ethnicity, family-size, region (Nuuk/districts) and comorbidity. Chosen co-variables were selected 'a priori' on the basis of existing literature and tested by separate inclusion in a univariable model, and only when significant ($p < 0.05$), included in the multivariable model.
Descriptive- and mortality analyses were based on the entire cohort of IPD-cases including the period 1973-2013. Mortality was considered related to IPD if the date of death occurred within 30 days after IPD-admission. Mortality analyses included Hazard-ratios calculated by Cox regression adjusting for age, sex, IPD-focus (blood and cerebrospinal fluid/meningitis), Charlson comorbidity-index score and calendar-period. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina)

Results

A total of 230 cases of IPD were identified during 1973-2013. The majorities of patients were Inuit (91.3%), were men (59.6%) lived in Nuuk (51.7%) and had a high Charlson comorbidity-score (46.8%) (Table 1). Short education (i.e. primary school) and low or very-low income dominated (Table 1). The overall age-standardized IPD-incidence-rates (IR) increased over time from 2.6/100,000 person-years (PY) during 1973 - 1980 to 22.6/100,000 PY during 2000 – 2010 (Figure 1). The age-specific IR were highest among children less than 2 years of age (59/100,000 PY) and adults aged 50-60 years (51.7/100,000 PY) and lowest among school-aged children (5/100,000 PY) during the period 2000 - 2010 (Figure 1). Cases were identified from almost all parts of the country with regional variations in IR, highest in Nuuk: IR 51.1 (95% CI 39.6-66.0) followed by Tasiilaq: IR 46.2 (95% CI 26.6-79.5) and lowest in Ilulissat IR 6.7 (95% CI 2.2-20.7). Within the districts, 80% of cases were from towns and 20% from rural settlements (data not shown). The frequency of IPD-cases varied across the seasons with bacteremia peaking during the winter whereas meningitis cases were evenly distribution across the season (Figure 2). A very low Charlson-score dominated among cases and none had a diagnosis of splenectomy or functional asplenia. Among children-cases almost 10% were born premature (< week 37) (Table 1).

Risk factors for IPD in children less than two years of age

We found no association between gender, ethnicity and the risk of IPD among children (Table 2). Crowding-related factors increased IPD-risk, however, results did not reach statistical significance. Infants with neonatal complications, including low gestational age (< 37 weeks), asphyxia and respiratory distress syndrome had an increased risk of IPD, although not significant in the adjusted analyses. Furthermore neurologic conditions and previous otitis media increased risk of IPD, but for otitis media the association was attenuated in adjusted analyses. The group of infections not related to the current IPD-episode remained associated with IPD regardless of adjustments.

Risk factors for IPD among adults > 18 years of age

Ethnicity was significantly associated with IPD, with Inuit having the highest risk (up to 4-times) and individuals with a mixed Inuit ethnicity twice the risk of IPD compared with non-Inuit (table 3). Moreover living in Nuuk increased the risk of IPD by 4-fold and being male was associated with an almost 40% increased risk of IPD (table 3). Surprisingly, living alone also increased the risk of IPD, whereas crowding-related factors such as family size or having young children (< 6 years) in the household did not. Neither socio-economic factors such as income nor educational level showed any significant association with IPD, except a trend of reduced risk with increasing income when income was included as a continuous variable in the analyses. A range of chronic underlying diseases were associated with increased risk of IPD (Table 3). In the adjusted analyses oncological, hematological and ophthalmological conditions remained significant risk factors for IPD (Table 3).

Mortality

From 1973-2013, 54 individuals died within 30 days after IPD-admission, yielding a total case-fatality risk of 23.5%. Males had higher 30-day mortality than females although not significant (table 4). The 30-day mortality-rate decreased over the study-period, with highest rates in 1990-<2000 and lowest rates occurring between 2010 and 2013. Overall, Inuit had higher mortality than individuals with mixed ethnicity and non-Inuit (Table 4). IPD-patients living in the districts had higher mortality –rate than Nuuk-patients, with a hazard rate (HR) of 2.34 (95% CI 1.07-5.13) for IPD-patients living in the southern region of Greenland compared with IPD-patients living in Nuuk (table 4 and figure 3). According to age groups, adults (aged 50-<65 years) had the highest mortality (almost 35%) whereas school-children and young adults had the lowest (9%). Mortality was twice as high among patients with Charlson-score ≥2 as with a score of 0. Moreover cases with meningitis suffered from twice the mortality-rate compared to cases with bacteremia.

When adjusting the analyses for Charlson score the increased mortality among Inuit-cases compared to individuals with a mixed ethnicity was attenuated and no longer significant (aHR 0.55; 95% CI 0.07-4.12) for cases with mixed ethnicity compared to Inuit origin. However, meningitis remained prognostic for a worse outcome of IPD compared to bacteremia cases (aHR 2.92; 95% CI 1.29-6.62). Also living in the districts, in particular South-Greenland were associated with increased mortality compared to Nuuk-citizens (aHR 2.07; 0.95-4.53).

Discussion

In this nation-wide case-control study of IPD study in a Greenlandic Inuit population covering a 40-year period, we found that the overall incidence of IPD increased almost ten-fold from the 1970s to the 2000s. The increase in incidence was based on increases in IR of both bacteremia and meningitis. This may though be biased by an increased attention on bacteremia combined with a lower threshold of performing microbiologic diagnostics, since a 4-fold increase in annual numbers of blood-cultures performed in Nuuk, occurred from 1990 to 2011 (figure 4). However, a true increase in IPD incidence may also have occurred since indications for performing lumbar puncture during the period is less likely to have changed, yet we did not have data on the annual number of cerebrospinal fluid analyzed. Overall the IPD incidence rates in Greenland are comparable to those of Denmark, Alaska and northern Canada prior to the introduction of conjugate pneumococcal vaccines (22.6 in Greenland versus 20, 20.6 and 31, all per 100.000 PY) [6,24]. The Greenland IR is most likely conservative, as the IR among persons from the capital Nuuk was markedly higher than among those living in the rural districts. This is likely due to the difference in access to microbiological service between Nuuk, where the only microbiological laboratory in Greenland is located, and the districts. The relatively low sensitivity of a positive blood-culture combined with long transportation time from the districts to Nuuk and subsequently long waiting time before receiving the results, makes blood-culturing less attractive for the clinicians in Greenland as a routine tool for decision making. An empiric treatment approach is thus often chosen, based on clinical data, ordinary biomarkers of infection and occasionally microscopy of cerebrospinal fluid. There was no difference in IPD risk among persons living in towns and rural settlements, and while this may be true it may also be that the expected underdiagnosing from the districts and even more from the settlements makes it difficult to estimate whether the risk of IPD depends on size of town/settlement of living.

As in other populations the highest IR was demonstrated among children < 2 years and among elderly, but the rate among adults and elderly increased already from age 30 years and peaked at ages 50 to 60 years. This is earlier than among Caucasian populations of most western countries where the increase occurs from age 40 and peaks among those aged > 65 years [29], but similar to some ethnic populations such as Native populations in Alaska and among African American in the US [7,30]. The reason for this relatively early age-

specific increased risk is likely multi-factorial but may include a higher level of comorbidity in this age-group as compared with adults of same age in western low-risk population as well as socio-economic challenges. We found that the group of adult IPD-patients aged 50 to 64 years had the highest proportion of high Charlson-scores ≥2 (27.8%), whereas among the elderly patients aged >65 years only 13.6% had a high Charlson score.

Risk factors

While obviously different for young children and adults due to the distribution of risk factors, main risk factors according to size of estimates were for both groups various comorbidities, and for children various birth-related conditions, although not all of neonatal factors were significant due to low sample size. Disease groups with increased risk of IPD among adults included oncological, hematological, infections not related to the IPD-episode, endocrinological, neurological and ophthalmologic conditions which for the majority are in line with many other studies [7,8,20,31,32]. Although epilepsy and seizure-disorders as such are not well-established risk factors for IPD, they have been described to increase risk of pneumococcal diseases in a cohort study based on three large health-care registers in the United States[33], possible due to increased risk of pneumonia due to aspiration. The relationship between glaucoma or iridocyclitis and IPD has not been described before. It is well established that the Inuit populations of the Arctic countries have 20 - 40 times the prevalence of primary angle closure glaucoma compared to the white population in the US [34], and so the results may partly be biased by the increased risk of IPD related to ethnicity, rather than the glaucoma in itself. Although the analyses were adjusted for ethnicity, some degree of residual confounding may exist. Since we only included the group of comorbidity necessitating hospitalizations, the patients with iridocyclitis may belong to those with severe manifestations requiring systemic treatment with steroids, which due to the immunosuppressing effects may contribute to an increased risk of IPD. However, our data could not distinguish between these effects. No IPD cases had been splenectomized, but splenectomy is generally a rare condition and in a small population like the Greenlandic such an association may not be revealed. Interestingly, compared with other specific comorbidities alcohol-related diseases were only marginally associated with IPD. A strong association between alcohol-intake and risk of IPD has been shown in other populations [32], but as we estimated alcohol intake indirectly through alcohol-related hospitalizations and not by the amount of alcohol consumed, the association may still exist in this population. Being of Inuit ethnicity increased the risk of IPD substantially compared with non-Inuit adults. The same trend was seen among children, although there were so few non-Inuit children with IPD in Greenland that the trend could not reach significance. The increased risk may rely both on genetic and

environmental factors, and while unadjusted confounding may explain a part, it was remarkable that the univariate estimates among adults did not change after adjustment for environmental factors and co-morbidity indicating a genetic component. Surprisingly, family size as a measure of crowding did not increase the risk of IPD, neither for children nor for adults. In contrast, living alone increased the risk for adults. A possible explanation for this finding could be that social factors related to living alone would increase the risk of IPD. However, the group of IPD-cases living alone was primarily adult women of mixed or non-Inuit origin, with a low level of comorbidities, very few records of alcohol-related hospitalizations and no records of lung diseases. In a population-based cohort study of almost 7000 men and women aged 45 to 74 years during a 10-year period, living alone was found to be an independent risk factor of overall mortality among men, but not women, despite adjustments for socio-demographic factors, comorbidities and health behavior factors such as smoking, alcohol consumption and physical activity [35]. They hypothesize that mental health or health-care seeking behavior may explain this association. [17]. Whether the increased IPD-risk related to living alone is caused by environmental exposures (work-related i.e. day-care institutions) or deprived mental health or other unmeasured confounders is speculative. However, our finding of a reduced risk of IPD with increasing income, although insignificant, supports that social factors influence the risk of IPD [36]. The effect, however, is not clear-cut as there was no association between educational status and risk of IPD. The study population is however, an elderly population born at a time in Greenland when longer educations were not readily available, so education may not be the best measure to use in an elderly Greenlandic population. Other studies have found risk of pneumococcal carriage and subsequently IPD to be associated with educational level, partly through deprived health-care seeking behavior [37,38], but our data indicate that this has no influence in Greenland where all health care is free. Unfortunately, we had only two measures of crowding (family size and number of young children in home), but no data on use of day-care centers, a well-known risk factor for respiratory tract infections and IPD [20,39]. We therefore cannot exclude the possibility of crowding as a risk factor, but so far this is not supported by our data.

Mortality

Overall we found age, comorbidity and clinical manifestation of IPD to affect the short-term mortality in Greenlanders. The overall 30-day mortality among Greenlanders (Inuit) following IPD-admission in Greenland was higher than Denmark (23.5% and 18% respectively) [24]. Furthermore among children less than 5 years, the mortality in Greenland was markedly higher 16% (95% CI 2.66-27.72) than among Danish children (3%)[24] the native populations of Alaska (1-2%)[7] and indigenous northern Canadian children (4%) [40]. In Alaska the highest mortality rate from IPD during 1986 to 1990 was observed among meningitis cases (18%), in contrast, Greenlandic meningitis-patients had a 30-day mortality of 28.5% (95%

CI 17.5-38.2) and Denmark (22%). Reasons for this obvious inequality in mortality across the region are unclear, but it is unlikely to be due to genetic susceptibility among Inuit since Greenlandic rates exceeds rates among the Inuit populations of Alaska and Canada. However, the threshold of performing blood-cultures and lumbar puncture may differ between the countries, with a relative high threshold in Greenland especially in the districts limiting the group of identified patients to only include the most severe cases and thus those with the poorest prognosis. In the US, practice guidelines for the management of infants and children with fever > 39 ˚C without a source includes blood-culturing [41], and thus milder IPD-cases are more likely to be identified. Moreover, the great distances and limited hospital facilities in the districts of Greenland appears to be of great importance for the prognosis, reflected by a 2-fold increased mortality among IPD-cases living in the southern district of Greenland as compared with Nuuk, whereas we found similar mortality-rates among Danish citizens (18%) and Nuuk-citizens (18%) where specialized hospital facilities are within close distance. The overall IPD-mortality in Greenland peaked during 1990 to 2000 and reached the lowest levels in 2010-2013, which probably is related to extra attention on the increased risk of pneumococcal diseases in Greenland and the establishment of national guidelines and international surveillance. This is of great importance, particularly in a country with relative frequent replacement of physicians particular in the districts since recruitment of health-care staff is a problem with numerous temporary employments.

Strengths and limitations

This study is, to our knowledge, the largest nation-wide study of IPD from an Inuit population covering a 40-year period using validated nationwide registers with detailed information on clinical samples, hospitalizations and demographic data. Thus, demographic and risk factor information of identified IPD cases and controls is almost complete and reliable with a fixed pre-IPD observational period for each person. There are a number of shortcomings, however. Firstly, notified cases of IPD in Greenland is most likely a conservative estimate given the logistic challenges in taking, using and interpreting blood cultures and other microbiological samples from district hospitals with a long distance to a microbiological laboratory. Also there may be bias in age, sex, and risk factor profile in IPD cases, as clearly less IPD cases are identified from coastal hospitals than cases living in Nuuk, and bias in taking of microbiological samples cannot be ruled out. Secondly, not all register information may be reliable such as e.g. information on income. Economy in Greenland, in particular in more remote towns and settlements, involves some element of non-official income such as that from hunting for one's own use, the degree of which may not

be reflected in official registers. Thirdly, we did not have access to direct information on important factors such as breast feeding, smoking and alcohol, as this is not contained in national registers. Instead, we had to rely on an indirect measure for alcohol use, namely alcohol associated co-morbidity, which may not be an accurate measure of alcohol use. Fourthly, only comorbidities resulting in hospitalizations were addressed, as out-patient contacts are so far not registered in nation-wide registers. This is important, as e.g. recurrent acute otitis media, which is highly prevalent in Greenland [42,43], may be a risk factor for IPD [44].

Conclusion

The overall IPD incidence rates in Greenland are comparable with other high-incidence countries in the Arctic in the pre-vaccine era, however, the incidence estimates are most likely conservative due to presumable limited microbiological diagnostics in the districts. The age-distribution of IPD is almost as in other countries except for increasing rates from age 35 years and peaking already from age 50 years, presumably due to a high degree of comorbidity in this age group. The pattern of risk factors is reflected by the degree of comorbidity, ethnicity and possibly socio-economic factors, whereas crowding is of less importance. Overall Inuit have a higher mortality from IPD than what is observed in other arctic countries and in Denmark. This is particularly noticeable among young children less than 2 years of age, and among middle-aged adults and those presenting with meningitis. A genetic importance of IPD risk among Inuit cannot be excluded; however, great differences in mortality rates were observed between the districts and Nuuk, indicating that the access to specialized hospital facilities within short distance is crucial for the prognosis of IPD.

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Table 1

Demographic and socio-economic characteristics of entire case-cohort (1973-2013) and of matched case-control cohort including Charlson comorbidity-score (1990-2013).

Demographic variables	All IPD-cases Period 1973 - 2013	Matched IPD-cases Period 1990 - 2013	Matched controls[a] Period 1990 - 2013
	n = 230 (%)	n = 201 (%)	n = 1952 (%)
Sex			
Female	93 (40.4)	83 (41.3)	910 (46.6)
Male	137 (59.6)	118 (58.7)	1042 (53.4)
Age in years median (Q1,Q3)[b]	44.8 (24.0-56.4)	47.2 (34.4-57.2)	47.6 (34.4-57.3)
Ethnicity[c]			
Greenlandic	210 (91.3)	185 (92)	1812 (92.8)
Mixed	11 (4.8)	10 (5)	87 (4.5)
Not Greenlandic	9 (3.9)	6 (3)	53 (2.7)
Region (Greenland only)			
Nuuk (capital)	119 (51.7)	106 (52.7)	427 (21.9)
Districts	111 (48.3)	95 (47.3)	1525 (78.1)
Settlements (incl. Nuuk)[d]			
No	201 (87.4)	176 (87.6)	1629 (83.5)
Yes	29 (12.6)	25 (12.4)	314 (16.1)
Family size (grouped)[e]			
Not alone	166 (72.2)	146 (72.6)	1543 (79.0)
Alone	57 (24.8)	50 (24.9)	334 (17.1)
Nr of children < 6 years of age[f]			
0	176 (76.5)	155 (77.1)	1464 (75.0)
1	33 (14.3)	30 (14.9)	312 (16.0)
2	13 (5.7)	10 (5.0)	88 (4.5)
3	1 (0.4)	1 (0.5)	12 (0.6)
4	0 (0)	0 (0)	1 (0.1)
Missing	7 (3.0)	5 (2.5)	75 (3.8)
Charlson-score[g] (grouped)			
Very low	58 (28.9)	156 (77.6)	1828 (93.6)
Low	49 (24.4)	19 (9.5)	88 (4.5)
High	94 (46.8)	26 (12.9)	36 (1.8)

Educational level[h]			
Short	93 (76)	93 (76)	953 (77)
Long	27 (22)	27 (22)	269 (22)
Missing	3 (2)	3 (2)	8 (1)
Household income[i]			
Very low	27 (25)	27 (25)	226 (21)
Low	39 (35)	39 (35)	354 (32)
Medium	20 (18)	20 (18)	214 (19)
High	24 (22)	24 (22)	292 (27)
Missing	0 (0)	0 (0)	14 (1)

Abbreviations: IPD: Invasive pneumococcal disease, Dkr: Danish kroner.

a. Control subjects matched on date of birth, and ethnicity
b. Q1: 25% percentile, Q3: 75% percentile
c. Control subjects matched on date of birth, but not ethnicity
d. Settlements: Nine controls could not be matched due to residence in research-stations or Sirius-patrol.
e. Family size: Registered as living alone or not in the Civil Registration System. Seven cases and 75 controls could not be categorized (residence in institutions or blocks)
f. Number of children less than 6 years of age living at the same address but not including the index person
g. Charlson comorbidity score: Including the age-component in left column and excluding the age-component in the case/controls since they are already matched on age. Very Low (score = 0), Low (score = 1), High (score = 2+)
h. Education analysis includes individuals > 15 years of age and only the period 2002 – 2013. The highest level of index person in the household is used.
i. Income analysis includes individuals > 15 years of age and only the period 2002 – 2013. The income-data at one year prior to IPD-diagnosis is used. Very low = < 75.000 Dkr./year, Low = 75.000 -<150.000 Dkr./year, Medium = 150.000 -< 225.000 Dkr./year and High = >225.000 Dkr./year

Table 2

Risk factors for Invasive Pneumococcal Disease among infants < 2 years of age in Greenland during 1990 – 2013.

Characteristics	Cases n = 16 (%)	RR (95% CI)	p	aRR (95% CI)	p
Sex					
Female	9 (56.3)	1 (ref)	0.53	1 (ref)	0.26
Male	7 (43.8)	0.72 (0.26-1.98)		0.49 (0.14-1.71)	
Ethnicity[a]					
Greenlandic	14 (87.5)	1 (ref)	1.0	1 (ref)	0.87
Mixed	2 (12.5)	1.01 (0.21-4.96)		1.56 (0.29-8.51)	
Not Greenlandic	0 (0)	<0.01 (<0.01->99.9)		-	
Birth order					
First child	7 (43.8)	1 (ref)	0.95		
Second child	6 (37.5)	1.14 (0.37-3.56)			
Third child	0 (0)	<0.01 (<0.01->99.9)			
Later child	3 (18.8)	0.72 (0.17-2.97)			
Region (Greenland only)					
Nuuk	6 (37.5)	1 (ref)	0.26		
Districts	10 (62.5)	0.53 (0.17-1.61)			
Settlements (exclusive Nuuk)					
No	9 (90)	1 (ref)	0.60		
Yes	1 (10)	0.56 (0.06-4.97)			
Family size (numbers)					
≤3 persons	2 (14.3)	1 (ref)	0.32		
4 to ≤ 6 persons	11 (78.6)	2.85 (0.61-13.3)			
7+ persons	1 (7.1)	1.18 (0.10-13.6)			
Children < 6 years at home[b]					
0	7 (50)	1 (ref)	0.90		
1	5 (35.7)	1.10 (0.33-3.65)			
2+	2 (14.3)	1.48 (0.29-7.63)			
COMORBIDITY[c]					
Pulmonal[d]					
No	14 (87.5)	1 (ref)	0.04	1 (ref)	0.22
Yes	2 (12.5)	**8.22 (1.13-60.1)**		5.58 (0.35-89.3)	

Infections[e]					
No	10 (62.5)	1 (ref)	0.002	1 (ref)	0.05
Yes	6 (37.5)	**7.88 (2.15-28.9)**		**4.86 (1.03-23.1)**	
Otitis					
No	14 (87.5)	1 (ref)	0.04	1 (ref)	0.87
Yes	2 (12.5)	**6.67 (1.11-39.9)**		1.23 (0.10-14.6)	
Neurological[f]					
No	13 (81.3)	1 (ref)	0.003	1 (ref)	0.02
Yes	3 (18.8)	**15.0 (2.51-89.7)**		**15.8 (1.47-171)**	
Neonatal conditions[g]					
No	13 (81.3)	1 (ref)	0.01	1 (ref)	0.31
Yes	3 (18.8)	**7.69 (1.51-39.2)**		3.00 (0.36-25.4)	
Apgar score (5 minutes)[h]					
0 – 7	3 (20)	1 (ref)	0.003		
8 – 10	12(80)	**30.0 (3.12->99.9)**			
BCG vaccinated					
Yes	7 (77.8)	1 (ref)	0.19		
No	2 (22.2)	3.43 (0.54-21.9)			
Gestational age (grouped)					
37+ weeks	12 (85.7)	1 (ref)	0.27		
19 - < 37 weeks	2 (14.3)	2.52 (0.49-12.8)			
Birth weight (grouped)					
2700+ grams	12 (85.7)	1 (ref)	0.47		
< 2700 grams	2 (14.3)	1.82 (0.37-9.02)			

Abbreviations: RR: Rate ratios, p: p-value, aRR: adjusted rate ratios, IPD: Invasive pneumococcal disease, BCG: Bacillus Calmette-Guerin vaccination.

a. Control subjects matched on age but not ethnicity
b. Number of children less than 6 years exclusive of the index person, living at the same address as the index person
c. Comorbidity, using data from the Greenlandic inpatient-register 1990 – 2013. Diseases are grouped by organ-system.
d. Pulmonal: Chronic obstructive lung disease, chronic bronchitis, asthma
e. Infections: laryngotracheobronchitis, bronchiolitis, influenzae and other respiratory tract infections. Exclusive otitis, tuberculosis and HIV.
f. Neurological: Unspecific seizure-disorder not related to fever and epilepsy.
g. Neonatal conditions: Respiratory stress syndrome, asphyxia, jaundice.
h. Apgar-score: A method to rapidly assess the health-condition of newborn children one- and five minutes after birth.

Table 3

Risk factors for Invasive Pneumococcal Disease among adults > 18 years of age in Greenland during 1990 – 2013.

Characteristics	Cases n= 168 (%)	RR (95% CI)	p	aRR (95% CI)	p
Sex					
Female	67 (39.9)	1 (ref)	0.10	1 (ref)	0.06
Male	101 (60.1)	1.32 (0.95-1.84)		1.41 (0.99-2.00)	
Ethnicity[a]					
Greenlandic	158 (94.0)	1 (ref)	0.005	1 (ref)	<0.001
Mixed	4 (2.4)	**0.56 (0.20-1.59)**		**0.39 (0.13-1.16)**	
Not Greenlandic	6 (3.6)	**0.27 (0.12-0.62)**		**0.23 (0.10-0.54)**	
Birth order					
First child	25 (35.7)	1 (ref)	0.26		
Second child	14 (20.0)	0.92 (0.46-1.87)			
Third child	11 (15.7)	0.83 (0.36-1.90)			
Later child	20 (28.6)	1.71 (0.86-3.40)			
Region (Greenland only)					
Nuuk	89 (53.0)	1 (ref)	<0.001	1 (ref)	<0.001
Districts	79 (47.0)	**0.25 (0.18-0.34)**		**0.23 (0.17-0.33)**	
Settlements (exclusive Nuuk)					
No	61 (77.2)	1 (ref)	0.28		
Yes	18 (22.8)	1.37 (0.78-2.41)			
Family size (numbers)					
≤3 persons	112 (67.5)	1 (ref)	0.86		
4 to ≤ 6 persons	48 (28.9)	1.09 (0.74-1.59)			
7+ persons	6 (3.6)	0.89 (0.37-2.13)			
Family size (grouped)					
Not alone	116 (69.9)	1 (ref)	0.01	1 (ref)	0.006
Alone (incl index pers)	50 (30.1)	**1.63 (1.13-2.36)**		**1.71 (1.16-2.52)**	
Children < 6 years at home[b]					
0	137 (82.5)	1 (ref)	0.80		
1	20 (12.0)	0.95 (0.57-1.60)			
2+	9 (5.4)	1.27 (0.59-2.73)			
Education[c]					
Short	93 (76.6)	1 (ref)	0.88		

Long	27 (22.0)	1.03 (0.64-1.67)			
Missing	3 (2.0)	-			
Income[d]					
Very low	27 (24.5)	1.32 (0.71-2.46)			
Low	39 (35.4)	1.22 (0.68-2.19)			
Medium	20 (18.2)	1 (ref.)	0.49		
High	24 (19.5)	0.85 (0.45-1.61)			
Missing	0 (0)	-			
Continuous index	-	0.86 (0.71-1.04)	0.12		
COMORBIDITY[e]					
Cardiac					
No	162 (96.4)	1 (ref)	0.38		
Yes	6 (3.6)	1.49 (0.61-3.65)			
Pulmonal[f]					
No	161 (95.8)	1 (ref)	0.04	1 (ref)	0.63
Yes	7 (4.2)	**2.38 (1.02-5.56)**		1.28 (0.47-3.49)	
Gastrointestinal[g]					
No	146 (86.9)	1 (ref)	0.01	1 (ref)	0.30
Yes	22 (13.1)	**1.90 (1.17-3.11)**		1.35 (0.76-2.4)	
Endocrinological[h]					
No	160 (95.2)	1 (ref)	<0.001	1 (ref)	0.07
Yes	8 (4.8)	**4.97 (2.07-11.9)**		2.71 (0.89-8.24)	
Neurological[i]					
No	154 (91.7)	1 (ref)	0.005	1 (ref)	0.32
Yes	14 (8.3)	**2.46 (1.32-4.59)**		1.49 (0.68-3.28)	
HIV					
No	167 (99.4)	1 (ref)	0.31		
Yes	1 (0.6)	3.24 (0.34-31)			
Oncological[j]					
No	155 (92.3)	1 (ref)	<0.001	1 (ref)	<0.001
Yes	13 (7.7)	**8.17 (3.81-17)**		**5.06 (2.06-12)**	
Hematological[k]					
No	164 (97.6)	1 (ref)	0.005	1 (ref)	0.06
Yes	4 (2.4)	**6.14 (1.72-22)**		4.90 (0.97-26)	
Infections[l]					

No	154 (91.7)	1 (ref)	0.001	1 (ref)	0.05
Yes	14 (8.3)	**2.79 (1.50-5.18)**		2.18 (0.99-4.83)	
Nephrological					
No	162 (96.4)	1 (ref)	0.34		
Yes	6 (3.6)	1.54 (0.64-3.72)			
Ophtalmological[m]					
No	162 (96.4)	1 (ref)	0.02	1 (ref)	0.02
Yes	6 (3.6)	**2.92 (1.15-7.41)**		**3.46 (1.26-9.50)**	
Vascular conditions					
No	165 (98.2)	1 (ref)	0.27		
Yes	3 (1.8)	2.03 (0.57-7.19)			
Alcohol-related[n]					
No	162 (96.4)	1 (ref)	0.10		
Yes	6 (3.6)	2.10 (0.87-5.10)			
Psychiatric					
No	157 (93.5)	1 (ref)	0.18		
Yes	11 (6.5)	1.57 (0.81-3.04)			
Trauma-related					
No	153 (91.1)	1 (ref)	0.33		
Yes	15 (8.9)	1.33 (0.75-2.34)			

Abbreviations: RR: Rate ratios, p: p-value, aRR: adjusted rate ratios, IPD: Invasive pneumococcal disease, HIV: Human immunodeficiency virus, dkr: Danish kroner,

a. Control subjects matched on age but not ethnicity
b. Number of children less than 6 years exclusive of the index person, living at the same address as the index person
c. Education analysis includes individuals > 15 years of age and the period 2002 – 2013. The total number of IPD-cases=123
d. Income analysis includes individuals > 15 years of age and the period 2003 – 2013. The income at the year prior to IPD-diagnosis is used. The total number of IPD-cases=110
   Very low = < 75.000 dkr./year, Low = 75.000 -<150.000 dkr./year, Medium = 150.000 -< 225.000 dkr./year and High = >225.000 dkr./year
e. Comorbidity, using data from the Greenlandic inpatient-register 1990 – 2013. Diseases are primarily grouped by organ-system.
f. Pulmonal: chronis obstructive lung disease, chronic bronchitis)
g. Gastrointestinal: Esophagitis, peptic ulcer, gastritis, pancreastitis)
h. Endocrinological: Diabetes
i. Neurological: epilepsy, stroke with long-term complications
j. Oncological: hematologic-, lung and gastrointestinal cancers
k. Hematological: anemia
l. Infections: primarily other bacterial pneumonia, influenza
m. Ophthalmologic: Glaucoma and iridocyclitis.
n. Alcohol-related diseases. Specific ICD-codes. 29109, 29129, 29199, 30309, 30319, 30320, 30328, 30329, 30390, 30399, 98099, DF10, DF100, DF1000, DF101, DF102, DF1024, DF1025, DF103, DF1030, DF104, DF105, DF106, DF108, DF109, DT51, DT510, DT519, DZ721 and DE512.

Table 4

The 30-day mortality and Hazard ratios after invasive pneumococcal disease admission, according to selected characteristics in Greenland during 1973-2013.

Factor	Cases (n)	Deaths (n)	Survivals (n)	Mortality[a] percent (95% CI)	HR[b] (95% CI)	p
Sex						
Female	93	18	75	19.3 (11.4-26.5)	1 (ref)	0.26
Male	137	36	101	26.3 (18.9-33.0)	1.4 (0.8-2.4)	
Ethnicity						
Inuit	210	53	157	25.2 (19.4-30.6)	1 (ref)	0.57
Mixed	11	1	10	9.1 (0-23.9)	0.3 (0.05-2.5)	
Other	9	0	9	0	-	
Age group (years)						
0 -< 5	31	5	26	16.1 (2.7-27.7)	0.7 (0.3-1.9)	0.06
5 -< 35	44	4	40	9.1 (0.5-17.0)	0.4 (0.1-1.2)	
35 -< 50	67	15	52	22.4 (12.7-31.0)	1 (ref)	
50 -< 65	66	23	43	34.8 (23.0-44.9)	1.6 (0.8-3.1)	
65 +	22	7	15	31.8 (10.4-48.1)	1.4 (0.6-3.5)	
Charlson group[c]						
Very low	58	8	50	13.8 (4.8-21.9)	1 (ref)	0.10
Low	49	12	37	24.4 (12.9-34.5)	1.9 (0.8-4.6)	
High	94	29	65	30.9 (21.2-39.3)	**2.3 (1.1-5.1)**	
Period[d]						
1973 - 1989	23	5	18	21.7 (4.5-35.9)	1.0 (0.4-2.6)	0.19
1990 - 1999	44	16	28	36.4 (21.5-48.4)	1.8 (0.9-3.2)	
2000 - 2009	130	28	102	21.5 (14.5-28.0)	1 (ref)	
2010 - 2013	33	5	28	15.2 (2.5-26.2)	0.7 (0.3-1.8)	
Region[e]						
Nuuk	119	22	97	18.5 (11.6-24.9)	1 (ref)	0.23
East	18	5	13	27.8 (5.5-44.8)	1.5 (0.6-4.1)	
South	27	11	16	40.7 (21.1-55.5)	**2.4 (1.2-4.9)**	
West	49	12	37	24.5 (11.9-35.2)	1.4 (0.7-2.7)	
North	17	4	13	23.5 (1.6-40.5)	1.3 (0.4-3.7)	
IPD-type						
Bacteremia	82	11	71	13.4 (6.0-20.2)	1 (ref)	
Meningitis	70	20	50	28.6 (17.5-38.2)	**2.2 (1.1-4.7)**	0.03
IPD by serotype[f]						
VT	105	15	90	14.3 (7.56-21)	1 (ref)	0.15

NVT	64	15	49	23.4 (12.9-33)	1.68 (0.82-3.44)

Abbreviation: IPD: Invasive Pneumococcal Disease, HR: Hazard Ratio, p: p-value for the respective factor, IPD-type: The manifestation of IPD.

a. Mortality: 30-day mortality after IPD-admission, estimated proportion by using the Kaplan-Meyer method.
b. HR: Hazard Ratio estimated by cox-regression
c. Charlson group: Charlson comorbidity score, including the age-component. Very Low (score = 0), Low (score = 1), High (score = 2+)
d. Period: Calendar-period
e. Region: Geographic region of Greenland, including Nuuk the capital as an independent region
f. IPD by serotype: IPD-cases grouped by serotypes included or not included in the 13-valent pneumococcal conjugate vaccine.

Figure 1

Age-specific incidence rates of invasive pneumococcal disease in Greenland, according to calendar period (1973-2013)

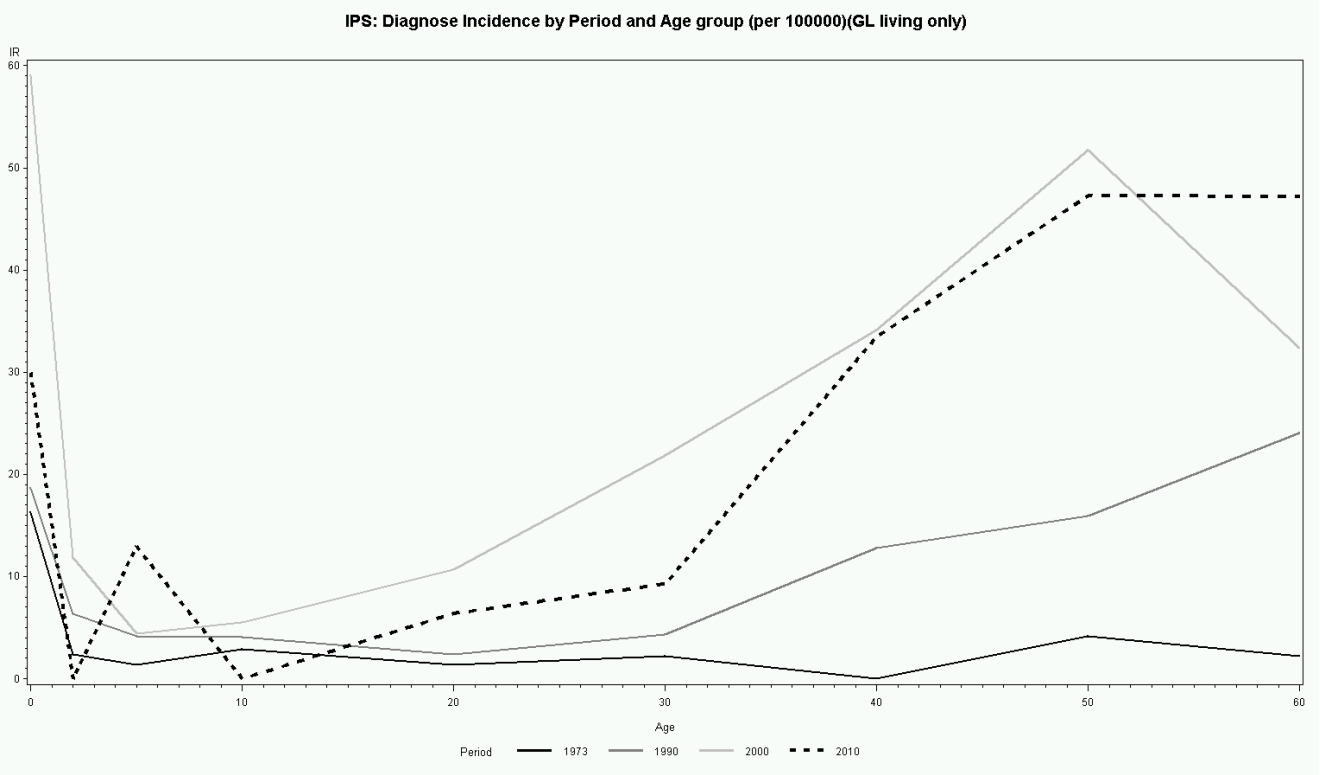


Figure 2

Number of cases of invasive pneumococcal disease according to type of manifestation (bacteremia and meningitis) and month of the year.

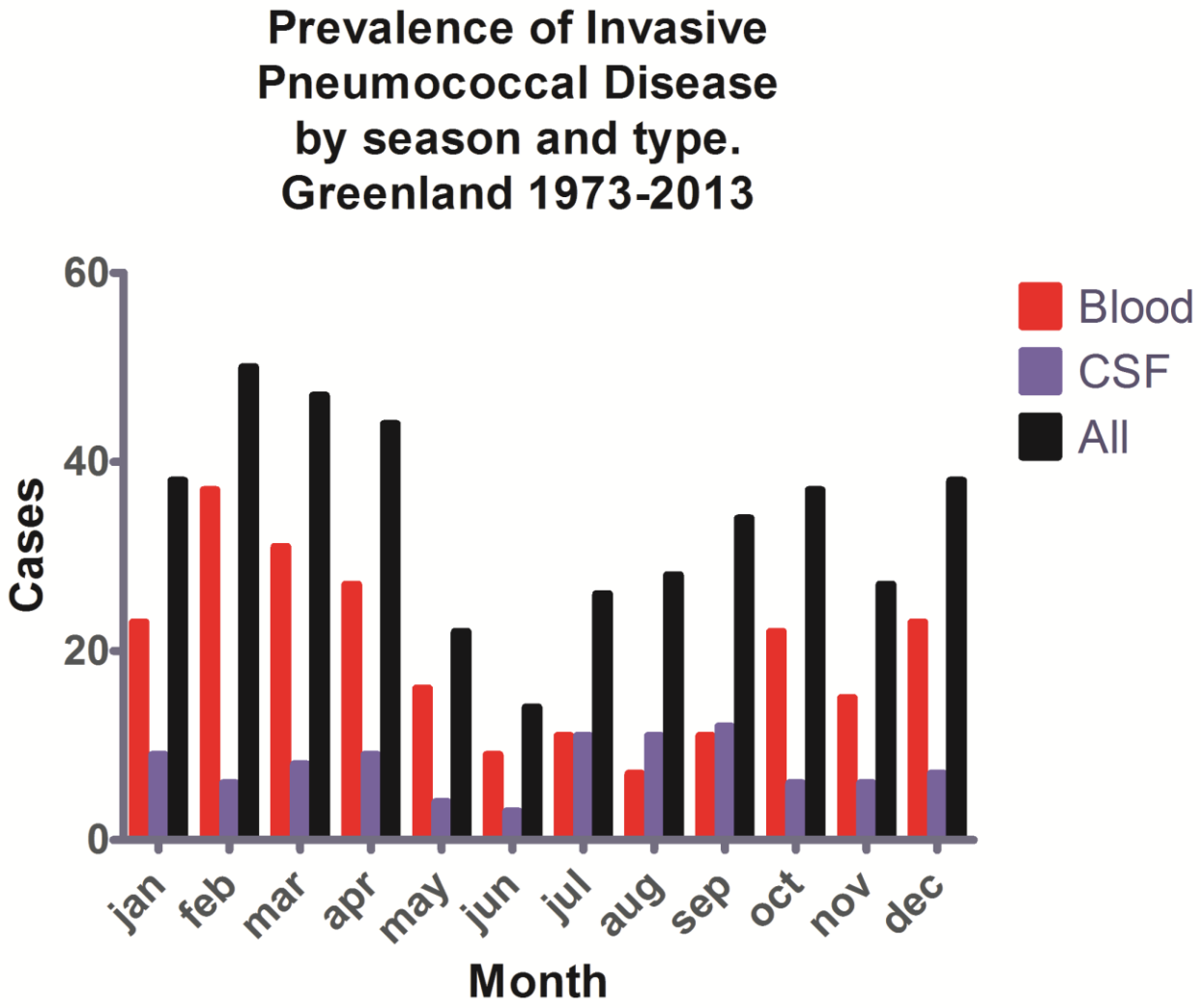
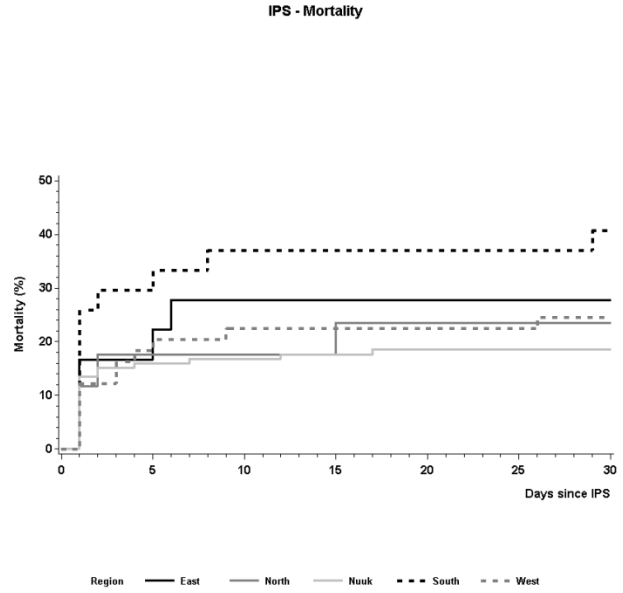


Figure 3

30-day mortality from invasive pneumococcal disease according to region in Greenland



30-day mortality from invasive pneumococcal disease in Greenland, according to age group.

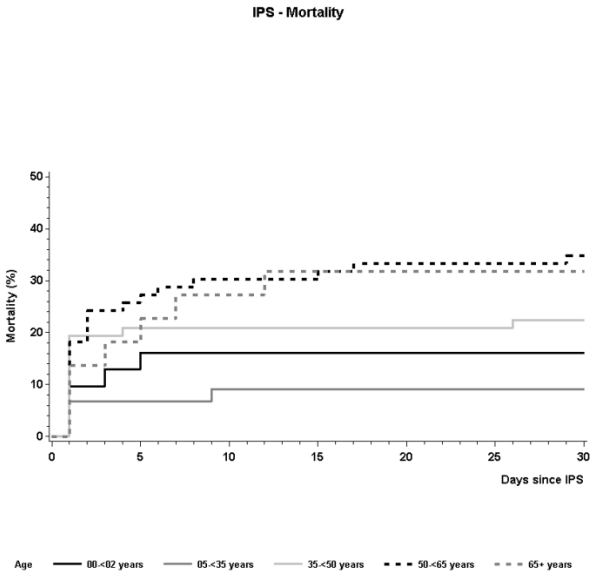
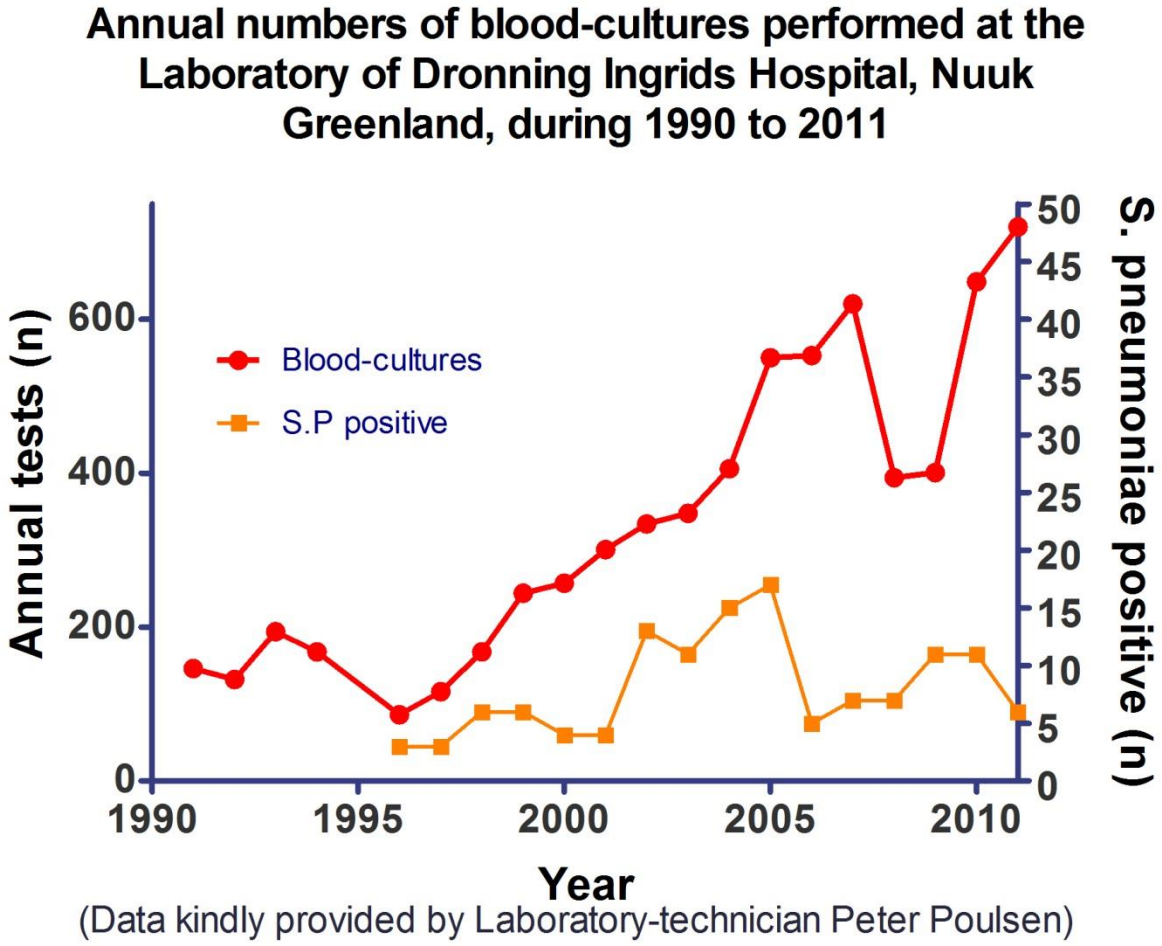


Figure 4.


Annual numbers of blood-cultures performed at the Laboratory of Dronning Ingrids Hospital, Nuuk Greenland, during 1990 to 2011
Annual tests (n)
S. pneumoniae positive (n)
Blood-cultures
S.P positive
0
200
400
600
0
5
10
15
20
25
30
35
40
45
50
1990
1995
2000
2005
2010
Year
(Data kindly provided by Laboratory-technician Peter Poulsen)

Effect of the 13-valent pneumococcal conjugate vaccine on naso-pharyngeal carriage by respiratory pathogens among Greenlandic children

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Running title: PCV-13 impact on bacterial carriage

Key-words: PCV-13, Nasopharyngeal carriage, Inuit, respiratory infections, pneumococcus

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ABSTRACT

Background In November 2010, Greenland introduced the 13-valent pneumococcal conjugate vaccine (Prevnar 13®; Pfizer/Wyeth - PCV-13) in the Children Vaccination Program. Recent studies indicate changes in rates of nasopharyngeal bacterial carriage post-PCV introduction. We aimed to evaluate the impact of the PCV-13 on nasopharyngeal carriage by four clinically relevant bacteria frequently associated with respiratory infections in children.

Method In 2013 a cross-sectional population-based study was conducted among Greenlandic children aged 0 to 6 years and data compared with a previous carriage study from 2011. PCV-13 status was obtained through nationwide registries. Nasopharyngeal samples tested for *Streptococcus pneumoniae,* Non-typeable Haemophilus influenzae (NTHi)*, Moraxella catarrhalis* and *Staphylococcus aureus* among others. Pneumococcal serotyping was performed by Quellung reaction and serotype-specific antisera. Statistical analysis included logistic regression models, adjusting for known risk factors.

Result A total of 377 nasopharyngeal samples were collected. Overall carriage rate of *S. pneumoniae* remained unchanged from 2011 to 2013 (51% and 56%, p=0.13), but significant serotype-shifts were observed both among vaccinated and unvaccinated. Carriage rate of *S. aureus* significantly declined among vaccinated (aOR 0.48, 95% CI; 0.25-0.91) whereas that of *M. catarrhalis* increased (aOR 1.52, 0.99-2.33).

Conclusion PCV-13 introduction in Greenland has likely led to shifts in nasopharyngeal pneumococcal serotype-distribution as well as reductions in *S. aureus* carriage and increasing carriage-rates of the important oto-pathogen *M. catarrhalis* among vaccinated children. Continued surveillance is warranted to clarify if these changes are temporary or persistent, and if these changes may have an impact on the pattern of respiratory and invasive diseases in Greenland.

INTRODUCTION

The Inuit population of Greenland suffers from high rates of respiratory infections and invasive bacterial diseases[1], causing higher morbidity and mortality than among non-Inuit [2–8].

Nasopharyngeal bacterial carriage is the essential precursor of these infections with *Streptococcus pneumoniae,* non-typeable Haemophilus influenzae (NTHi)*, Moraxella catarrhalis*, and S*taphylococcus aureus* as the most clinically relevant bacteria [9–12]. Besides causing auto-infections within the host, the nasopharyngeal colonization is also a source of transmission to other individuals. Greenlandic children are frequently colonized by these agents, with acquisition shortly after birth and frequent co-colonization by multiple species [13]. The disease burden from respiratory tract infections in Greenland children is among the highest reported [4] , primarily acute and chronic otitis media with frequent long-term sequelae [14].

The Inuit-populations of Alaska and Canada share the high risk of respiratory infections as well as invasive pneumococcal disease (IPD)[15–17]. In Greenland, Inuit have four-times as high risk of invasive pneumococcal disease compared with non-Inuit [18].

The 7-valent pneumococcal conjugate vaccine - Prevnar ® by Pfizer/Wyeth (PCV-7) introduced in the childhood immunization program in the US in 2000 and gradually widespread in many countries, has proven efficient in reducing disease-rates caused by the 7 serotypes included in the vaccine, so-called vaccine-types (PCV-7 VT). Furthermore, the PCV-7 has proven to be able to prevent PCV-7 VT nasopharyngeal carriage and thus interrupt the chain of transmission to other individuals including non-vaccinated resulting in herd immunity. However, the overall effect on pneumococcal carriage rates has been counterbalanced by increasing rates of pneumococcal serotypes not included in the vaccine i.e. non-vaccine type (PCV-7 NVT) a phenomenon known as replacement [19–21]. Recent PCV-evaluating studies now indicate that the carriage rates of other co-colonizing pathogens such as *S. aureus* and *NTHi* may also be changed after PCV introduction [22,23]. These observations have subsequently raised the concern of an altered disease pattern after widespread use of PCV, given the polymicrobial pathogenesis of respiratory diseases. In fact, the Finnish Otitis Media study observed higher rates of acute otitis media (AOM) caused

by NTHi among PCV-7 vaccinated children in a randomized controlled trial compared with a control group of unvaccinated children [24]. Also *S. aureus* related AOM has been observed in higher proportions among PCV-7 vaccinated children compared with unvaccinated children [25]. A possible mechanism for the observed changes may be the dynamic nature of the nasopharyngeal microbial composition, where the balance may be skewed due to a PCV-related clearance of VT pneumococci which leaves the nasopharyngeal niche vacant to be occupied by other opportunistic microbes [26].

In 2000 the PCV-7 was introduced in the Children Vaccination Program in Alaska. In the following years the overall incidence rates of IPD in the childhood population of Alaska were reduced, but among the native Alaskans this was counterbalanced by a dramatic increase in rates of IPD caused by non-vaccine types (NVT), resulting in an even bigger disparity in IPD-rates among the Native Alaskan and the general US-population[27]. In 2010 in Alaska, the PCV-7 was replaced by the successor the 13-valent pneumococcal conjugate vaccine (PCV-13) including 6 additional serotypes, which resulted in further reductions in IPD-rates, particularly IPD caused by PCV-13 serotypes but also declining rates of IPD caused by non-PCV-13 serotypes [28]. However, this may be counterbalanced since increases in rates of colonizing non-PCV-13 serotypes were observed in a carriage-study of PCV-13 impact on pneumococcal colonization conducted within the same population [29]. Greenland introduced the PCV-13 (Prevnar 13®; Pfizer/Wyeth) in the childhood immunization program in September 2010 [30].

As the Inuit populations of Alaska, Northern Canada and Greenland are closely related and share similar living conditions [31], we aimed to describe possible changes in nasopharyngeal carriage rates by four clinically important bacteria among Greenlandic children aged 0 to 6 years from 2011 to 2013 following the introduction of the PCV-13 in Greenland 2010.

METHODS

A cross-sectional study was conducted in October to December 2013 and data compared with data from a previous cross-sectional study conducted between October and December 2011 [13]. Since 1972 all citizens of Greenland has been given a unique personal identification-number registered in the Civil Registration System (CRS). The daily updated CRS contains vital information about place and date of birth, gender, birth order, siblings, parents, current and earlier addresses [32], and the unique personal identification number allows for accurate linkage between other national registers. Through the CRS-registry, we identified all children aged 0 to 6 years living in October 2013 in the towns of Tasiilaq (East Greenland) or Sisimiut (West Greenland) including their surrounding settlements, and invited the children via their parents to participate. After written and oral informed consents were obtained from parents or caretakers, the parents completed a questionnaire by the assistance of an interpreter regarding birthplace, number of siblings, day-care institution attendance, breastfeeding, recent antibiotic use, domestic tobacco exposure, recent respiratory tract infections (otitis media, tonsillitis, pneumonia or ear-discharge within the last three months or rhinitis within the last week), hospitalizations within the last three months, self-experienced housing standard, number of rooms in house-hold exclusive of bathroom and kitchen, number of children less than five years sleeping in the same room, in-house water supply and heating source. Ethnicity was defined from the parents' place of birth. If both parents were born in Greenland, ethnicity was defined as Inuit; one parent born in Greenland was defined as a 'mixed Inuit' ethnicity, and no parents as 'other', which is mainly Danish (Caucasian) ethnicity. Data on PCV-13 doses were obtained from local medical files. The PCV-13 is administered as a 2+1 schedule given at 3- and 5 months of age, with a booster at 12 months of age. During the introduction of the vaccine, a catch-up campaign was initiated, offering three vaccinations for children aged 3 to 11 months, and two vaccinations for children aged 12 to 23 months [30].

Nasopharyngeal sampling

Children were sampled at the local day-care institutions and schools or invited to the health-care center to participate. Briefly, the WHO protocol for pneumococcal carriage-studies [33] was followed, sampling from the posterior nasopharynx with Minitip Flocked Nylon Swabs (FLOQSwabs™) and using Skim milk-Tryptone-Glucose-Glycerin medium (STGG) which has proven useful for the study of respiratory pathogens including *S. pneumonia*, NTHi and *M. catarrhalis* [34]. Samples were temporarily stored (up to 3 weeks) at - 20 ˚C before shipping to Denmark for storage at -80 ˚C. The same procedure was used for the study in 2011 [13]

Laboratory analysis

Swab samples were tested at Statens Serum Institut, Copenhagen, Denmark (SSI), for the presence of *S. pneumoniae,* non-typeable *H.influenzae* (NTHi)*, M. catarrhalis* and *S. aureus* using standard culture methods. Bacterial identification was based on colony morphology by conventional microbiologic procedures and verified by MALDI/TOF mass spectrometry [35]. All isolates were tested for antimicrobial susceptibility using the disk diffusion test and EUCAST breakpoints [36]. Pneumococci were identified based on α-hemolysis, optochin sensitivity and capsular reaction (Quellung). Non-typeable pneumococci were identified using bile solubility-test.

Pneumococcal group-determination was performed directly on the serum-broth enriched NP-samples by Pneumotest latex® agglutination and serotypes identified with *Quellung* [37] reaction by the use of type-specific antisera from the Statens Serum Institut, Copenhagen, Denmark [38,39]. To increase the likelihood of detecting low-density carriage and multiple pneumococcal serotype carriage, we added 50µl of the nasopharyngeal swab samples to a 2ml serum-ox broth and incubated in CO_2, 37˚C for 24 hours, before plating, which previously has shown efficient in increasing the detection level of *S. pneumoniae* in nasopharyngeal swab samples [39].

Statistical analyses

The study was designed to demonstrate potential changes in pneumococcal carriage after the introduction of the PCV-13 as the primary outcome, and changes in NTHi*, M. catarrhalis* and *S. aureus* as secondary outcomes. Based on figures from 1996, which were the most recent ones from Greenland [40] carriage rates were estimated to be 67%, 42% and 8% for NTHi*, M. catarrhalis* and *S. aureus,* respectively. Given these estimates sample sizes of 350 in 2011 and 2013, respectively, with 80% power and a significance-level of 5%, would be sufficient to detect a minimal difference in carriage between the years of 10%, 11% and 7%, respectively. Differences in prevalence rates were tested by chi-square tests, with a p-value < 5% considered significant. Associations between nasopharyngeal carriage by the four bacteria and PCV-13 vaccination were estimated by multivariable logistic regression analyses. Possible confounders were tested by separate inclusion in basic adjusted models with adjustments for age (one-year intervals), sex, PCV-13 vaccination and sampling-year. Only when significant in a basic model at a 5%-level, it was included in the more adjusted models. The fully adjusted models included adjustments for age, sex, ethnicity, region (East/West), current day-care attendance, having siblings in a day-care, recent respiratory infections (otitis media, ear-discharge, tonsillitis or pneumonia within the last three months or rhinitis within the last week). Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina). For analytical purposes we grouped pneumococcal serotypes in vaccine-types (VT), i.e. serotypes included in the PCV-13 and non-vaccine types (NVT), not included in the PCV-13. In case of potential repeated measurements from individuals appearing in both of the two cross-sectional studies, we did a robustness analyses using a general estimation equation (GEE) [41] to account for correlated data.

RESULTS

A total of 377 children participated in 2013 with demographic characteristics as shown in Table 1. Eighty-six children also appeared in the previous cross-sectional study from 2011 [13]. Testing the effect of repeated measurements on bacterial colonization only minimal changes in estimates and confidence intervals were

observed, and interpretation of results was unchanged. The distribution of most baseline characteristics in this study was comparable with those of the previous study from 2011 [13], such as age, ethnicity, sex, in-home crowding, recent antimicrobial usage and recent otitis media. However, some significant differences were seen, including proportions of the children sampled in East- and West-Greenland, day-care attendance, in-house smoking and proportions of PCV-13 vaccinated. When testing for potential risk factors for carriage in 2011 and 2013 the following variables were identified: age, sex, ethnicity, the year of sampling, PCV-13 vaccination, geographic region (East-/West-Greenland), daycare attendance, having siblings attending a daycare and experiencing an episode of respiratory infections within the last three months. We also tested for potential effect modifications or interactions between the different co-variables, but found no significant interactions and overall estimates were not affected.

S. pneumoniae

The overall carriage-rate of pneumococci in 2013 were 56%, and among serotypes most frequently identified were 6B, 11A, 15A, 15B, 21, 23A, 23B, 23F, 35B and 35F (figure 1). Three years after PCV-13 introduction, the overall pneumococcal carriage rate was basically unchanged compared with 2011 (Table 2). However, among PCV-13 vaccinated children, serotype-specific changes were observed with significantly reduced carriage-rates of VT-serotypes aOR 0.43 (95% CI: 0.20-0.90) and increasing rates of NVT aOR 1.63 (95% CI: 1.07-2.48) compared with unvaccinated children and controlling for year of sampling, age-group, sex, geographic region of sampling, current day-care attendance, having siblings attending a day-care and recent respiratory infections. Furthermore, when adjusting for PCV-13 vaccination, the odds of carrying VT in 2013 was significantly reduced compared to 2011 aOR 0.44 (95% CI: 0.24-0.82) whereas odds of NVT carriage was increased aOR 1.36 (95% CI: 0.97-1.90). By age-group the reduction in VT-carriage dominated among children at 2 to 5 years of age, whereas the increase in NVT was seen predominantly among children aged 1 to 5 years (Figure. 2).

NTHi

The average carriage rate among children < 3 years was 47% (95% CI: 42% to 53%), and 40% (95% CI: 35% - 45%) among children ≥ 3 years. Overall, NTHi-carriage remained stable during the period (Table 1), with an insignificant increased odds of carriage aOR 1.29 (95% CI: 0.84-1.98) compared with unvaccinated children (Table 3). This increase was primarily seen among children aged 3 to 6 year-olds (Figure. 2).

M. catarrhalis

Average carriage rate among children < 3 years was 66% (95% CI: 61% - 71%) and among children ≥ 3 years 37% (95% CI: 32% to 41%). Overall carriage-rates were unchanged during the period (52% versus 49%). In the crude analysis PCV-13 vaccination was associated with increased odds of *M. catarrhalis* carriage OR 1.72 (95% CI 1.14-2.58) and when fully adjusted for confounders, the association was attenuated although still indicating a positive association (aOR 1.52, 95% CI 0.99-2.33). The increase in carriage was primarily observed in 0 < 1 year-olds and 2 < 3 year-olds (Figure.2).

S. aureus

Among children less than 3 years average carriage rate was 12% (95% CI: 8% - 15%) and among children ≥ 3 years 8% (95% CI: 6% to 11%). Among vaccinated children there was a significant reduction in carriage rate of *S. aureus* during from 2011 to 2013 , most noticeable among 1 to 5 year-olds (Figure.2).

DISCUSSION

To our knowledge, this is the first study concurrently to estimate PCV-13 impact on nasopharyngeal carriage rates of *S. pneumoniae, M. catarrhalis, NTHi and S. aureus*. Furthermore, the analyses included important confounder control for bacterial carriage. In line with previous PCV-7 carriage studies we confirmed a substantial pneumococcal serotype shift from VT to NVT among vaccinated individuals three years after PCV-13 introduction in Greenland. In a double-blind randomized placebo-controlled trial, Obaro et al. were among the first to report significant reductions in carriage rates of vaccine serotypes in Gambian infants vaccinated with a penta-valent PCV, counterbalanced by increasing carriage rates of NVT [42]. In the following years several studies have confirmed these findings [26]. In our study we also observed a cohort-effect showing reductions in VT carriage independent of PCV-13 vaccination, which may indicate herd-immunity, but also may be due to other unmeasured confounders. Furthermore, NVT-carriage rates increased independently of PCV-13 vaccination. We found no signs of interaction between year of sampling and PCV-13 vaccination, so our findings point towards an independent period effect. This may be due to reduced VT serotype carriage rate as well as increased rates of NVT serotypes circulating in the community after three years of PCV-13 use, a change in exposure to these serotypes both among vaccinated and unvaccinated. Reasons for these relatively early changes, observed after only three years of vaccination, may be due to the catch-up campaign during the PCV-13 introduction. So far, only one previous study has addressed the PCV-13 impact on pneumococcal colonization among Inuit [29]. This study from 2012 was conducted in Alaska and included the Native population, and reported reductions in carriage-rates of the six additional serotypes included in the PCV-13 replacing the PCV-7 vaccine, previously used in Alaska. The effect was observed both among vaccinated and unvaccinated children indicating a herd immunity effect [29]. Furthermore, an increase in colonizing NVT was observed. The authors were, however, not able to control for potential confounders such as day-care attendance or underlying medical conditions except for recent antimicrobial use. The increases in NVT carriage observed in both the Alaskan and the present study indicate that the increase in invasive disease by NVT types observed in Alaska following introduction of the

PCV 7 vaccine [43] may also appear following introduction of the PCV-13. Moreover, we have recently described the mortality from IPD in Greenland during 1973-2013, and found the short-term mortality among patients with IPD caused by NVT to be higher than IPD caused by VT, which may subsequently counterbalance the beneficial effect of the vaccine [18]. These findings warrant continued surveillance of nasopharyngeal carriage and invasive pneumococcal diseases in the Arctic.

Besides the apparently vaccine induced serotype shift significant changes in carriage of other co-colonizing clinically important pathogens were observed. Carriage of *M. catarrhalis* increased among PCV-13 vaccinated children. This may be due to natural fluctuations in rates of *M.catarrhalis* circulating in the community; however, bacterial interactions may also be a part of the explanation. We have previously reported observations of specific associations between the colonizing pathogens; including a positive interaction between *M.catarrhalis* and NVT pneumococci [13]. Given the anticipated increases in carriage of NVT post PCV-13 introduction, the balance between the colonizing bacteria in the nasopharynx may change in a direction with higher carriage-rates of *M. catarrhalis*. Whether increasing carriage rates of *M. catarrhalis* lead to changes in disease-rates is not clarified. However, studies of PCV-7 impact on acute otitis media (AOM) have shown increases in proportions of *M.catarrhalis* and NTHi in middle ear fluid from PCV-7 vaccinated children with AOM, indicating an association [44–47]. A scenario of increasing carriage-rates of *M.catarrhalis*, being a similar important oto-pathogen in young children [11], is concerning especially since the Greenlandic population suffers from a very high burden of otitis media [4] We observed a significant reduction in carriage by *S. aureus* among PCV-13 vaccinated children. Whether this is due to random fluctuations or temporary changes in the native flora of the nasopharynx due to bacterial interactions is speculative, but an inverse relationship between *S. pneumoniae* and *S. aureus* has been reported previously. In a randomized controlled trial on the effect of PCV-7 on nasopharyngeal carriage Van Gils et al. found a negative association between pneumococci (both PCV-7 serotypes and non-PCV-7 serotypes) and *S. aureus* [48]. The mechanism behind this association has not been clarified, but may include the production of hydrogen peroxide by pneumococci, which in vitro have been shown to kill *S*

aureus [49]. The present study was not designed to specifically sample from the anterior nasal cavity where *S. aureus* resides. However, when entering the nasopharynx, the niche is passed twice, giving the opportunity to detect current *S. aureus* colonization.

Strengths and limitations

To our knowledge this is the first study to estimate PCV-13 impact on four clinically relevant respiratory pathogens in a high-risk population burdened by high frequencies of respiratory tract infections and IPD. The study was based on two consecutive cross-sectional studies using the exact same design, sampling method, transport medium and laboratory techniques, which minimize study design bias. Furthermore, the study is population-based, based on random sampling from the CRS, a register updated on a daily basis, which minimize selection bias. We made a large effort in obtaining valid data from registers and questionnaires on potential confounders for carriage. However, some differences in baseline characteristics between the two cross-sectional studies were present, including proportions of children sampled in the two regions. Since the regions are separated by large distances, local clustering in carriage proportions may have biased the results, however, we accounted for this by adjusting for region in the multivariable analyses. In 2013 the proportions of children attending a day-care center and children being exposed to in-house smoking were smaller than in 2011. This could have created bias towards overestimating the effect of the vaccine, since both of the factors have been shown to increase the risk of *S. pneumoniae* carriage [50]. We did, however, include these factors in the multivariable analyses to limit the potential confounding effect on the results. Unfortunately, we were not able to get baseline data before the introduction of PCV-13 in Greenland. Yet, only a minority of the cohort of children was vaccinated during the first cross-sectional study making potential confounding by indirect herd immunity minimal. Moreover, since we only have three years of study time post-PCV-13 introduction, we are unable to determine if the observed changes reflect a transitional- or steady state. Finally our data were collected from two separate regions of Greenland and may not be generalizable to all Greenlanders.

Conclusions

PCV-13 introduction in Greenland 2010 has led to significant shifts in nasopharyngeal pneumococcal serotype-distribution with NVT replacing VT pneumococci and thus no consequent impact on the overall carriage rate of pneumococci. Other clinically important co-colonizing pathogens have also changed in carriage rates in the study period from 2011 to 2013, dominated by an increase in the important oto-pathogen *M. catarrhalis* and reductions in *S. aureus* carriage among vaccinated children, most likely as a result of the PCV-13 introduction. Continued surveillance is warranted to clarify if these changes are temporary or persist in the long-term. Furthermore, it is essential to monitor the clinical significance of these changes including the invasive potential of the emerging NVT.

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Figure 1. Proportions of nasopharyngeal pneumococcal serotype distribution among Greenlandic children aged 0 – 6 years in 2013 and compared with serotype distribution in 2011 [13]. Arrows indicates serotypes included in the 13-valent pneumococcal conjugate vaccine.

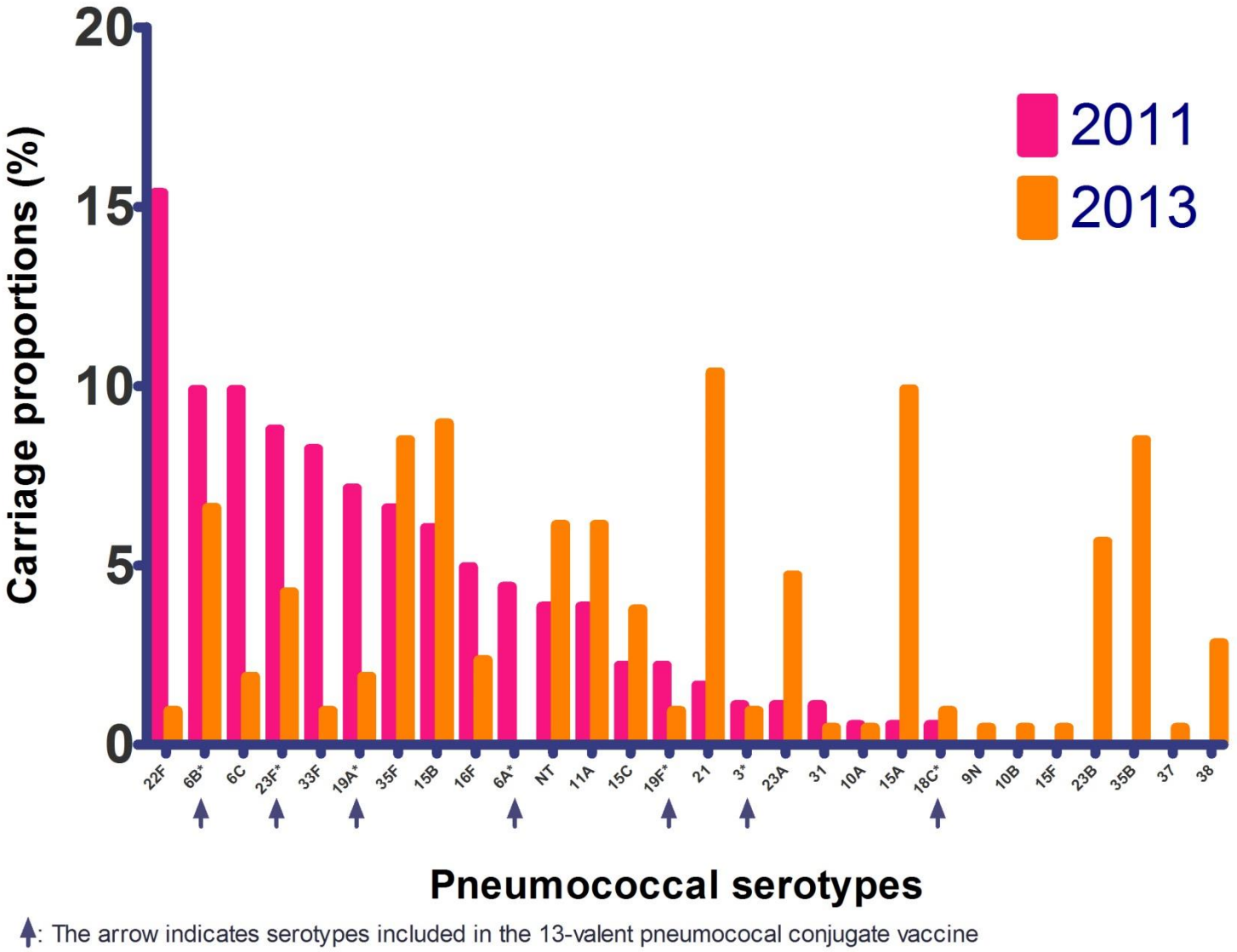
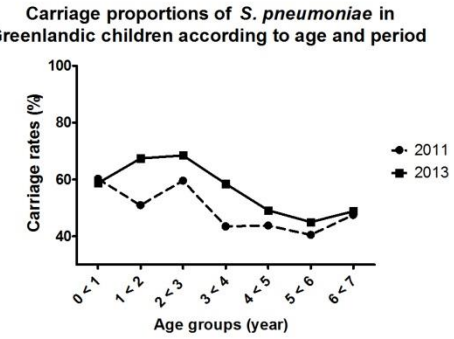
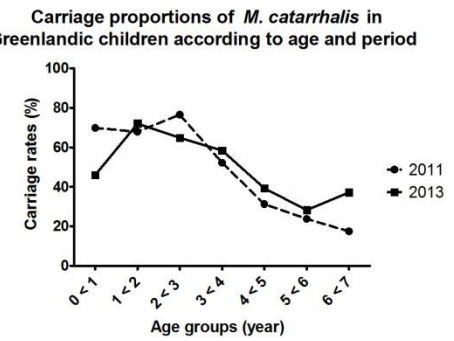
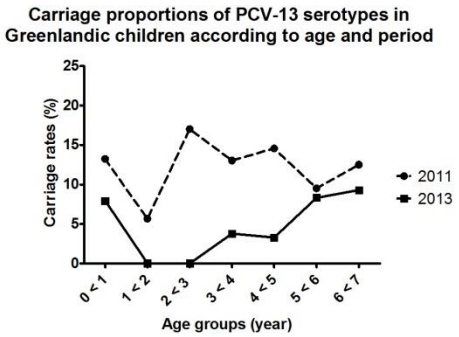
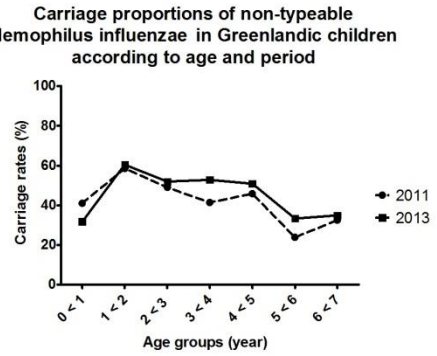


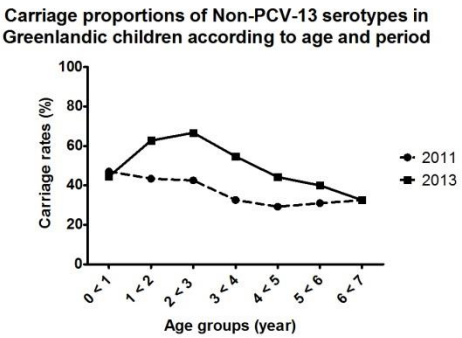
Figure 2: Carriage patterns of *S. pneumoniae*, PCV-13 serotypes, Non-PCV-13 serotypes, Non-typeable Hemophilus influenzae, *M. catarrhalis* and *S. aureus*, according to age-groups and period. Dotted lines represents carriage rates from a previous study conducted in 2011 [13].











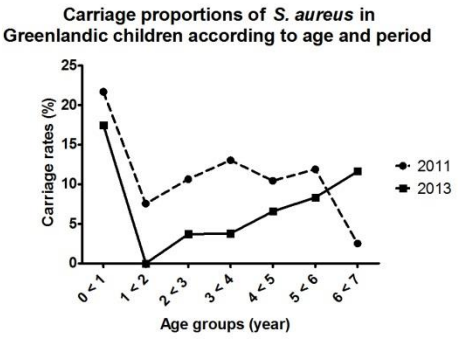


Table 1: Demographic characteristics of study population 2013 (n=377 children) and comparison population 2011 (n=352) [13]). Unselected children aged 0-6 years living in two regions of Greenland.

Variable	Level	Year 2013 n = 377 (%)	Year 2011 n = 352 (%)	p[a]
Gender	Male	192 (51)	179 (51)	0.98
Age, years	Median (Q1;Q3)	3.50 (1.68; 5.12)	2.85 (1.08; 4.84)	0.34
Ethnicity	Inuit	343 (91)	320 (91)	0.35
Region of Greenland[b]	East-coast	186 (49)	128 (36)	**<0.001**
	West-coast	191 (51)	224 (64)	
Daycare attendance (current)	yes	232 (62)	260 (74)	**<0.001**
Having siblings in DC[c]	yes	134 (36)	139 (39)	0.29
Inhouse smoking[d]	yes	39 (10)	67 (19)	**0.002**
Sharing bedroom with children < 5 years of age	yes	210 (56)	210 (58)	0.45
Number of persons per room[e]	0	23 (6)	14 (4)	0.41
	1	181 (48)	167 (47)	
	2	167 (44)	163 (47)	
Resp. infection < 3 months[f]	yes	229 (61)	206 (58)	0.43
Antibiotics < 3 months[g]	yes	53 (14)	58 (16)	0.39
PCV-13 vaccinated[h]	yes	212 (56)	127 (36)	**<0.0001**
***S. pneumoniae* detected**	yes	212 (56)	178 (51)	0.13
VT-pneumococci detected	yes	18 (5)	44 (12)	**<0.001**
NVT-pneumococci detected	yes	185 (49)	137 (38)	**0.003**
***S. aureus* detected**	yes	29 (8)	38 (11)	**0.04**
NTHi detected	yes	168 (45)	152 (42)	0.54
***M. catarrhalis* detected**	yes	183 (49)	186 (53)	0.38
Any of the bacteria detected[i]	yes	317 (84)	290 (82)	0.55

Abbreviations: DC: Day-care center, AOM: Acute otitis media, PCV-13: the 13-valent pneumococcal conjugate vaccine, S. pneumoniae: *Streptococcus pneumoniae*, VT-pneumococci: Pneumococcal serotypes included in the PCV-13, NVT-pneumococci: Pneumococcal serotypes not included in the PCV-13, S. aureus: *Staphylococcus aureus*, NTHi: non-typeable Haemophilus influenzae, M.catarrhalis: *Moraxella catarrhalis*.

a. P-value based on chi-square test for difference
b. Region: East (Tasiilaq, Kuummiut, Sermiligaaq, Kulusuk), West (Sisimiut, Sarfannguaq)
c. Having siblings attending a day-care institution
d. Tobacco smoke inside the house
e. Number of persons per room: (0= less than 1 person per room in household), (1= 1 to <2 persons per room), (2= ≥2 persons per room)
f. Respiratory infections: Any episode of rhinitis, acute otitis media, ear-discharge, tonsillitis or pneumonia within the last three months prior to nasopharyngeal sampling
g. Having received treatment with antimicrobial drugs within the last three months
h. PCV-13 vaccinated: Vaccinated with ≥1 dose of the 13-valent pneumococcal conjugate vaccine
i. Any bacteria: The detection of either *S. pneumoniae*, *M. catarrhalis*, NTHi or *S. aureus* in the nasopharyngeal sample

Table 2

Crude- and adjusted odds ratios for bacterial nasopharyngeal carriage by *Streptococcus pneumoniae*, non-typeable Haemophilus influenzae, *Moraxella catarrhalis* or *Staphylococcus aureus*, among Greenlandic children aged 0 to 6 years in 2013 compared with data from a cross-sectional study in 2011 [18].

	PCV-13				Year			
	No	Yes			2011	2013		
Bacterium	**1 (ref.)**	**OR[a] (95% CI)**	**aOR[b] (95% CI)**	**p**	**1 (ref.)**	**OR[a] (95% CI)**	**aOR[b] (95% CI)**	**p**
S. pneumoniae	1 (ref.)	1.31 (0.88-1.95)	1.19 (0.78-1.82)	0.41	1 (ref.)	1.23 (0.90-1.68)	1.18 (0.84-1.65)	0.33
VT	1 (ref.)	**0.44 (0.22-0.92)**	**0.43 (0.20-0.90)**	0.02	1 (ref.)	**0.42 (0.23-0.75)**	**0.44 (0.24-0.82)**	0.01
NVT	1 (ref.)	**1.65 (1.11-2.46)**	**1.63 (1.07-2.48)**	0.02	1 (ref.)	**1.45 (1.06-1.99)**	1.36 (0.97-1.90)	0.07
NTHi	1 (ref.)	1.17 (0.78-1.75)	1.29 (0.84-1.98)	0.24	1 (ref.)	1.07 (0.78-1.48)	1.10 (0.79-1.54)	0.58
M. catarrhalis	1 (ref.)	**1.72 (1.14-2.58)**	1.52 (0.99-2.33)	0.06	1 (ref.)	0.83 (0.60-1.16)	0.82 (0.58-1.16)	0.27
S. aureus	1 (ref.)	**0.51 (0.27-0.94)**	**0.48 (0.25-0.91)**	0.03	1 (ref.)	0.70 (0.42-1.18)	0.62 (0.35-1.07)	0.09
Any bacterium	1 (ref.)	1.58 (0.88-2.84)	1.43 (0.76-2.68)	0.27	1 (ref.)	1.13 (0.74-1.74)	1.04 (0.65-1.66)	0.87

Abbreviations: PCV-13: the 13-valent pneumococcal conjugate vaccine, OR: Odds-ratio, aOR: adjusted Odds-ratio, p: p-value for adjusted OR, CI: Confidence interval, S. pneumoniae: *Streptococcus pneumoniae*, VT: pneumococcal serotypes included in the 13-valent pneumococcal conjugate vaccine. NVT: pneumococcal serotypes not included in the 13-valent pneumococcal conjugate vaccine. NTHi: non-typeable Haemophilus influenzae, M. catarrhalis: *Moraxella catarrhalis,* S. aureus: *Staphylococcus aureus*. Any bacterium: Odds of carrying either *S. pneumoniae*, NTHi, *M. catarrhalis* or *S. aureus* (grouped).

a. Odds ratios mutually adjusted for year of sampling and PCV-13 vaccination, as well as age groups (1 year intervals) and sex.
b. Odds ratios mutually adjusted for year of sampling and PCV-13 vaccination, as well as age groups (1 year intervals) and sex. Additional adjustments: region (East-/West-Greenland), recent respiratory infection (otitis media, ear-discharge, nasopharyngitis, tonsillitis or pneumonia within the last three months), current day-care attendance (yes/no), having siblings in a day-care (yes/no) and ethnicity ('Inuit', 'mixed Inuit/other ethnicity' or 'non-Inuit').