Early life predictors for atopic dermatitis in infancy

Eva Maria Rehbinder

University of Oslo, Institute of Clinical Medicine

Department of Dermatology, Oslo University Hospital

ORAACLE

Oslo Research group of Asthma and Allergy in Childhood; the Lung and Environment

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Table of Contents

1 Preface

1.1 Acknowledgments

I was still in kindergarden when I decided to become a doctor. I struggled with atopic dermatitis and had regular appointments with professor Georg Rajka (Head of dermatology department at Rikshospitalet, Oslo and a giant in atopic dermatitis research) who helped me deal with my disease. I think that this was one of the reasons why I thought about medicine in the first place and especially dermatology. When Kristin Ryggen, the head of the dermatology department at St. Olav´s hospital, decided to give me a chance, I was thrilled. Although I had to be away from my family and could see my daughter, Elisa (1,5 years at that time) only in the weekends for 7 months, I loved every bit of the job. Kristin was a fair and supportive leader as well as being a strong female role model. My colleagues in Trondheim were all amazing, however Kristine Skjetne who started out at the same time as me will always have a special place in my heart.

Kristian and Elisa finally joined me in Trondheim, but when Thilda was born in 2013 we decided to move back to Oslo during my maternity leave. Fortunately, a research and teaching fellowship in dermatology opened at the University of Oslo. I applied, Petter Gjersvik believed in me, and I got the position.

I was presented with an exciting, new and ambitious project, the PreventADALL study. Karin C. Lødrup Carlsen, an energetic, inspiring and clever pediatrician and professor in the field of asthma and allergic disease, was the project leader and head of the ORAACLE (Oslo Research group of Asthma and Allergy in Childhood; the Lung and Environment) research group. Linn Landrø, a dermatologist was going to be the post doc and local PI in Oslo and I became the first PhD student on this project. My PhD project was initially supposed to be on skin microbiome and atopic dermatitis, with Karin as my main supervisor and Linn and Peter Gaustad, professor in microbiology as my co-supervisors, however as you can see from the title it changed along the way.

It took almost a year before we got the REK approval on the 9th of December 2014, and during this time we were very busy preparing for the launch, making procedures and planning all the logistics, such an elaborate study requires. In this period I met many dedicated and very friendly researchers that were part of the planning, initiation and execution of PreventADALL. In Oslo I got to know Kai-Håkon Carlsen, Annetine Staff,

Guttorm Haugen, Monica Hauger Carlsen, Berit Granum, Unni Nygård, Petter Mowinckel, Pål Fugelli and Geir Håland to mention some. In Stockholm, Gunilla Hedlin, Björn Nordlund, Cilla Söderhäll, and Anna Asarnoj with their team made a tremendous effort to set PreventADALL in motion at Karolinska Hospital and in Østfold, Sigrid Sjelmo, Bente Kvenshagen, Magdalena Vaernesbranden, Katrine Dønvold Sjøborg and later Jon Lunde and Christine Monceroy Jonassen were pivotal for the establishment and running of PreventADALL at Østfold Hospital Trust.

Karin, you are unstoppable and without you the PreventADALL project with all it´s 19 PhD students would never have happened. I feel very lucky to be one of them and to have you as my supervisor. Thank you for your dedication to public health and to me and I look forward to all that is ahead of us.

With Linn, I was lucky to have a friend as my colleague and supervisor. Your diplomatic, perceptive and pragmatic way is inspiring, and it has been great working so closely with you. Thank you for all your valuable contribution to my PhD and that I can talk to you about anything and everything.

The PreventADALL Oslo team started to grow with Liv Julie Sørdal, Hrefna Katrín Gudmundsdóttir, Oda Lødrup Carlsen, Thea Aspelund Fatvik, Live Nordhagen, Karen Eline Stensby Bains, Ina Kreyberg, Mari Kjendsli, Malén Gudbrandsgard and Katarina Hilde in the beginning. Further expanding with Angelica Johansen Winger, Vibeke Dyrseth, Kristine Wedum Davanger, Kristine Eikenæs, Khaledeh Samimi, Ingvild Essén, Carina Madelen Saunders, Peder Annæus Granlund and Kim Advocaat Endre. Some have stayed longer, some shorter. We have shared many laughs, we have had fruitful discussions, and made each other better. We have made endless PreventADALL kits together, recruited pregnant women like there was no tomorrow and we have examined a multitude of babies. I´m also very grateful for all the work hours the teams in Stockholm and Østfold have dedicated to PreventADALL.

Thank you, so much to all the families that wanted to be included in our study, did the interventions, are answering the questionnaires and are coming to the follow-up investigations. You are the best! The PreventADALL study could not have been possible without the contribution of numerous funding bodies, and I am sincerely grateful for this.

Håvard Skjerven took over as local PI in Oslo after Linn and I really appreciate his mind, sense of humour and that we can be frank with each other. Riyas Vettukatil is relentless in managing the PreventADALL database and he continues to impress me everytime we talk and meet. Tonje Reier-Nilsen, my lively and sharp friend and colleague in ORAACLE, thank you for being you.

Joining the Oslo team later, putting many work hours into the project, are also other accomplished and fun people: Sofie Rabo Carlsen, Åshild Wik Despriée, Hilde Aaneland, Elke Maes, Johanne Uthus Hermansen, Asima Locmic, Anine Lie, Andrea Dystvold Hansen and Marius Kurås Skram.

At the University, Teresa Løvold Berents, a bright and kind dermotologist that was already a research fellow when I started has been my mentor. She did her PhD also in atopic dermatitis, and she included me very fast into the university universe. I love her enthusiasm, and I miss her when I haven´t talked to her for a while.

I feel very privileged that Petter Gjersvik, professor and head of the dermato-venerological education, have shared his wisdom with me and for all the support he has given me. It has been inspiring to be part of the educational team, including Jan-Øyvind Holm, Jon Anders Halvorsen, Daniel de la Rosa Carillo, Kristin Bergersen, Anne Olaug Olsen and my dear Astrid Lossius who came after Teresa and is also doing her PhD on atopic dermatitis.

In the planning phase of the PreventADALL study I was in charge of making the procedures for the microbiome sampling and a passion for bugs grew inside of me. Peter Gaustad, Ben Marsland, Petri Auvinen, Johannes Hov and Marius Trøseid were my highly competent advisors. Later I got to know the brilliant, Knut Rudi who supervised and gave me access to his lab at NMBU, where I could do the amniotic fluid work with the expert help from Inga Angell, and where I also met Morten Nilsen for the first time. Thank you!

Leiv Sandvik, my statistical genius has supervised me judiciously through my third and fourth paper. Thank you! In Ben´s lab in Lausanne I have collaborated with Céline Pattaroni, Alexis Rapin and Niki Ubags on skin microbiome and I look forward to the continuation of this work.

Special thanks to Karin, Riyas and Håvard for your work on paper I, to Angelica and Linn for your work on paper II, to Knut and Annetine for your work on paper III, and to Kim and Leiv for your work on paper IV and to all my co-authors for all your efforts into making my papers as good as possible.

I have met many more people in the years as a PhD student that have made an impact and that have been involved in the PreventADALL study. The list is too long to mention all these names, but I´m very grateful for their effort, all these meetings, all their help and encouragement!

At the dermatology ward in Oslo University Hospital, Jon Anders Halvorsen and Jorun Hagen Rønsen gave me a job. Daniel de la Rosa Carillo welcomed me and took care of me in my new position as a resident, combining clinical work and research and Jan Sezary Sitek is now leading me steadily towards my next goals. Thank you! I truly enjoy working with all my colleagues at the dermatology ward and I look forward to better getting to know everyone. I´m especially happy that the devoted and lovely Guro Sunniva Bjørnevaagen, Eva Astrid Tønsberg and Anne Sofie Brandstorp-Boesen now are guiding me through my residency, and that the passionate and cunning Kristine Bø has become my clinical supervisor.

I would like to thank all my family and friends for your love, kindness and support. You know who you are. ❤️
Kristian, you are my rock and together with our daughters Elisa (9) and Thilda (6) you make me a better person. Your patience and backing have been too good to be true.
I love you.

1.2 Summary of thesis

1.2.1 Background

Atopic dermatitis (AD) and other allergic diseases have reached epidemic numbers, with an AD incidence of up to 15 % already in the first year of life. Although parental atopy has been established as the most important single factor predicting offspring AD, genetic background cannot explain the increase in AD prevalence across the globe. Seeking an understanding on how prenatal and perinatal factors could either predict or explain the onset of AD in early infancy would be important to better understand AD pathomechanisms, optimise therapy as well as possibly select infants for primary prevention strategies.

1.2.2 Aims

The objective of this thesis was to identify prenatal and perinatal factors that predicts impaired skin barrier function and AD in early infancy in order to better understand the nature of AD and possibly select infants for potential primary prevention. We aimed to explore mode of delivery and a potential amniotic fluid microbiome, as well as determining prevalence for dry skin and if it is associated with increased transepidermal water loss (TEWL), and if dry skin or increased TEWL predicts AD in early infancy.

1.2.3 Methods

The study cohort was recruited from the Preventing Atopic dermatitis and Allergies in children (PreventADALL) study. The PreventADALL study, a general population-based, multicenter, two-by-two factorially designed, randomised controlled interventional and explorative birth cohort study enrolled pregnant women at the 18-week fetal ultrasound investigation, and their healthy babies born at gestational age (GA) 35.0 or later. Infants were randomised to skin intervention, food intervention, both interventions, or control. The main outcomes for the interventions are assessed at 12 months for AD and at 36 months for food allergy. In this thesis the focus is on the exploratory part of the study. We included 1150 mother-child pairs that were

randomised to food intervention only or control for the outcomes dry skin, TEWL and AD, which was assessed at the 3- and 6 months investigations. Atopic dermatitis, in this thesis, was defined as the presence of eczematous lesions, excluding differential diagnoses to AD. High TEWL was defined as TEWL > 90th percentile, equalling 11.3 g/m^2/h. Extensive electronic questionnaires at 18- and 34-week pregnancy and obstetric charts recorded potential predictive factors. Logistic regression analysis was used to identify significant predictors. Amniotic fluid in term pregnancies delivered by caesarean section (CS) was successfully sampled from 65 women. We selected 10 samples from elective CSs, where all were sampled in the same operating room, with intact amniotic membranes (non-ROM (rupture of membrane) group) prior to labour as well as including all 14 with on-going labour and ruptured amniotic membranes (ROM group) as positive controls. Amniotic fluid was analysed by highly sensitive digital droplet (dd) PCR and sequencing techniques as well as bacterial culturing.

1.2.4 Results

Of the 2701 pregnancies (2697 women) included at a mean 18.7 (range 15.7 - 22.7) weeks GA, 2397 children (11 twins) (52.7% boys) were included in the mother-child cohort born at mean 39.2 GA. The mother´s mean age (min-max) was 32 (18-42) years at inclusion, over 50 % had more than 4 years of higher education, almost all were married (41.2%) or lived with their cohabiting partner (55.9%). From the 2701 fetuses, 88.7% (n=2397) were included at birth, 52.7% were boys and 16.4% were delivered by CS. At least one doctor diagnosed allergic disease was reported by 42% of the mothers, and at least two by 20.1%, while AD was reported by 19.8 %.

Maternal allergic disease was the only significant prenatal predictor for high TEWL at 3 months of age (OR: 1.80, confidence interval (CI) 95%: 1.08-3.01; p=0.025). The following prenatal factors predicted AD at 3 months of age: maternal allergic disease (OR: 1.61, CI 95%: 1.02-2.55; p=0.041), and multiparity (OR: 1.63, CI 95%: 1.03-2.57; p=0.037).

The only significant perinatal predictor for high TEWL was birth during winter season (OR: 2.02, CI 95%: 1.31-3.14; p=0.002), while female sex was the only protective factor (OR: 0.61, CI 95%: 0.40-0.93; p=0.022). For AD at 3 months of age, elective CS (OR: 2.50, CI 95%: 1.19-5.25; p=0.016) was the only significant perinatal predictor.

In amniotic fluid from the non-ROM group, the median (min-max) concentration of prokaryotic DNA (16S rRNA gene copies/ml; ddPCR) (664 (544-748)) was similarly low as in the negative controls (596 (461-679)), while the ROM group had more than 10-fold higher levels (7700 (1066-251430)) (p = 0.0001, by Mann-Whitney U-test). By anaerobic culturing 50% of the ROM samples had detectable bacterial growth, in contrast to none of the non-ROM samples. Sanger sequencing of the ROM samples identified bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections.

The prevalence of dry skin at 3 months was 59%. Dry skin without AD was found in 47%, 13% had AD, 96% of these having dry skin, and 40 % had unaffected skin. Infants with dry skin on cheeks and extensor surfaces of the extremities had significantly higher mean TEWL ($g/m^2/h$) of 9.5 (95% CI: 8.4, 10.6) compared to those with dry skin on extensors and not cheeks 7.9 (6.9, 8.8), (p=0.025), and significantly lower than those with AD 12.5 (10.9, 14.0), (p <0.0001). Predictive factors for dry skin were delivery> 38 gestational weeks (OR: 2.46, CI 95%: 1.60-3.79; p<0.0001) and increasing paternal age, in particular >37 years (OR: 1.96, CI 95 %: 1.16-3.13; p=0.012). Dry skin without AD at 3 months was predictive for AD at 6 months, ($OR_{adjusted}$: 1.92, 95% CI: 1.21-3.05, p=0.005), while high TEWL at 3 months was not.

1.2.5 Conclusions

In a general population of 3 months old infants, significant predictors for high TEWL were maternal allergic disease, birth during winter season and male sex, while for AD it was maternal allergic disease, multiparity and elective CS. In elective CSs before rupture of amniotic

membranes and labour, amniotic fluid was sterile, in uncomplicated term pregnancies. Dry skin was present in 59 % at 3 months regardless of AD and in 47% without AD, with cheeks and extensor surfaces of the extremities most commonly affected. Mean TEWL was significantly higher in infants with dry skin than in those with unaffected skin, especially in those with concurrent presence of dry skin on cheeks and extensors. Increasing GA at birth and increasing paternal age were significant predictors for dry skin at 3 months, which in turn predicted AD at 6 months of age.

1.3 Abbreviations

AD	Atopic dermatitis
AF	Amniotic fluid
BMI	Body Mass Index
CI	Confidence Interval
CS	Caesarean section
EASI	Eczema Area and Severity Index
FLG	*Filaggrin* gene
GA	Gestational age
OR	Odds Ratio
POEM	Patient Oriented Eczema Measure
ROM	rupture of membrane
RCT	Randomised controlled trial
SD	Standard Deviation
TEWL	Transepidermal water loss

1.4 List of papers

Paper I

Carlsen, K. C. L, *Rehbinder, E. M, *Skjerven, H. O, Carlsen, M. H, Fatnes, T. A Fugelli, P, Granum, B, Haugen, G, Hedlin, G, Jonassen, C. M, Landrø, L, Lunde, J, Marsland, B. J, Nordlund, B, Rudi, K, Sjøborg, K, Söderhäll, C, Staff, A. C, Vettukattil, R, Carlsen, K. H. *Equal contribution. **Preventing Atopic Dermatitis and ALLergies in Children - the PreventADALL study.** Allergy. 2018; DOI: 10.1111/all.13468

Paper II

Rehbinder, E. M, *Lodrup Carlsen, K. C, *Staff, A. C, Angell, I. L., Landrø, L, Hilde, K., Gaustad, P, Rudi, K. *Equal contribution. **Is amniotic fluid of women with uncomplicated term pregnancies free of bacteria?** Am J Obstet Gynecol. 2018; DOI: 10.1016/j.ajog.2018.05.028

Paper III

*Rehbinder, E. M, *Winger, A. J, Landrø, L, Asarnoj, A, Berents, T. L, Carlsen, K. H Hedlin, G, Jonassen, C. M, Nordlund, B, Sandvik, L, Skjerven, H. O, Söderhäll, C, Vettukattil, R, Carlsen, K. C. L. *Equal contribution. **Dry skin and skin barrier in early infancy.** Br J Dermatol. 2019; DOI: 10.1111/bjd.17626

Paper IV

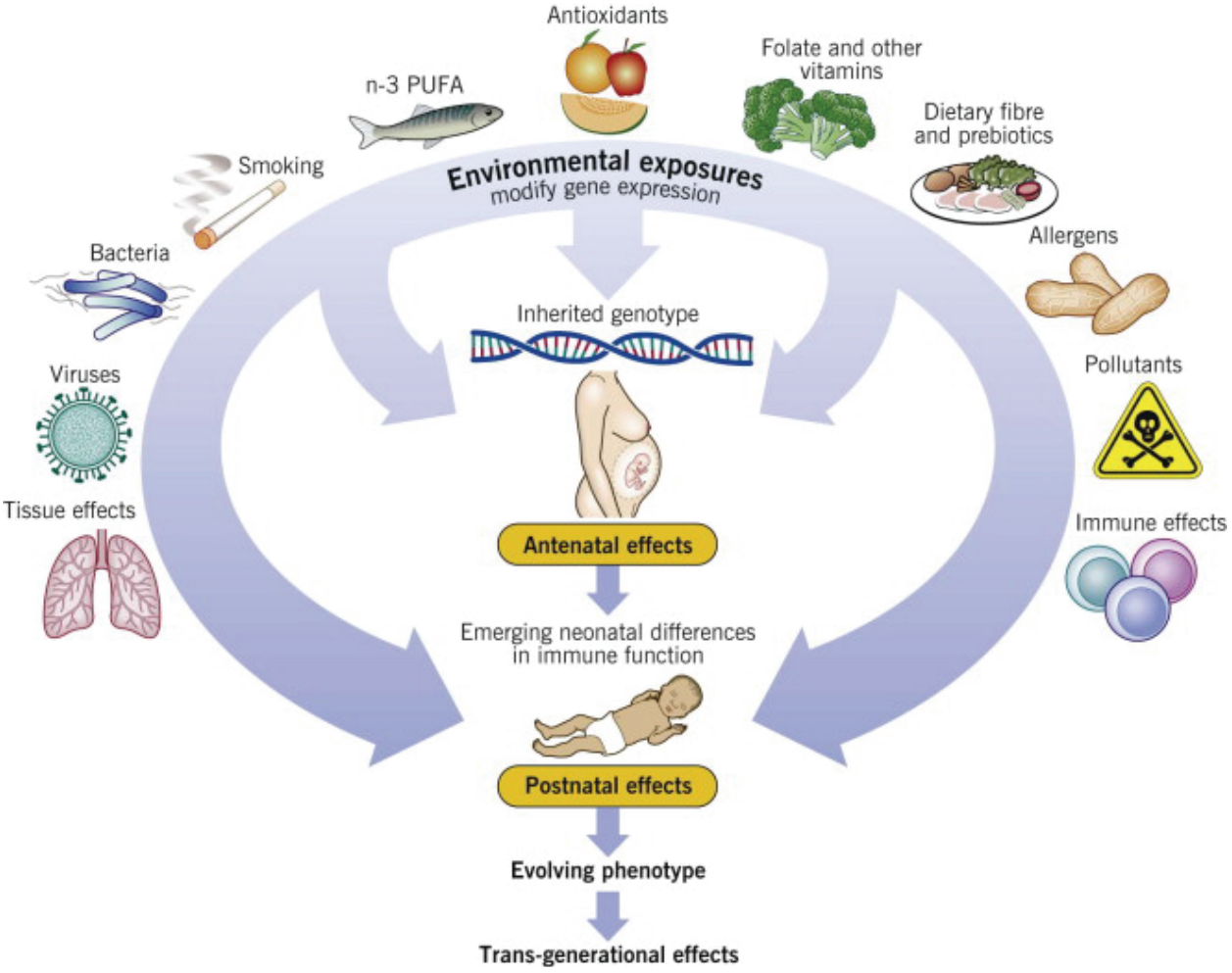
Rehbinder, E. M, Endre, K.A, Carlsen, K. C. L, Asarnoj, A, Bains, K.E.S, Berents, T. L, Carlsen, K. H, Gudmundsdóttir, H.K, Haugen, G, Hedlin, G, Kreyberg, I, Nordhagen, L, S, Nordlund, B, Sandvik, L, Saunders, C, M, Skjerven, H. O, Söderhäll, Staff, A.C, Vettukattil, R, Værnesbranden, M.R, Landrø, L. **Predicting skin barrier dysfunction and atopic dermatitis in early infancy.** Manuscript accepted 17.09.19 in JACI: In Practice

2 General Introduction

Allergic diseases such as atopic dermatitis (AD), asthma, food allergy and allergic rhinitis have over the last 50 years reached epidemic numbers affecting both young and old in industrialized as well as developing countries (1, 2). Identifying risk factors for optimal management as well as establishing effective primary prevention strategies are in demand to reduce the burden of disease.

More than one allergic disease co-exist more often than by chance in the same individual (3), pointing to their co-morbidity and a possible common origin. A genetic susceptibility to allergic diseases (4) has long been recognized, but the dramatic increase in prevalence is likely due to environmental changes involved in immune dysregulation (5). Several environmental factors have been proposed as shown in Figure 2-1, such as reduced diversity of environmental and gut microbiota (6), increased exposure to environmental toxins (7), exposure to tobacco or nicotinic products (8) and reduced food diversity (9). These factors can further interact with genes, causing reversible and irreversible epigenetic modifications possibly leading to the development of inflammatory conditions (10). The skin, enveloping our body and being and an interface to the surrounding environment is biologically more susceptible in infancy to irritants, toxins and infections (11, 12) compared to later in life. An optimal barrier capacity is not established for at least one year following birth (11-13), which is highly dependent upon the genetic predetermination in combination with the modifying effect of the environmental exposure (11). Allergic diseases often start with AD, food allergy or both in early infancy, followed by wheeze or asthma and allergic rhinitis in childhood. The debate around this concept of the atopic march has been ongoing for years (14) implying that the manifestation of one allergic disease may lead to another and indicating a causal relationship (15).

Figure 2-1 Early gene–environment interactions in the pathogenesis of allergic disease. A wide range of environmental factors, acting antenatally or postnatally, influence the maturation of immunologic competence and thus modulate risk for development of allergic diseases. In addition to effects on early gene expression patterns, some of these factors could modify local tissue milieu during early immune programming. (Reprinted with permission from Holt PG, Sly PD, Prescott SL. Early life origins of allergy and asthma. In: Holgate ST, Church MK, Broide DH, Martinez FD, eds. Allergy: principles and practice. 4th edn. London: Elsevier; 2012.)



2.1 Allergic diseases; definitions, epidemiology, diagnosis and pathogenesis.

Allergic diseases afflicting 40% of those younger than 70 years are the most common non-communicable diseases (NCDs) in childhood and have large individual and societal impact (16, 17). Allergic diseases encompass asthma, allergic rhinitis, AD, food allergy and other less prevalent diseases, such as allergic reactions to drugs or venoms. Allergic rhinitis had the first observed increase in incidence already from the end of the 19th century (2). The frequency of all these disorders has increased worldwide, particularly since the 1960-70ies, and mainly in children (2, 6, 18). This generation born during the period has now themselves become parents

and multigenerational studies are important for studying the possible impact of cross-generational transmission of allergic disease risk through genetics, epigenetics and shared environment (19).

2.1.1 Atopic dermatitis

Atopic dermatitis is an inflammatory skin condition, characterized by an intense itch. The first known description of itchy dermatologic skin conditions is found in the Papyrus Ebers from Egypt, dating back to 1500 B.C., being the oldest known translated document on ancient medical practice (20). Ancient Chinese traditional medicine describes entities consistent with infant eczema (21) and the earliest mentioning of an atopic syndrome was by the Roman historian Suetonius of emperor Augustus (born 63 B.C.) describing symptoms of itchy rash, seasonal rhinitis and chest tightness (22). Avicenna, one of the most prominent physicians of the middle-ages described several conditions consistent with modern knowledge of AD and dry skin. The condition had gone through many names such as; morbis cutaneis, porrigo larvalis, teigne muqueuse, strophulus confertus, lichen agrius, Besnier´s prurigo and many more, before the term eczema, originitating from the Greek ekzein meaning to erupt or boil over, was introduced by the two English physicians Willan and Bateman in the early 19th century (21).
In the beginning of the 20th century von Pirquet introduced the term allergy as an abnormal reaction to any substance. Not long after, in 1923 the pioneers in immunology research, Coca and Cooke proposed the term atopy from the greek atopia meaning “out of place” or “strangeness.” Ultimately, atopic dermatitis introduced in 1933 by Wise and Sulzberger, replaced the numerous names from the past, although still leaving atopic eczema and eczema in use (21).

This lack of consensus concerning the nomenclature has led to an ongoing debate and some confusion. The European Academy of Allergology and Clinical Immunology (EAACI) proposed a nomenclature in 2001, further revised by World Allergy Organization (WAO) in 2004

supporting the term eczema for when IgE sensitization is unknown and atopic eczema/atopic dermatitis when IgE sensitization is confirmed (23). Despite this recommendation from the WAO, the nomenclature has continued to be inconsistent and a theoretically based terminology, that is not easy to understand neither for patients nor for clinicians unfamiliar with allergology. Atopic dermatitis, atopic eczema (AE) and eczema has continued to be used interchangeably, however a systematic review from 2016 found that the term most commonly used was AD, followed by eczema and then AE. Interestingly, the term eczema was more often used in conjunction with eczematous disorders such as “hand eczema”, “dyshidrotic eczema” and “contact eczema” than when having AD in mind (24). Eczema is therefore considered by many experts to be an ambiguous name, and due to the need for a uniform nomenclature, AD has recently been recommended as the preferred term (24, 25). In this thesis the term AD will be used when referring to the disease.

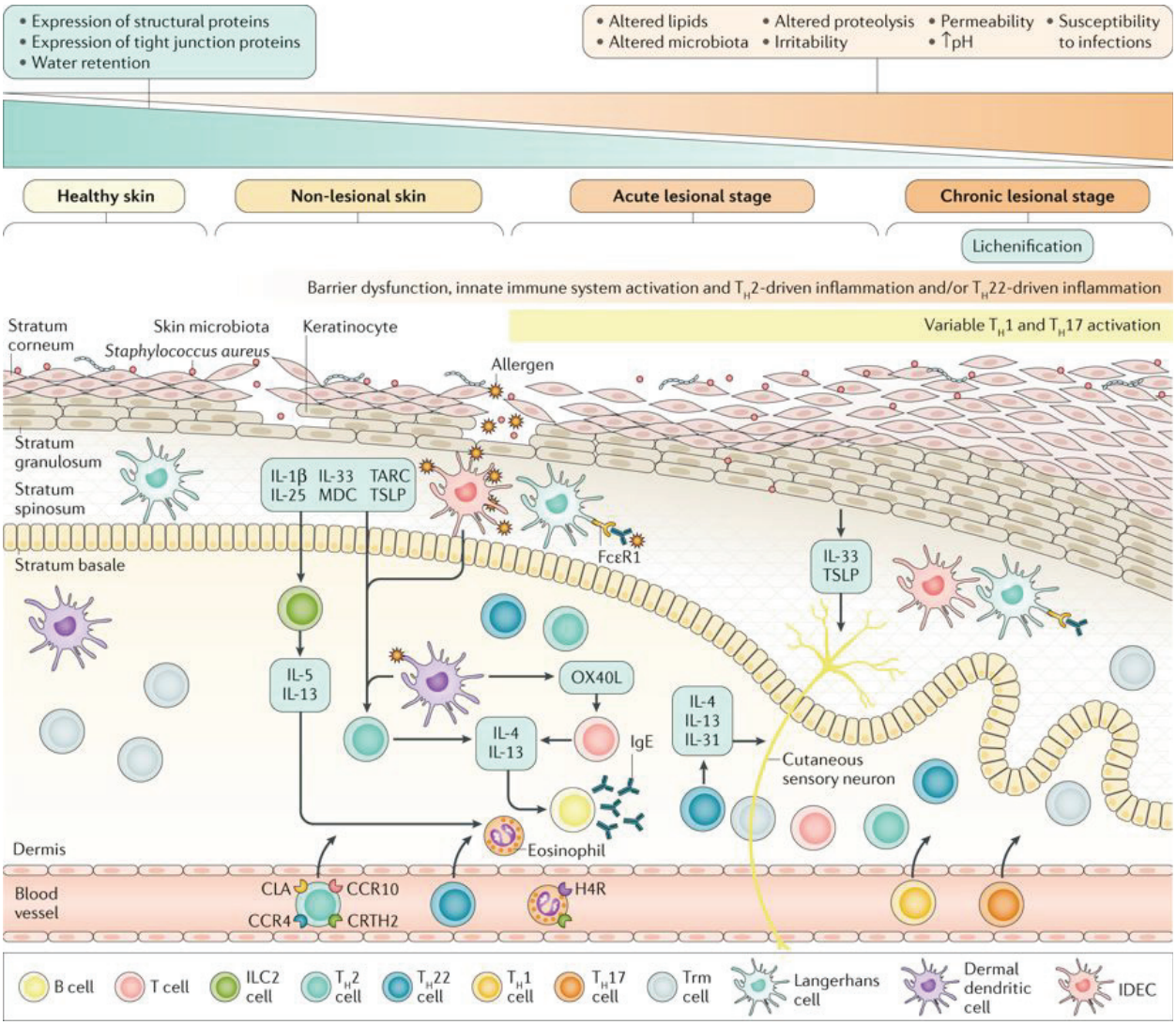
Atopic dermatitis is the most common inflammatory skin disease in the developed world (16). In children, the global prevalence varies greatly from around 1% up to 25%, with the highest prevalence rates in Sweden, Latin-America and Africa based on the phase three International Study of Asthma and Allergy in Childhood (ISAAC) study reported in 2009, including 165 centres from 65 countries (26). The incidence of AD peaks in infancy, but can appear for the first time at any age (16). In young children the incidence has been increasing during the last decades in industrialized countries, including the Nordic, but now seems to be settling around 15-20% (1, 27, 28). However, the prevalence in developing countries is still on an increase (26, 29). Also, several large American epidemiological studies report higher prevalence rates, around 7-10%, of adult AD than previously reported (1, 16, 30).

Diagnosing AD can be challenging, especially in infants and in mild cases, where itch, the cardinal feature of AD may not be apparent (31). In infants, the skin inflammation commonly

appears on the cheeks and extensor surfaces, while in toddlers and later in childhood the areas of predilection for the eczematous lesions are the flexures of elbows and knees. Hands, face and neck are also often affected, especially in the adult population (32). Many different diagnostic criteria have been developed for AD, however the most widely used and validated are the Hanifin and Rajka diagnostic criteria (33) and the UK Working Party diagnostic criteria (34). The presence of eczema on predilection areas, itch, the chronic and relapsing nature, family history of allergic disease, the presence of other allergic diseases in the patient and dry skin are the most important features and criteria for diagnosing AD.

Three fundamental elements are involved in the pathogenesis of AD, skin barrier disruption, immune dysregulation and an imbalanced skin microbiota (16, 32), as seen in Figure 2-2. In atopic skin the expression of epidermal barrier proteins and lipids are reduced, which again reduces the expression of tight-junction proteins (35). A barrier disruption can be in response to inflammation and scratching, but can also be a primary defect. The most commonly reported epidermal protein in relation to AD is filaggrin (36), a filament aggregating protein that binds keratin fibers in epithelial cells and strengthens the skin barrier by hydration, keeping the pH acidic and protecting against UV radiation. Single loss-of-function mutations in the gene encoding filaggrin *(FLG)* are observed in approximately 10 % of the Caucasian population and they have a three-fold risk of developing AD (16, 36). Previously, AD was considered to be a primary immunologically driven disease with secondary barrier disruption. Recent evidence favours a primary barrier dysfunction (16, 36) supporting the outside-inside hypothesis (37), where the entry of external allergens and irritants offsets a complex immunological reaction as seen in Figure 2-2, driving the inflammation and further disrupting the barrier as well as promoting a colonization with *Staphylococcus aureus* (38).

Figure 2-2 In atopic skin, epidermal barrier disruption as well as microbial dysbiosis stimulate keratinocytes to express chemokine and cytokines activating and driving the type 2 immunity, which further exacerbates pruritus directly through sensory neurons. Reprinted by permission from Copyright Clearance Center´s RightsLink service: Springer Nature Reviews Disease Primers, Atopic dermatitis, Weidinger et al. 2018.



The mainstay treatment of AD is directed towards skin barrier enhancement with daily emollients, and topical anti-inflammatory agents containing corticosteroids or calcineurin inhibitors, as well as local antiseptic agents in case of superinfection (39). Ultraviolet-therapy (39) and systemic anti-inflammatory treatment is reserved for patients with moderate to severe disease (40). Patients with AD have increased risk of several comorbidities (16, 41, 42); other allergic diseases, infections, cardiovascular disease, autoimmune disease and mental health disorders such as attention deficit and hyperactivity disorder (43), depression and anxiety. Atopic dermatitis reduces the quality of life considerably for the patients themselves as well as for the caregivers (16, 44, 45). The economic burden of AD is significant (32, 44, 46), both on an individual and societal level.

2.1.2 Food allergy, asthma and allergic rhinitis

Global documented food allergy prevalence is up to 10%, and usually between 5-8%, however it depends on age, the food in question as well as geographical location, with highest prevalence in children less than 5 years old from Australia, Canada, United States, United Kingdom and Finland (18, 47, 48). The mechanisms leading to food allergy are unknown. However, a current dual-allergen exposure hypothesis suggests that transcutaneous sensitisation to allergens through an impaired skin barrier may offset an allergic immune response, particularly in the absence of allergens first being introduced via the gastrointestinal tract (49, 50). This may lead to a disruption of the immunological and clinical tolerance to the food consumed, resulting in IgE-mediated reactions, diagnosed by clinical history ranging from mild unspecific symptoms to anaphylaxis. The diagnostic investigations include a careful medical history, documentation of allergic sensitisation via skin prick test or serum specific IgE and preferably an oral food challenge test. The immunological response in non-IgE mediated food allergies is less clear, which also makes the diagnostics more demanding (47).

Asthma, characterized by chronic airway inflammation, is one of the most common chronic diseases, affecting up to 20% of children in the age of 6-7 years (18, 51). The adult prevalence varies considerably among countries from 1 to 21%, and the global burden of asthma symptoms has increased by 30% in the last 20 years (52). Asthma is defined by a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, as well as variable expiratory airflow limitation (53).

Allergic rhinitis steady increase in prevalence for the last decades has not yet come to a halt, and up to 25 % of all children and 40 % of adults are subjected to this disorder of the nose (18, 54). Symptoms consist of sneezing, nasal obstruction and mucous discharge often accompanied by

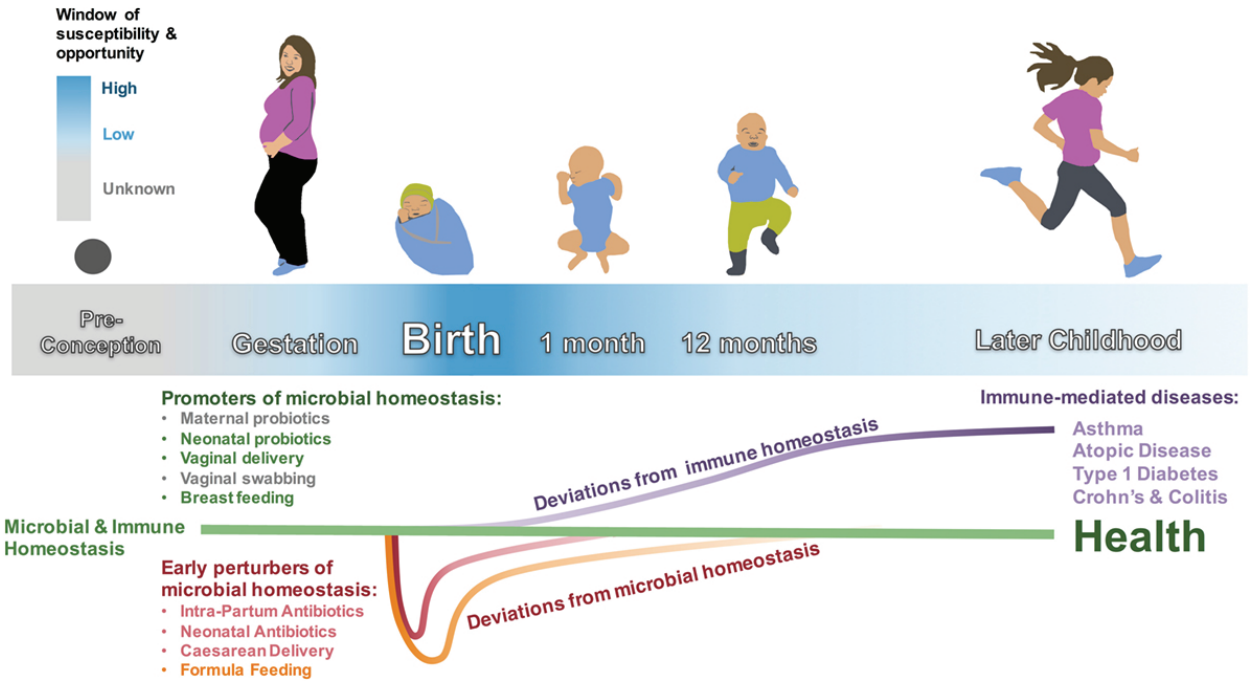
allergic conjunctivitis, tiredness and reduced concentration induced by an IgE-mediated inflammation after allergen exposure (54, 55).

2.2 Allergic disease and early microbial colonization

Over the last decades, with the discovery of culture independent techniques such as sequencing, the human microbiome research has made an effort to map and identify microbial communities living in and on the body surface (56, 57). Ongoing research is now focusing on how the human microbiome, and especially how the gastrointestinal microbiome plays a role in the development of health and disease (58). The establishment of the human microbiota has been considered to start during birth, with the amniotic membranes working as a barrier to the maternal live microbes in an uncomplicated pregnancy (59). The sterile womb paradigm has been challenged (60), by findings of a placental microbiome (61), as well as linking placental microbiota to adverse birth outcomes (62). There have also been suggestions of a unique amniotic fluid microbiome in uncomplicated pregnancies, with overlapping bacterial phylotypes identified in corresponding placenta and meconium (63). Although, some of the recent reviews point to an intrauterine colonization (64-66), there are still arguments for a sterile womb, including contamination bias in molecular studies (67) as well as the existence of germ-free mammals (60, 68). In addition, the delivery mode appears crucial for the infants' first colonization, with vaginal bacteria predominating among the vaginally born infants and bacterial skin commensals among the infants delivered by caesarean section (CS) (69).

There is substantial evidence pointing towards a critical window of opportunity in early infant life influencing the development of the microbiota and its interaction with the immune system (70-73), as outlined in Figure 2-3.

Figure 2-3 Microbial and immune homeostasis from preconception to early childhood. The window of susceptibility and opportunity represents the period around birth when promotors of microbial homeostasis have the largest effect on correcting microbial dysbioses, with an unknown extention into gestation and possiby even preconception. Reproduced with Creative Commons Attribution License (CC BY) from Amenyogbe N, Kollman TR and Betn-Othman R (2017) Early-Life Host-Microbiome Interphase: The Key Frontier Immune Development. Front. Pediatr. 5:111. Doi: 10.3389/fped.2017.00111



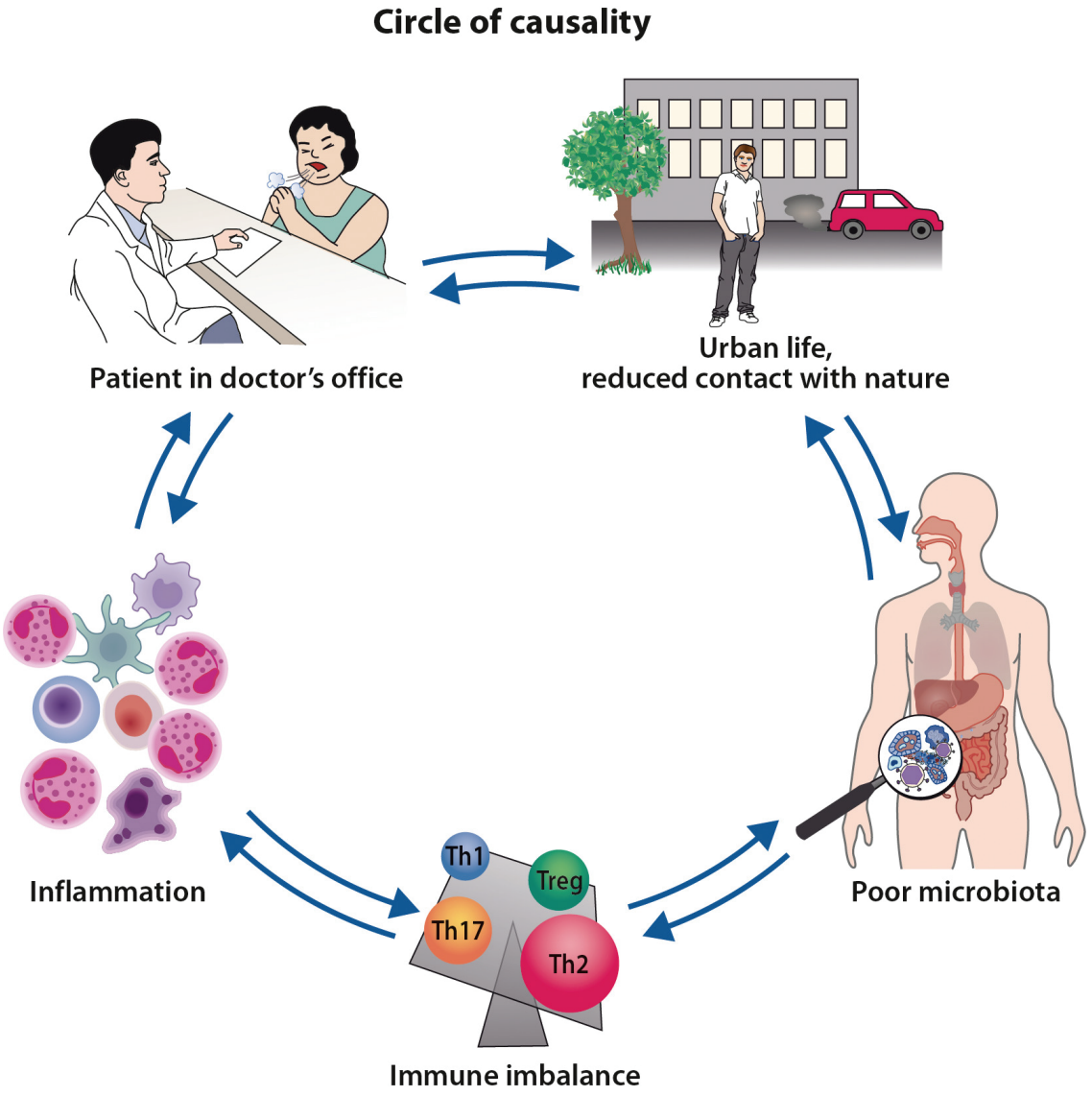
The maternal gut microbiota influences the development of the offspring immune system, and a maternal gut dysbiosis has been associated with immune-mediated diseases in the offspring, including allergic disease (73-76). Bacterial gut metabolites known as short chain fatty acids (SCFAs) can modulate the immune system both in utero and after delivery, most likely by increasing the epithelial barrier function and exerting anti-inflammatory effects (70, 72), also in the airways (77). Caesarean delivery is associated with allergic disease, especially asthma and food allergy (73, 78, 79). Caesarean section has also been linked to a reduced infant gut microbial diversity (80), which in turn has been associated with allergic disease (81). Reduced gut diversity has also been associated with allergic disease regardless of mode of delivery (82, 83).

Breastfeeding is beneficial for many reasons in infant development, including for the infant gut microbiota promoting the growth of *Lactobacilli* and *Bifidobacteria* (71-73), suggesting a

protective effect against allergic diseases as seen in studies with unpasteurized farm milk consumption (66, 72). There is no doubt that breastfeeding when feasible is superior to formula-feeding in promoting infant health, but the evidence concerning allergic diseases show conflicting results (84). Antibiotic exposure may negatively impact a healthy gut microbiota and can lead to changes in the immune function (71), especially early in life (80, 85). Antibiotics used during pregnancy as well as in infancy, and even pre-conceptually have been found to increase the risk of allergic disease (72, 73, 86-88).

Another important aspect in the development of the human microbiota is the early-life environmental exposure to microbes, linking the epidemics of allergic disease with modern time urbanisation which decreases environmental biodiversity (89), illustrated in Figure 2-4. Several studies have found association between urban upbringing, reduced human microbial diversity and allergic disease (6, 89, 90). While small-scale agricultural life style (6, 91) and green environment (90) has been linked to a decrease in allergic disease development (89). Although urban life leads to less biodiversity, exposure to microbiota from home pets as well as pests such as cockroaches and mice in the first year of life has been found to reduce the prevalence of atopy and wheezing in some studies (92) while others have failed to show any significant impact on allergic disease development (93). Also, there may be differential effects related to dog exposure which early in life has been found protective for AD, and cat exposure that may have the opposite effect (94).

Figure 2-4 The circle of causality from the megatrend of urbanisation to increase in non-communicable inflammatory diseases, or the other way around from symptoms to background factors. Reproduced with Creative Commons Attribution License (CC BY) Haahtela, Allergy, DOI: (10.1111/all.13763)



Strachan was one of the first to propose the "hygiene hypothesis"(95), suggesting that the decrease in family size, higher standard and increase in personal cleanliness were implicated in the allergic disease epidemics. The "biodiversity" hypothesis has later been proposed and developed further focusing on the early exposure to diverse organisms priming the immune system to identify and distinguish between harmful and harmless agents (96). Future studies will identify more precisely the composition and the timing of microbial exposure and if targeted microbial exposure, probiotics, prebiotics or synbiotics have a role in the prevention of allergic disease. The nomenclature used in microbiome research (97) is outlined in Table 2-1.

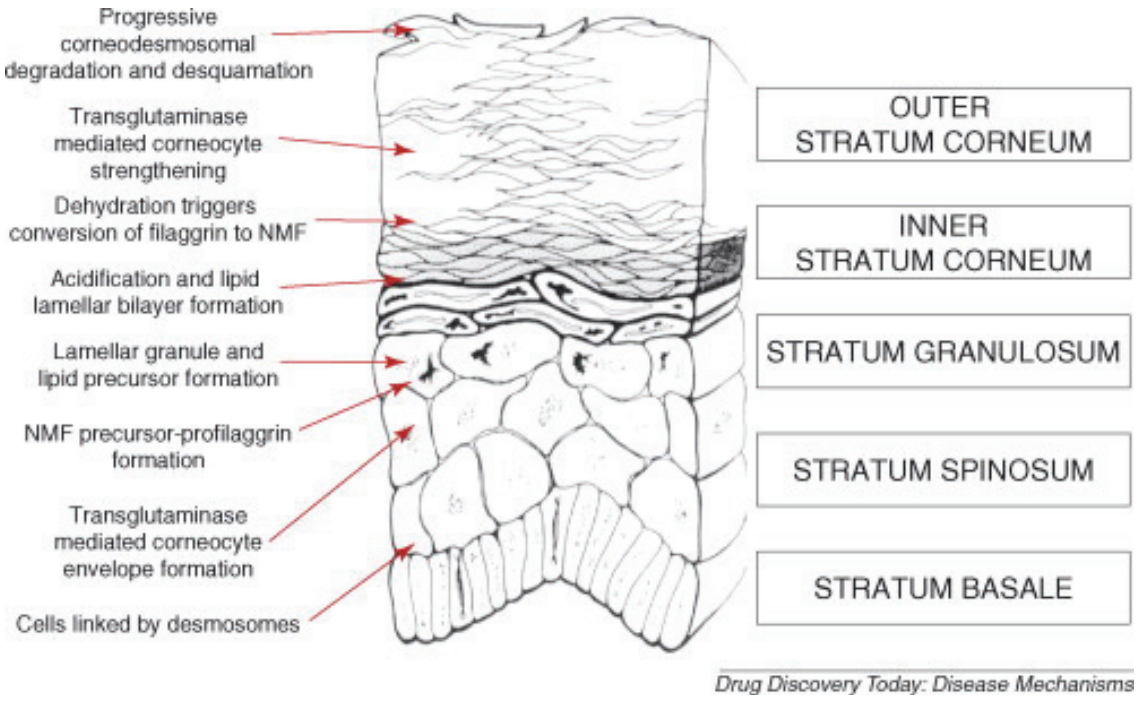
Table 2-1 Nomenclature used in microbiome research.

Microbiome	A microbial community occupying a well-defined ecological niche and having distinct physicochemical properties. This refers to the micro-organisms involved, their genomic elements as well as their spectrum of activity.
Microbiota	A community of micro-organisms (bacteria, archeae, virus, fungi,protozoae) that occupy a well-defined ecological niche.
Diversity	A calculated index that incorporates measures of microbial richness and species distribution.
Dysbiosis	Descriptive term for imbalance in a microbial ecosystem; for example, dysbiosis of the intestinal or respiratory tract associated with a disease state compared with health.
Probiotics	Live microorganisms, when administered in adequate amounts confer a health benefit for the host.
Prebiotics	Mainly non-digestible food components that benefit the host by selectively stimulating the growth and activity of microorganisms.
Synbiotics	Use of a combination of prebiotics and probiotics producing synergistic health benefits.

2.3 Skin barrier, transepidermal water loss and dry skin in atopic dermatitis.

A dysfunctional skin barrier is thought to be critical to the early onset and severity of AD, mainly due to alterations in skin barrier properties in the outermost layer of the epidermis, the stratum corneum (32, 98, 99). The epidermis consists of four layers (100, 101) as depicted in Figure 2-5, all serving important functions to preserve the physical barrier (100). The dermis, mainly composed of connective tissue, is tightly connected to the epidermal stratum basale, contains blood vessels, hair follicles, glands and sensory neurons, while the innermost layer of the skin, the subcutis contains adipose cell, fibroblasts and macrophages. In these three layers immune cells interact and communicate with other cells as well as the skin microbiota, forming a complex immunological barrier (100).

Figure 2-5 Typical structure of the epidermis and crucial steps in formation of stratum corneum (reprinted with permission from A.V. Rawlings,P.J. Matts,C.D. Anderson,M.S. Roberts, Skin biology, xerosis, barrier repair and measurement, Drug Discovery Today: Disease Mechanisms, 2008).

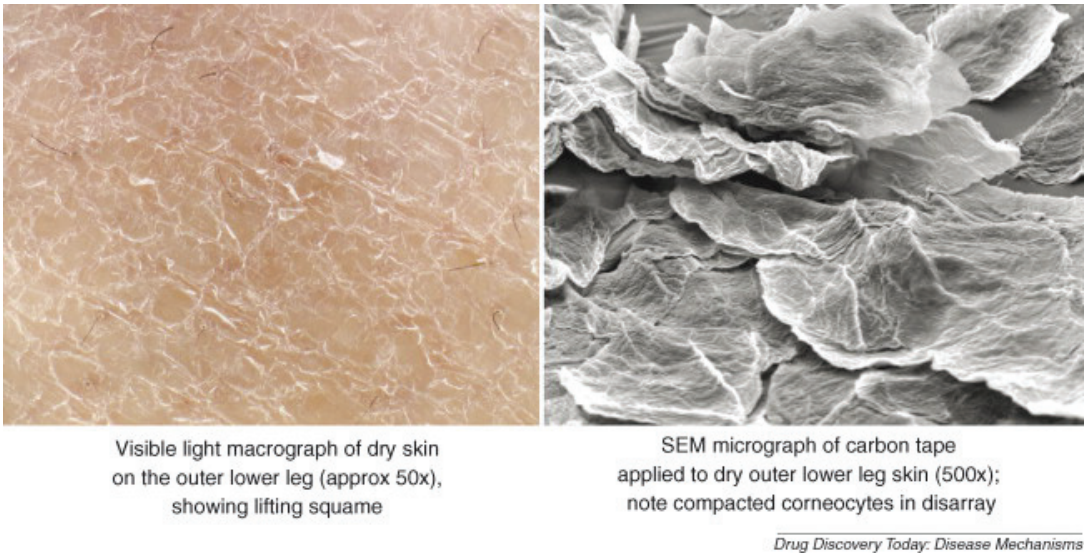


During the first months of life the newborn skin adapts rapidly to the new environment. In full-term newborns, born around 40 weeks of gestation, the skin has a good water-holding capacity during the first few days demonstrated by low TEWL values. This is probably due to water binding moieties provided by the vernix (102). Post-term neonatal skin having less vernix may experience longer direct exposure to amniotic fluid, which can disrupt the stratum corneum lipid bilayer (102, 103), and promote post-term skin dryness and higher TEWL values. After the first few days, infant skin water holding properties decreases and TEWL increases, followed by an gradual increased hydration, especially until 3 months of life (104). The skin hydration properties stabilizes around 12 months of age and resembles that of older children and adults (105). However, the optimization period of the skin barrier function can even last up to around two years of age, and is largely dependent on the infant genetics in combination with the exposure to environmental factors such as, climate, soaps and other irritants (11, 12, 102). The anatomical sites of TEWL measurements varies greatly both in infants (106) and in adults (107), and the level of TEWL varies among the different anatomical skin areas. One of the most

common sites of measurement, the volar forearm, has been found in infants to be among the sites displaying most TEWL variation (106). A recent study compared TEWL measurement on the lateral upper arm to the volar forearm and found a possibly better differentiation in TEWL values between no AD, possible AD and AD when measuring on the lateral upper arm (108).

Dry skin may be a condition in itself or a sign of disease and is one of the cardinal features and a diagnostic criterion for AD (33, 34, 109). Dry skin can be observed clinically as accentuation of skin markings and fine scales, and demonstrated in electron microscope as accumulation of corneocytes due to inefficient degradation of corneodesmosomes (101) presented in Figure 2-6. Dry skin is associated with higher TEWL in adult AD patients compared to healthy controls (110). There is limited documentation on the manifestation of dry skin in infancy, particularly at AD predilection sites, and its association to TEWL without the presence of AD. In a Swedish case-control study clinically dry skin was observed in 40% of ninety-nine healthy two-year old children and in all the 221 children with AD (111). In another Swedish study, dry skin was reported in 37% of 1922 eight-year-old children through questionnaires (112); 17 % also had AD manifestations and 94 % of those with AD also had dry skin. A German study reported dry skin in 25% of healthy young adults (113), while an Indian study found dry skin in 14% of the 100 healthy children up to 12 years of age (114).

Figure 2-6 Common dry skin (reprinted with permission from A.V. Rawlings,P.J. Matts,C.D. Anderson,M.S. Roberts, Skin biology, xerosis, barrier repair and measurement, Drug Discovery Today: Disease Mechanisms, 2008).



A dysfunctional skin barrier in AD patients has been found to increase TEWL, in both lesional and non-lesional skin (98, 110, 115). A possible way of detecting infants with an impaired skin barrier prior to developing AD has been proposed using measures of TEWL (116-118). These findings support the hypothesis that skin barrier dysfunction is a primary predisposing factor for AD development (37, 99, 115). Caucasian children with *FLG* null-mutations often exhibit AD in the most exposed areas such as cheeks (119) and extensor parts of extremities, especially dorsal part of the hands, compared to wild-type, and in a small study of 3 months old infants *FLG* loss-of-function mutations was associated with increased TEWL, not only in children with AD, but also in children without AD (120).

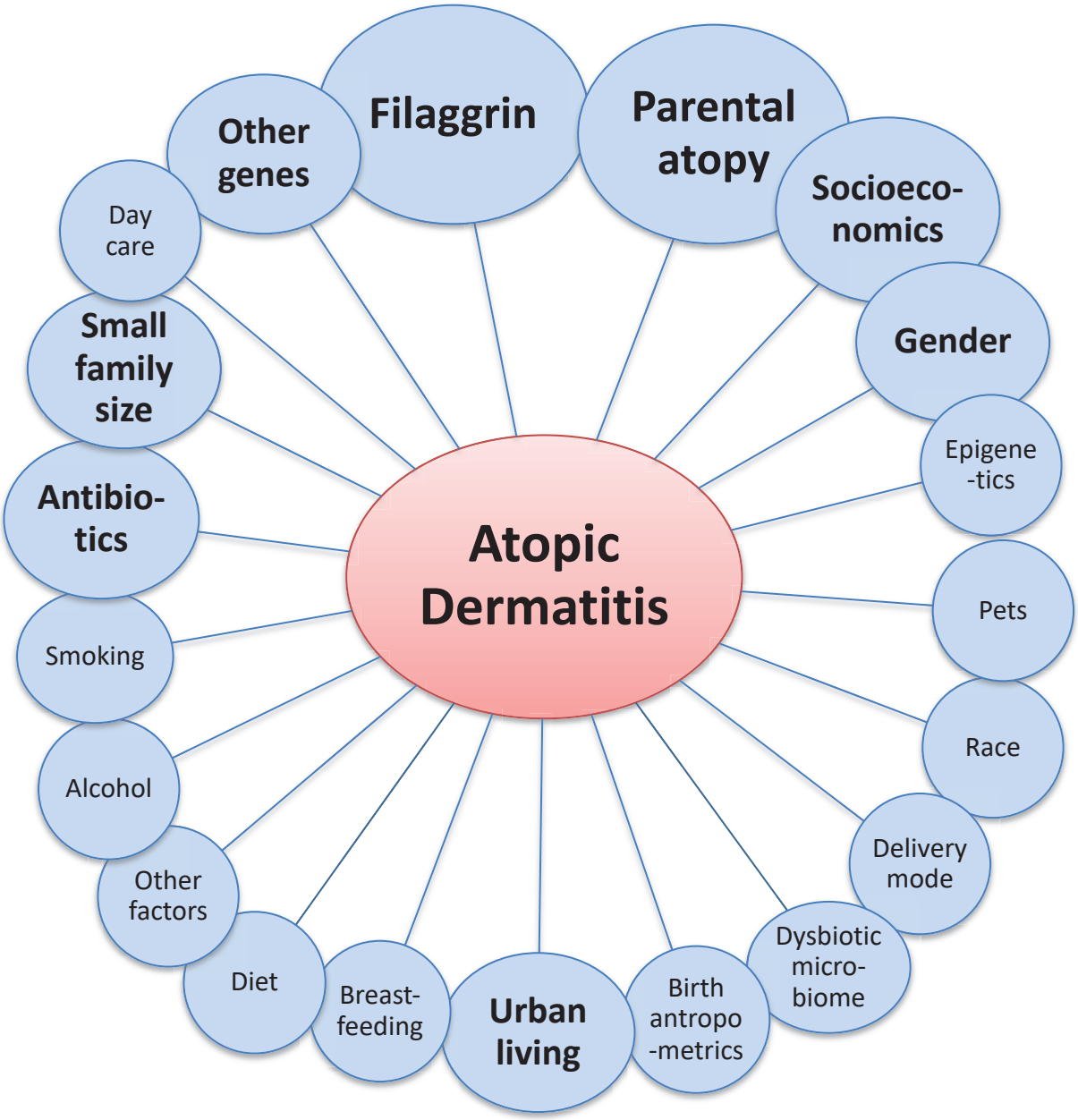
2.4 Prediction of atopic dermatitis and skin barrier dysfunction

In order to distinguish between clinical AD phenotypes and identify individuals eligible for potential preventive strategies as well as personalized medicine there is an imperative need to determine predictors for AD. Observational biomedical research is often conducted to detect risk factors for a certain disease, either to look at risk stratification or prediction or on the other hand to assess causality. Some risk factors can be both predictor and/or explanatory factors, making

the term risk factor imprecise, and which may lead to confusion (121). In this thesis when addressing risk stratification and using prediction models, the term predictor or predictive factor is therefore used. The term "risk factor" may imply both a predictor and an explanatory factor. A predictor can in many cases also be an explanatory factor, but would require a different methodological approach to determine. In order to identify significant predictors for such a heterogeneous disease as AD, large longitudinal observational cohort studies are needed to find potential risk factors that can be included in a prediction model. Many risk factors have been studied and it is not always clear whether these risk factors are predictors, if they address causality or both. An overview of the many pre, peri- and postnatal risk factors studied (16, 94, 122) can be found in Figure 2-7. I have focused on the risk factors for AD with high level of evidence and it is beyond the scope of this thesis to elucidate further.

The first description of heritability of AD dates back to when Emperor Augustus' grandson and great grand-nephew experienced the same dry, itchy patches as him more than 2000 years ago (22). Familial allergic disease has later become a well-established predictor as well as explanatory factor for offspring AD, with any parental allergic disease increasing the risk for offspring AD 1,5 fold while parental AD increases it 3 to 5-fold depending if one or both parents have the disease (16, 122, 123). Several genes have been identified and linked to AD development; most of them being involved in skin barrier development and immune regulation, and although they have provided important insight into the pathogenesis of the disease they have small effect sizes (123). Until present, the only identified genetic predictor is the gene encoding filaggrin, with 3-fold increase in AD prevalence in individuals carrying the *FLG* null-mutation (16, 36). Although AD is closely associated with a dysfunctional skin barrier and dry skin, there are to our knowledge no previous studies that have investigated possible predictors for reduced skin barrier through increased TEWL or infant dry skin.

Figure 2-7 Prenatal, perinatal and postnatal risk factors studied for atopic dermatitis. The size of the font express the level of evidence. The largest font has high level of evidence, medium font has medium level of evidence and smallest font has low level of evidence often with conflicting results.

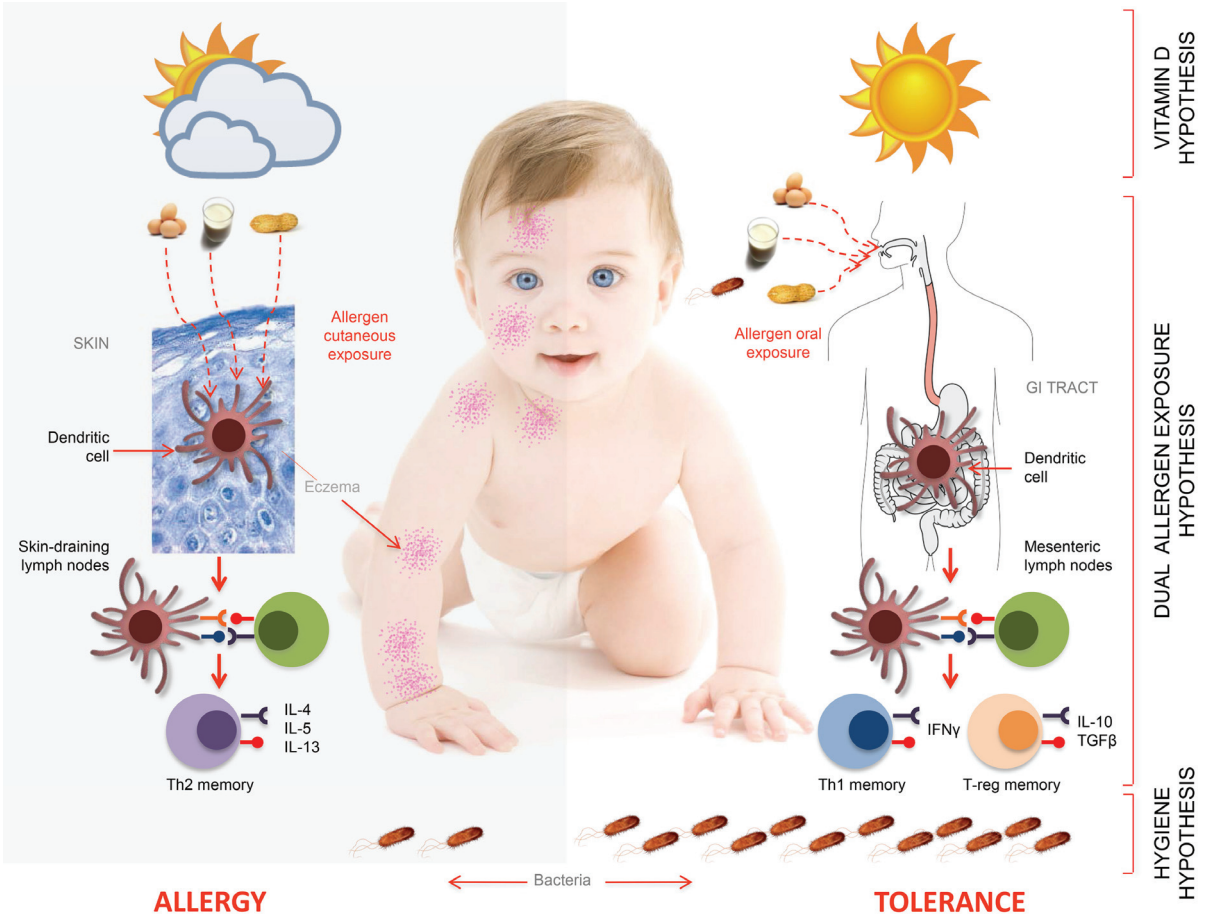


2.5 Atopic dermatitis; A role in the atopic march and in preventing allergic disease?

Recent evidence points to AD in infancy predisposing to food allergy development (124), as well as asthma (125) and allergic rhinitis (115). Increased TEWL during early infancy has been associated with as well as predicting AD development later in childhood (116-118). These findings support the hypothesis that skin barrier dysfunction is a primary predisposing factor for AD development (37, 99, 115), making skin barrier therapy a possible target for primary prevention of AD. A few small clinical trials in high risk infants (less than 130 infants in each)

suggest that regular emollients from early infancy may reduce AD (126, 127), and a pilot study from 2012 demonstrated significantly reduced xerosis and a tendency towards reduced AD using a paraffin-emulsion based bath oil to 6-week old infants with xerosis (128). The promising results of these studies have led to larger clinical trials aiming to prevent AD and possibly the atopic march with skin barrier therapy starting in early infancy (129-131). Aggressive treatment of early manifestations of eczema combined with oral introduction of egg, significantly reduced the incidence of egg allergy (132). Strategies for prevention of food allergy might be primary, before the onset of IgE-sensitization or secondary before clinical food allergy, but after the already established IgE-sensitiziation (49). The dual allergen exposure hypothesis, illustrated in Figure 2-8, suggest that early oral exposure to food allergens promotes tolerance, whereas cutaneous exposure to food proteins through a disrupted skin barriers may facilitate allergic sensitization and lead to food allergy (49, 50). This has been observed in infants with AD (133, 134), as well as those with increased TEWL regardless of AD (135). Primary prevention of allergic disease should therefore start early and target both AD and food allergies (50).

Figure 2-8 The dual allergen exposure hypothesis. Reproduced with Creative Commons Attribution License (CC BY) Du Toit et al. JACI 2016, 10.7196/SAMJ.2017.v107i10.12418



3 Objective and specific aims of the thesis

In order to better understand the nature of AD and possibly select infants for potential prevention strategies of AD and other allergic diseases the objective of this thesis was to identify predictors for dysfunctional skin barrier and AD in early infancy.

Specific aims

1. To identify prenatal factors that predicts impaired skin barrier function or AD in early infancy. (Paper I and IV)
2. To identify perinatal predictors for impaired skin barrier function and AD in infancy, also exploring mode of delivery and a potential amniotic fluid microbiome. (Paper I, II and IV)
3. To determine the prevalence and predictors for dry skin in early infancy. (Paper I, III and IV)
4. To determine if dry skin is associated with increased transepidermal water loss, and if either one of these factors predicts AD in infancy (Paper I and IV)

4 Methods and subjects

4.1 Study design

The thesis is based upon data obtained in the PreventADALL study, an international, multicenter, prospective, general population-based birth cohort study of 2396 mother-child pairs recruited in pregnancy, with a 2x2 factorially designed randomised clinical trial of two primary prevention interventions (skin care and early food introduction) in infancy. The PreventADALL study is planned for follow-up of the offspring into adult age, and currently the project period is defined from 2015-2044.

The study has two main arms; one exploratory with NCD outcomes, and one randomised clinical trial for primary prevention of AD, food allergy and other allergic outcomes. The present thesis concerns the exploratory arm of the study. *Recruitment* of the *pregnant women* included a letter of invitation enclosed in the appointment letter to the 18- gestational week ultrasound screening investigation at Oslo University Hospital, Oslo and Østfold Hospital Trust, Kalnes (both Norway). In Stockholm, several maternity hospitals and outward ultrasound clinics were approached in order to inform about the study and to facilitate recruitment at a visit closely after the 18-weeks ultrasound investigation for inclusion at Karolinska University Hospital, Stockholm (Sweden). Further written (brochures and detailed study description with the consent form) and oral study information was provided prior to mothers-to-be signing informed consent. *Maternal inclusion criteria:* All consenting mothers-to-be at the 18-weeks ultrasound investigation with sufficient language skills (Scandinavian language), gestational age (GA) 16-22 weeks. *Maternal exclusion criteria:* Plans to move outside reasonable travel distance from any of the participating hospitals within the first year of the offspring's life, pregnancy with three or more fetuses or severe fetal malformations or illness observed before or at the 18-week ultrasound examination.

Children born to the included mothers were identified on a daily basis at all the maternity wards and were included within 24 hours of birth or as soon as possible thereafter, following renewed maternal consent as well as partners consent.

Newborn inclusion criteria: Live-born infants of at least GA 35.0 weeks (including twin pregnancies) and maternal/parental willingness to further participate in the study. *Newborn exclusion criteria:* severe neonatal cardiac, pulmonary, neurologic, dermatologic disease or other severe diseases, plans to move outside reasonable travel distance from any of the participating hospitals within the first 12 months of life of the child.

Randomisation into four similar size groups with no intervention, skin care only, early food introduction only or both interventions respectively, was done with a stepped wedge design. Randomisation, pre-set at the onset of the study and unknown to the women until inclusion of the baby, was based upon postal codes and changed every three months to ensure similar intervention groups in all areas across all seasons.

The *skin intervention* from week 2 through 8 months of age consisted of combined oil baths (0.5 dl bath oil per 8 L/water) for at least five minutes, at least 4 days/week, as well as daily facial cream (Ceridal ®). The *food intervention* was complementary to the recommended breastfeeding and consisted of systematic early introduction of peanut, cow's milk, wheat and egg between three and four months of age. The minimum required intake of each food was tasting at least four days per week at least until 6 months of age, and thereafter encouraged to continue on a regular basis. A *weekly electronic diary* was completed for each child from week 2 through 26 of life, recording skin care, infant feeding and symptoms of allergic diseases. Any surplus of bath oil was assessed at 3- and 6 months visit and finally at 9 months of age (e-mail). *Adverse events* were recorded up to 12 months of age and acted upon if appropriate. Any (possible) allergic reaction to food allergens was investigated, diagnosed, treated according to general guidelines and recorded. The children were offered direct access to the local paediatric department for relevant investigations and treatment when needed in case of allergic reactions or allergic disease

symptoms. *Investigations* performed in the mothers-to-be and their children up to the child's third year of life are listed in Table 4-1, with similar investigations planned for future offspring follow-up studies throughout childhood and into adulthood. *Participant information* includes electronic questionnaires and diaries, as well as registered data during investigations, as outlined in Table 4-1.

Table 4-1 The investigations in mothers-to-be and their children up to 36 months of age in the PreventADALL study. The coloured boxes for E-diary weekly and for breast milk indicate sampling time. Reproduced with Creative Commons Attribution License (CC BY) Carlsen et al. Allergy 2018 DOI: 10.1111/all.13468

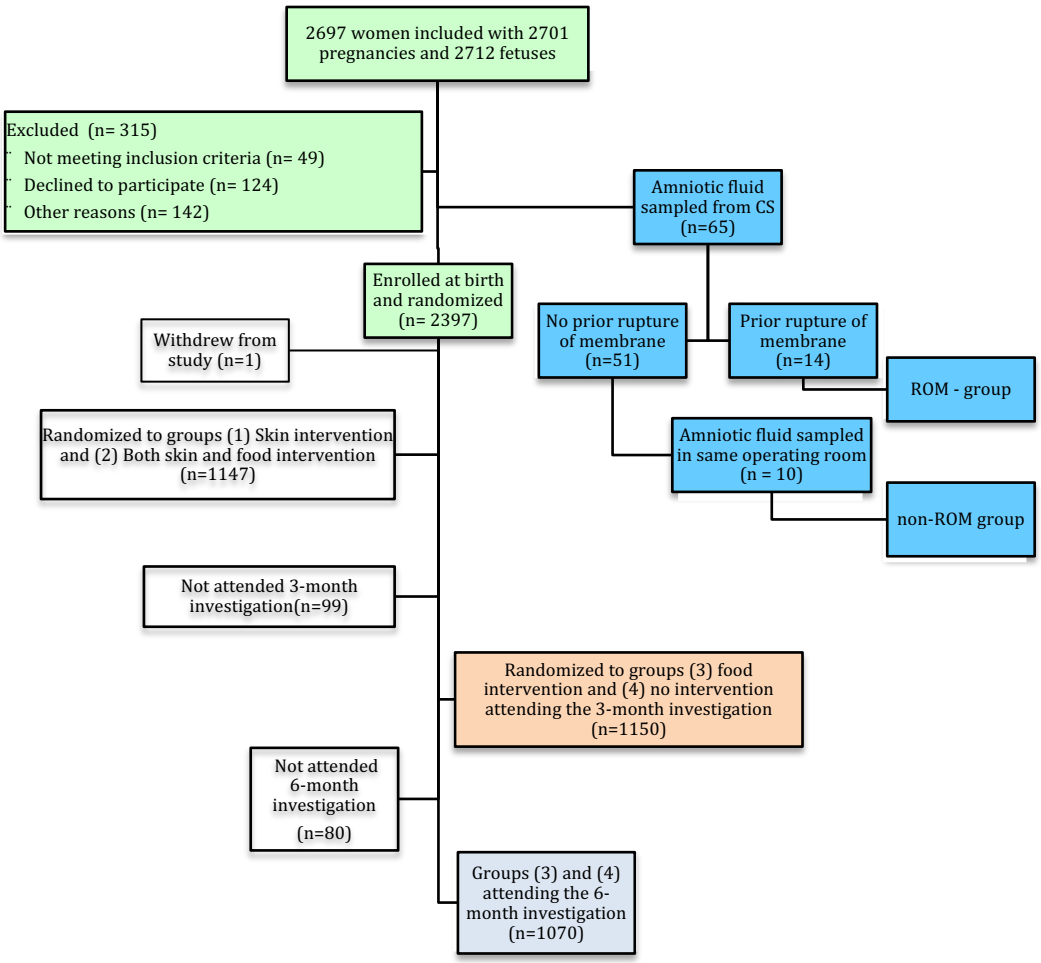
		Mother, gestational week off-spring				Months of age of child								
Assessment		**18**	**30**	**34**	**Birth**	**Newborn**	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**36**
E-Questionnaire	general	x		x			x	x	x	x	x	x	x	x
E-food frequency Questionnaire	total habitual diet	x												
E-Diary weekly	food, skin care, symptoms													
Foetal Ultrasound	foetal growth	x	x											
Physical assessment	general						x	x		x				x
Anthropometric measures	somatic size and growth	x				x	x	x		x		x		x
Skin scoring	dry skin and atopic dermatitis						x	x		x		x		x
Trans Epidermic Water loss	skin barrier assessment						x	x		x				x
Lung function							x			x				x
Skin prick test	allergic sensitisation							x		x				x
Blood pressure		x					x	x		x				x
Biological sampling														
Serum		x			x (Cord blood)		x			x		x		x
E-swabs skin/nares	microbiome analyses				x	x	x	x		x				x
Faeces	microbiome analyses	x				x	x	x		x				x
DNA/RNA blood	genetics/epigenetics	x			x (Cord blood)					x		x		x
Urine		x			x maternal		x	x		x				x
Saliva	cortisol and other investi	x					x			x				x
Vernix caseosa					x									
Placenta					x									
Amniotic fluid					x									
Breast milk														
Guthrie cards	epigenetics					x								

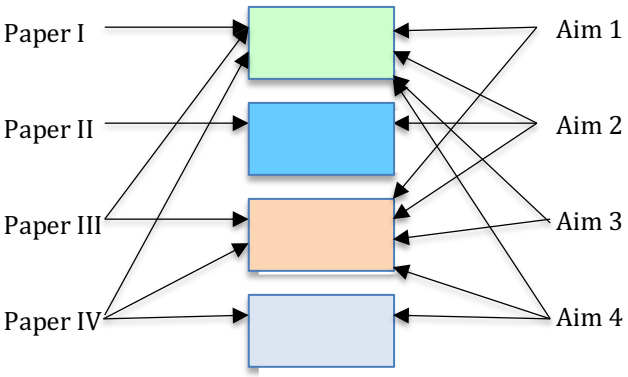
A *biobank* was established in December 2014 at Oslo University Hospital, with fully traceable samples electronically recorded in the MedInsight system (Oslo University Hospital, Oslo, Norway). At enrolment, all participating women provided signed consent after thorough written and oral study information. Upon inclusion of the baby, signed consent was renewed by the mother as well as signed by the baby's father (or co-habiting mother in case of mother-mother pairs). The study was approved by the Regional board for Medical Ethics – Health region South-East B in December 2014 (2014/518) and Stockholm, Sweden in March 2015 (2014/2242-31/4), as well as registered at clinicaltrial.gov ***NCT02449850.***

4.2 Subjects

The study subjects in this thesis are all from the PreventADALL birth cohort and are presented in Figure 4-1.

Figure 4-1 The flow chart shows the number of women and children included into the PreventADALL study, as well as the study subjects in this thesis. The colours of the boxes in the flow chart, are matched with papers I-IV and the specific aims of the thesis 1-4 using arrows and the corresponding colours. The boxes with no colour are not included in this thesis, however still part of the PreventADALL study.





A total of 2697 women with 2701 pregnancies were enrolled around 18-week pregnancy, 2149 in Norway and 552 in Sweden between December 9th 2014 and October 31st 2016. Four women were included twice with singleton pregnancies, and 17 were twin pregnancies (one in Sweden). The majority lived with their husbands (41.2%) or cohabiting partner (55.9%), 1.9% were single mothers, whereas 1% had other living arrangement. The mean (SD) estimated fetal GA at enrolment was 18.7 (min-max 15.7 - 22.7) weeks.

Mean maternal and paternal age (min-max) was 32 (18-42) and 34 (21-72) years, respectively, and most parents were born in Norway (67% of mothers and 64% of fathers) or Sweden (22% mothers and 20% of fathers). The majority of mothers (57 %) and almost half of their partners (48 %) had more than four years of college or university education, whereas 11% of the mothers and 21% of their partners had the compulsory 9-10 years schooling as their highest educational level. At least one doctor diagnosed allergic disease was reported by 42% of the mothers, and at least two by 20.1%, while 19.8 % reported AD in contrast to only 10.2 % of the fathers.

The mother-child cohort included 2383 mothers, of whom three were enrolled by the same mother but with two different pregnancies, and 2397 children (52.7% boys) including 11 twins. One mother-child pair withdrew after randomisation. The mean (min-max) GA of the newborns was 39.2 (35.6 - 42.9) weeks, and 16.4% were delivered by CS.

For the sub-populations of the women where we analysed amniotic fluid in paper II, the baseline characteristics are presented in Table 4-2, and the baseline characteristics of the 1150 3-months and 930 6-months old infants included in paper III and IV are presented in Table 4-3.

Table 4-2 Baseline characteristics for women undergoing caesarean section (CS) where amniotic fluid has been sampled without rupture of amniotic membranes (non-ROM) and with prior rupture of amniotic membranes (ROM).

Characteristics	non-ROM n= 10	ROM n= 14
Mothers:		
Age, yrs: mean (SD)	34.4 (3.6)	33.1 (3.6)
Pregnancy complications		
Clinical chorioamnionitis	0	4
GBS in urine	0	1
Antibiotics antepartum	0	5
Antibiotics intrapartum	0	14
Indications for CS:		
Maternal request	6	
Heart disease mother	1	
2 previous CS	1	
Breech and/or large for GA	1	1
Breech and fetal growth restriction	1	
Slow progression of birth		7
Fetal distress		2
Chorioamnionitis		4
ROM, hours: median (range)	-	14 (2-36)
GA at CS, weeks: mean (range)	39.1 (2.1)	40.5 (4.4)
Birth weight, g: mean (SD)	3548.6 (546.4)	3749.0 (578.7)

Table 4-3 Baseline characteristics of 1150 3 months old infants and 930 6 months old infants where those with atopic dermatitis at 3 months of age were excluded.

Characteristics	3 months investigation (N=1150)	6 months investigation (N=930)
Age mother (years), mean, (SD, min-max)(N=1150)	32.6 (4.1, 21.0-48.0)	32.5 (4.1, 21.0-47.0)
Age father (years), mean, (SD, min-max)(N=983)	34.8 (5.3, 21.0-72.0)	34.8 (5.3, 21.0-72.0)
Mother Nordic origin N (%)(N=1046)	955 (91.3)	783 (91.7)
Father Nordic origin N (%)(N=1026)	916 (89.3)	749 (89.5)
Education mother, > 4 years of University, N (%) (N=1040)	611 (58.8)	506 (59.6)
Education co-parent, >4 years of University, N (%) (N=1001)	497 (49.7)	412 (50.4)
Family income N (%)(N=1032)		
Low	153 (14.8)	123 (14.6)
Middle	751 (72.8)	620 (73.6)
High	128 (12.4)	99 (11.8)
Single mother N (%)(N= 1038)	17 (1.6)	
BMI, mother at 18 weeks of pregnancy, mean, (SD, min-max)(N=1132)	24.8 (3.7, 17.2-41.4)	24.8 (3.6, 17.2-39.5)
≥ 1 previous parity N (%)(N=1046)	430 (41.1)	335 (39.2)
Any allergic disease mother, N (%) (N=1046)	673 (64.3)	543 (63.6)
Atopic dermatitis mother, doctor diagnosed N (%)(N=1046)	216 (20.7)	169 (19.8)
Asthma mother, doctor diagnosed N (%)(N=1046)	187 (17.9)	151 (17.7)
Allergic rhinitis mother, doctor diagnosed N (%)(N=1046)	221 (21.1)	176 (22.6)
Food allergy mother, doctor diagnosed N (%)(N=1046)	137 (13.1)	116 (14.4)
Any allergic disease father, N (%) (N=1048)	522 (49.8)	416 (48.8)
Atopic dermatitis father, doctor diagnosed N (%)(N=1048)	116 (11.1)	87 (11.2)
Asthma father, doctor diagnosed N (%)(N=1048)	144 (13.7)	118 (14.3)
Allergic rhinitis father, doctor diagnosed N (%)(N=1048)	243 (23.2)	198 (25.4)
Food allergy father, doctor diagnosed N (%)(N=1048)	94 (9.0)	75 (9.2)
Lifestyle during pregnancy		
Any alcohol intake N (%)(N=914)	64 (7.0)	48 (6.5)
Any use of tobacco/nicotine l N (%)(N=1128)	121 (10.7)	93 (10.2)
Any smoking N (%)(N=1128)	51 (4.5)	36 (3.9)
Any snus use N (%)(N=1128)	76 (6.7)	63 (6.9)
Live rural N (%)(N=1046)	90 (8.6)	80 (9.4)
Exposure to humidity/mould N (%)(N=984)	136 (13.8)	114 (14.1)
Pets in general N (%)(N=1046)	250 (23.9)	207 (24.2)
Cat, no dog N (%)(N=1046)	90 (8.6)	74 (8.7)
Dog, no cat N (%)(N=1046)	123 (11.8)	103 (12.1)
Cat and dog N (%)(N=1046)	15 (1.4)	14 (1.6)
Pets other than cat and dog N %)(N=1046)	22 (2.1)	16 (1.9)
Caesarean section, N (%)(N=1137)	176 (15.5)	133 (14.4)
Elective N (%)(N=1137)	64 (5.6)	45 (4.9)
Acute N (%)(N=1137)	112 (9.9)	88 (9.6)
Gestational age at birth (weeks), mean (SD, min-max) (N=1128)	39.3 (1.7, 35.0-42.9)	39.3 (1.7, 35.0-42.9)
Female gender N (%) (N=1146)	530 (46.2)	440 (47.5)
Birth weight (kg), mean, (SD, min-max) (N=1114)	3.6 (0.5, 1.9-5.1)	3.5 (0.5, 1.9-5.1)
Born during winter season (October – March) N (%)(N=1146)	631 (55.1)	513 (55.3)
Clinical investigation		
Age (days), mean (SD, min-max) (N=1145)	93 (8.4, 55-150)	190 (13.2, 146-248)
Weight (kg), mean, (SD, min-max) (N=1118)	6.3 (0.8, 4.2-9.3)	8.1 (1.0, 5.2-12.3)
Length (cm), mean, (SD, min-max) (N=1125)	61.9 (2.3, 51.0-70.9)	68.5 (2.7, 52.0-82.7)

4.3 Methods

4.3.1 Recruitment and enrolment

An invitation to participate in the PreventADALL was enclosed with the appointment letter to all women scheduled for a routine ultrasound investigation at 18-week pregnancy at the participating study centers. At the ultrasound investigation the midwives asked all women for potential participation. The women who were willing to participate in the study were provided with a print out of standard as well as extra ultrasound measures, before attending an enrolment visit with the study team. The enrolment visit consisted of reading, discussing and signing the informed consent form, followed by a brief interview of contact details and pregnancy information, measures of height, weight and blood pressure, blood and urine samples, instructions on how to respond to questionnaires, and home sampling of fecal and salivary specimen. A copy of the national antenatal health record was retained for information on pregnancy development. The GA was estimated from ultrasound measures of femur length at enrolment (136).

4.3.2 Questionnaires at 18 weeks and 34 weeks of gestational age

Electronic questionnaires included information of health and diseases in the index mother and child, as well as in the family, details on life-style and physical activity during pregnancy, environment, stress and quality of life, as well as nutritional and food diversity in the off-spring and habitual diet in mothers (during pregnancy). All questionnaires, diaries and registration forms were developed by the study team in close collaboration with the University Center for Information Technology (USIT) at the University of Oslo - for electronic responses via computers, tablets and smart phones. The integrated frontend Electronic form (nettskjema.uio.no) tool administrating online data collection was used both for registering data obtained at the study visits by study personnel and the diaries/questionnaires completed by the mothers. Swift delivering of fully Pretty Good Privacy (PGP) encrypted answers to the projects

secure infrastructure was ensured. This includes a set of virtual machines (VMs) for managing / post-processing data and dedicated secure storage within the central TSD system (137).

4.3.3 Inclusion at birth

Newborn children of participating mothers were included at the maternity ward within the first 1-2 days of life, alternatively as soon as possible in case the mother-child left the maternity ward before inclusion. The baby enrolment visit included renewed written consent extended also to the baby's father/co-mother whenever possible, swabs for microbiome analyses of skin and nares, anthropometric measures and appropriate information and training related to group randomisation as well as instructions for home sampling. In case the infant was randomised to the group with skin intervention, bath oil and emollient were provided for the first three months, and parents were thoroughly instructed in safe handling of the baby, including hands-on demonstrations. Skin swabs and vernix were collected by midwives within 10 minutes after delivery, cord blood from late clamping and placenta samples were stored in the fridge for maximum 24 hours, and collected for further storage at -86 ^{0}C in the PreventADALL biobank.

4.3.4 Skin assessment at 3 and 6- months investigations

Study health care personnel were trained to assess the skin by visual inspection and palpation, represented in Figure 4-2. Observations of dry skin, presented as scaling and roughness, were recorded for 11 predefined anatomical skin areas as seen in Table 4-4 based on a previous publication (138). Severity of dry skin was recorded in terms of no, mild, moderate or severe dry skin in line with the principles of the Dry skin/Ichtyosis and Severity Index (DASI), but without their score of erythema (139). *Mild dryness* was categorized by barely visible scaling, slight roughness when stroking the skin; *moderate dryness* was categorized by clearly visible scaling with or without fissures, roughness when stroking the skin; *severe dryness* categorized by abundant scaling and present fissures, as well as very rough skin when stroking the skin.

Figure 4-2 Examination of skin at 6 months follow-up visit. Picture taken by mother at Oslo University Hospital and reprinted with parental signed consent.

In this thesis, AD was defined as the presence of eczematous lesions observed by study personnel and excluding differential diagnosis to AD by a trained medical doctor. The UK Working Party diagnostic criteria (109) were used for the diagnosis of AD at 3 and 6 months follow-up visits, however very few met these criteria and we therefore chose a more clinical and broader approach for AD diagnosis at this early age. In the PreventADALL study, from 12 months visits and beyond the Hanifin and Rajka diagnostic criteria (33) were used in addition to the UK Working Party diagnostic criteria. The severity of the eczema was assessed by Eczema Area and Severity Index (EASI)(140) as well as Patient Oriented Eczema Measure (POEM)(141). Severity is not included as an outcome in this thesis.

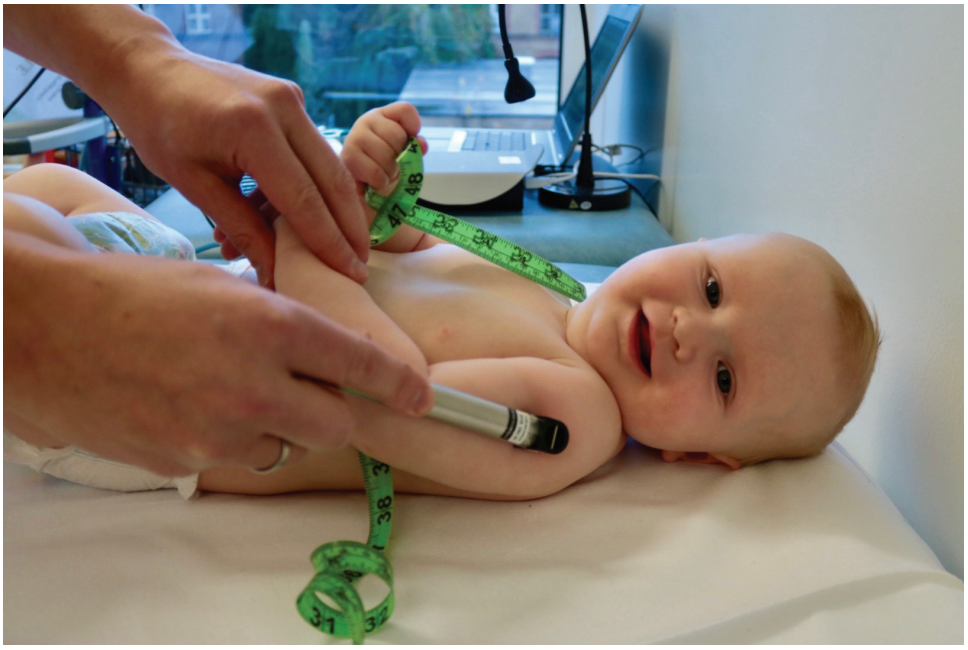
Table 4-4 Skin areas examined for mild, moderate or severe dry skin at the clinical investigations.

Dry skin and eczema: Mark x where relevant, or write unsure if that is the case.					
	Mild dryness	Moderate dryness	Severe Dryness	Eczema, not infected	Eczema, infected
Left lateral upper arm					
Left elbow crease					
Scalp					
Cheeks					
Head and neck (excluding scalp and cheeks)					
Truncus					
Extensors arms/legs					
Flexors arms/legs					
Flexors elbows and knees					
Dorsal hands/fingers/wrists					
Palmar hands/fingers/wrists					
Feet (incl. soles and ankles)					
Diaper area					

4.3.5 Transepidermal water loss measurement at 3 and 6- months investigations

Infants were undressed for 15 minutes for acclimatisation prior to the TEWL measurements using an open chamber DermaLab USB (Cortex, Hadslund, Denmark). Parents were instructed not to bathe the infants or use any emollients within 24 hours prior to the examination. Three measurements were performed on the left upper lateral arm as illustrated in Figure 4-3. Measurements required a calm child, a room temperature as close to $22^{O}C$ as possible with windows and doors shut, noting ambient temperature and humidity.

Figure 4-3 Transepidermal waterloss measurement on left lateral upper arm at 6 months follow-up investigation. Photo by Mari Kjendsli at Oslo University Hospital, reprinted with parental signed consent.



4.3.6 Amniotic fluid sampling and analysis

Amniotic fluid was collected in a sterile manner during elective (planned, with no ongoing labour) or acute (labour already started) CS, after uterotomy, by aspiration of amniotic fluid through intact amniotic membranes using a sterile 19G needle and 10 ml syringe. The amniotic fluid samples were left at 4ºC for maximum 24 hours and subsequently aliquoted to volumes of 4 ml into 1-2 sterile Cryotubes 4.5 ml SI 363452 tubes (Sigma Aldrich®, USA) and 0.5 ml into 1 sterile tube containing 1ml Aimes medium (ESwab Copan 490CE;Thermo Fischer Scientific, USA). These vials were stored at -80 ºC until further analysis. Negative controls were sampled from two different operating rooms using sterile containers with NaCl (9mg/ml, 100 ml iv infusion, B. Braun), using the same sampling and aliquoting procedure as the amniotic fluid samples. In addition, two negative controls from the PCR water used in the laboratory were included.

Extraction of DNA from 1 ml of amniotic fluid was done manually by mag™ midi kit (LGC Genomics, UK) following the manufacturer's recommendations. Quantification of prokaryotic 16S rRNA gene copies in the amniotic fluid samples was done using ddPCR (Bio-Rad,

USA)(142). All reactions were performed on a 2720 Thermal Cycler (Applied BioSystems, USA) and the droplets were quantified using the Bio-Rad Quantisoft software. The baseline was set manually with a fluorescence threshold of 15 000 Relative Fluorescence Units (RFUs).
For aerobic and anaerobic culturing amniotic fluid in Aimes medium was suspended in liquid Brain Heart Infusion (BHI) medium. Tubes for anaerobic culturing were prepared in a closed jar using Thermo Scientific™ Oxoid AnaeroGen 3.5L Sachets (USA). The samples were incubated at 37°C for 48 hours and 10µl from each sample was plated out on BHI agar for aerobe (48 hours) and anaerobe (120 hours) incubation at 37°C. DNA was extracted manually by mag™ midi kit (LGC Genomics, UK) following the manufactures recommendations from all the cultures as well as from the bacterial colonies found on the BHI plates after incubation. Amplification by PCR was performed on DNA from all the liquid culture samples, using 1xHotFirePol®DNA polymerase Ready to load (Solis BioDyne, Estonia. The size of the PCR products was determined using gel electrophoresis with a 1,5% agarose (Sigma Aldrich, Germany and DNA concentrations were measured on the QubitTM fluorometer (Life Technologies, USA). Sanger sequencing was performed to identify the cultured bacteria by GATC BioTech, Norway. Illumina sequencing was used for direct culture independent characterization of the taxonomic composition of the microbiota. Resulting sequences were analysed using the open source QIIME bioinformatics pipeline (143) and OTUs were defined at 97% similarity and taxonomy was assigned based on >97 % identity using the SILVA database (144).

4.4 Definitions and outcomes

4.4.1 Unaffected skin and dry skin

Unaffected skin was defined as no eczema and no dry skin. Dry skin included all infants with observed dry skin on at least 1/11 predefined skin areas Table 4-5, regardless of eczema. Dry skin only was defined as only dry skin and no eczema. Dry skin only was further sub-categorized

into dry skin on cheeks, extensors surfaces of the extremities (extensors) and both cheeks and extensors.

4.4.2 Atopic dermatitis

In this population of very young infants, diagnosing AD is challenging, as the disease has often not yet manifested completely and will subsequently rarely meet the diagnostic criteria for AD. For these infants aged up to 6 months we therefore did not use any diagnostic criteria and chose a proxy for AD, defined as the presence of eczematous lesions observed by study personnel at the clinical investigations, excluding differential diagnosis to AD Table 4-5. In paper III and in paper IV the same outcome was called possible AD and eczema respectively.

Table 4-5 Outcome definitions for unaffected skin, dry skin and atopic dermatitis in this thesis.

Unaffected skin	No eczema and no dry skin at clinical investigation.
Dry skin	The presence of skin scaling and roughness both by visual inspection and palpation in at least 1/11 predefined skin areas at clinical investigations.
Atopic dermatitis	The presence of eczematous lesions, excluding differential diagnosis to AD by a trained medical doctor.

4.4.3 Transepidermal water loss

Transepidermal water loss was used as a continuous variable as well as divided into quartiles. For the prediction model in paper IV we defined high TEWL as TEWL > 90th percentile, which was (11.3 g/m^2/h).

4.4.4 Potential predictors for dry skin, high transepidermal water loss and atopic dermatitis.

The potential predictors are given in Table 4-6, followed by a brief description of each factor.

Table 4-6 The variables used to analyse potential predictors for dry skin, high TEWL and atopic dermatitis were either from Questionnaires reported by mothers at 18 weeks (Q18w) or 34 weeks (Q34w) of pregnancy, from the 18 weeks of pregnancy inclusion visit (Incl18w) or from birth records (BR).

Potential predictors	Variable explanation
PRENATAL FACTORS	
Age mother	Calculated from mother's date of birth and date of Incl18w where it was reported
Age father (years)	Calculated from father's date of birth and date of Q18w where it was reported
Mother Nordic origin and Father Nordic origin	Birth country of mother and father was reported in detail in Q18w, computed into dichotomous variable: Nordic (Norway, Sweden, Denmark, Finland, Island) and other
Education mother and education partner	Reported in in details in Q18w, computed into dichotomous variable: more than 4 years of University or up to 4 years of University/College.
Family income	Reported as household income beforet taxes in details in Q18w, computed to three categories: Low (<600 000 NOK/year), Middle (600 000 – 1400 000NOK) and High (>1400 000NOK)
Single mother	Reported as marital status in Q18w, and computed into dichotomous variable: single or not
BMI, mother	Calculated from height and weight recorded at the Incl18w by study personnel
Multiparity	≥ 1 previous delivery reported by mother in 18 weeks questionnaire
Allergic disease mother and father	Reported in Q18w for mother and in Q34w for father as ever had any of the following: atopic dermatitis, asthma, food allergy, allergic rhinitis, anaphylaxis or urticaria
Doctor diagnosed atopic dermatitis, asthma, allergic rhinitis or food allergy in mother and father	Reported in Q18w for mother and in Q34w for father as ever had doctor diagnosed: atopic dermatitis, asthma, allergic rhinitis or food allergy respectively
Lifestyle during pregnancy	
Alcohol intake	Reported in Q18w and Q34w, computed into dichotomous variable: alcohol intake during pregnancy (unknown amount) and no alcohol intake after known pregnancy
Tobacco use in general, smoking and snus use	Reported in Q18w and Q34w, computed into 3 dichotomous variables: tobacco use in general, smoking and snus use during pregnancy (unknown amount) and no tobacco use in general, no smoking and no snus use after known pregnancy
Live rural vs urban	Reported in Q18w and computed into dichotomous variable rural (countryside not in village and countryside in village) and urban (city, suburbs and town)
Exposure to humidity/mould	Reported in details in Q18w, computed into dichotomous variable: exposure to humidity/mold and no exposure.
Pets in general	Reported in Q18w, computed into dichotomous variable: pets and no pets
Pets, more details	Reported in Q18w, computed into variable with 5 categories: Not pets, Cat and no dog, Dog and no cat, Cat and dog, Pets except cat and dog
PERINATAL FACTORS	
Caesarean section (CS)	Reported in BR and computed into three categories No CS, elective (planned) CS and acute (emergency) CS
Gestational age at birth (weeks)	Calculated from femur length at ultrasound investigation reported in Incl18w, and date of birth
Female gender	Reported in BR
Birth weight (kg)	Reported in BR
Born during winter season	Reported at newborn inclusion and defined as birth from 1st of October to 31st of March, giving to equal 6 months season periods of winter and summer.

4.4.5 Amniotic fluid; non-ROM group and ROM group

All amniotic fluid samples were collected from women with term CSs.

non-ROM group

All 10 samples from elective CSs with no prior rupture of membranes, none of these having started labour and all sampled in the same operating room.

ROM group

All 14 samples with prior rupture of membranes and on going labour were used as positive controls for the non-ROM group. They were sampled in two different departments.

4.5 Statistical methods

Categorical variables are presented as numbers and percentages. Continuous variables are presented as means, standard deviation (SD) and min-max.

Parametric statistical methods based on normal distribution were used when comparing TEWL in subgroups as the deviation from normality was considered as moderate. TEWL measurements ($g/m^2/h$) were available in 1046 (91%) of the 3 months old infants. The significant mean TEWL difference between the sub-categories of unaffected skin, dry skin extensors and dry skin cheeks and extensors could not be explained by temperature or humidity, we therefore included measures from the whole range of humidity recorded (6% - 73%, mean 29%, SD 12.7). In line with international recommendations we included only measurements performed in room temperature between 20 and 25°C (145).

When comparing continuous variables we used independent sample t-test, and when comparing categorical variables we used chi-square test.

We used logistic regression analysis to investigate the associations between selected parental and pregnancy- related variables and the outcome variables dry skin, AD or high TEWL. A cut-off p-value of 0.2 was chosen for the univariate logistic regression analysis, followed by multivariate

regression analysis. Only variables with less than 15% missingness in the complete-case univariate analysis were included in the multivariate analysis. In each regression model the assumption underlying multivariate logistic regression analysis were checked and found to be adequately met.

In order to investigate the impact of dry skin and high TEWL at 3 months of age on AD at 6 months of age, the following three regression models were performed: Model 1: Unadjusted. Model 2: Adjusted for the variables found to be significantly associated with dry skin, high TEWL and AD at 3 months of age in the multivariate regression model 2. Model 3: Variables from model 2 together with variables significantly associated with AD at 6 months from univariate logistic regression analysis.

The nonparametric data (ddPCR results) were calculated using Independent Samples Mann-Whitney U Test.

Statistical significance level was set to 5 %. All the analyses were performed using IBM© SPSS© statistics version 25 (Chicago, IL, U.S.A.).

4.6 Ethical considerations

In this large prospective study including randomisation to one of four groups, including none, one or both interventions, we have a particular focus on the ethical aspects of study participation. The PreventADALL study was approved by The Regional Committee for Medical Research Ethics in South-east Norway (2014/518) and Stockholm, Sweden (2014/2242-31/4). The study is approved with a wide consent within non-communicable diseases. Given the many interventional design, the relatively frequent study visits, bio-sampling and questionnaire responses, it was essential that all women and their partners had a full understanding of what the study entailed. Study information was therefore crucial prior to enrolment, thus the enrolment visit around 18 weeks of GA started with mothers reading a four-page detailed information sheet, followed by

study personnel informing and discussing the implications of study participation with both parents, whenever possible and subsequent signing of the informed consent form by the index mother.

When the babies were born, parents were approached by study personnel, repeating the information and opening for reflection and discussion about implications of the study participation, after which both mother and father/co-mother signed a new consent form for the child. Randomisation allocation was revealed to parents first after the new consent sheet was signed. The study participants were thoroughly informed that all investigations and data collections were voluntary and that they could withdraw from the study at any time without giving any reason for the withdrawal.

Throughout the follow-up time of the children, parents have had free access to study personnel. Parents were encouraged to contact the study team in case of any suspected allergic disease development, in which case appropriate medical measures would be investigated.

5 Results

Prenatal and Perinatal factors that were selected for the multivariate prediction models for dry skin, high TEWL and AD based on the univariate analysis are represented in Table 5-1.

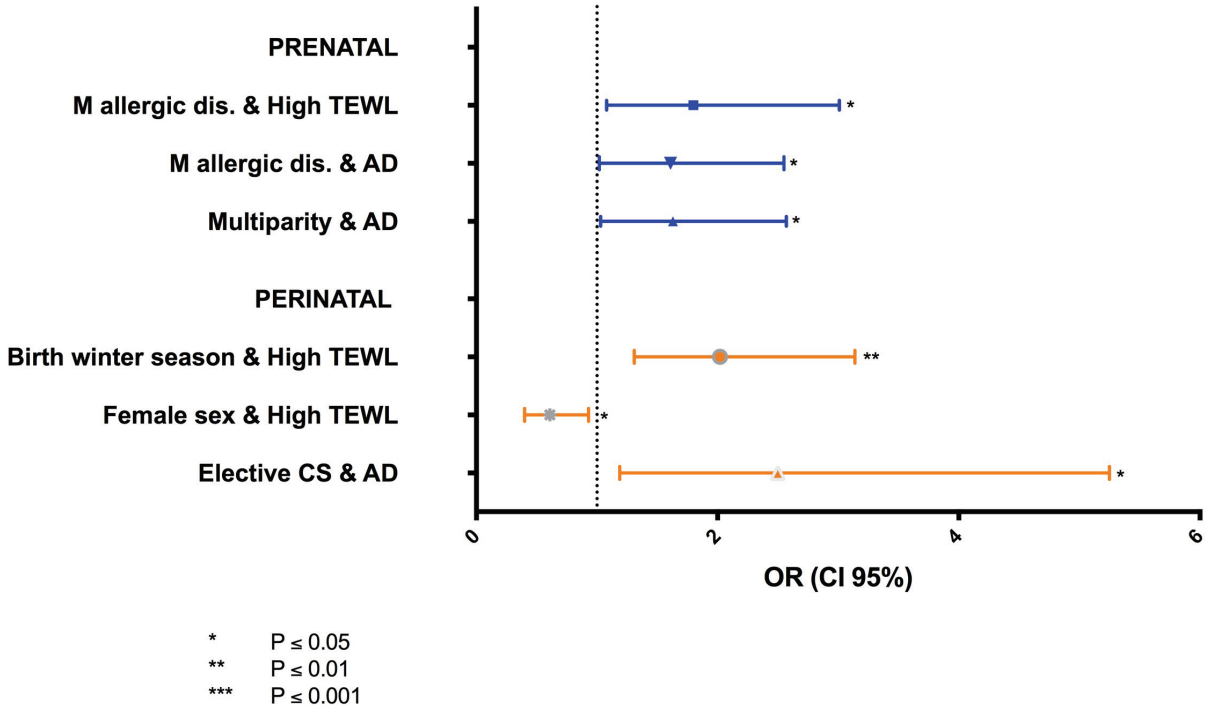
Table 5-1 Potential predictors for dry skin, high TEWL or atopic dermatitis with a p-value <0,2 in the univariate logistic regression analysis are marked in red for potentially increasing the risk and green for a potentially decreasing the effect. Crossed boxes are those with > 15% missingnes.

Potential predictors	Univariate analysis with p-value < 0.2		
PRENATAL FACTORS	Dry skin	High TEWL	Atopic dermatitis
Increasing age mother			
Increasing age father (years)			
Mother Nordic/Father nordic origin			
Higher education > 4 years mother			
Higher education > 4 years partner			
Increasing family income			
Single mother			
BMI, mother			
Multiparity			
Any allergic disease mother			
Any allergic disease father			
Atopic dermatitis mother			
Atopic dermatitis father			
Asthma mother			
Asthma father			
Allergic rhinitis mother			
Allergic rhinitis father			
Food allergy mother			
Food allergy father			
Lifestyle during pregnancy			
Alcohol intake			
Smoking			
Snus use			
Rural living			
Exposure to humidity/mould			
Pets in general			
Pets, more details			
Cat, no dog			
Dog, no cat			
Cat and dog/Other pets			
PERINATAL FACTORS			
Elective caesarean section			
Acute caesarean section			
Increasing GA at birth (weeks)			
Female sex			
Increasing birth weight (kg)			
Born during winter season			

5.1 Prenatal predictors for impaired skin barrier function or atopic dermatitis. (Papers I and IV)

High TEWL (> 90th percentile/ > 11.3 g/m^2/h) at 3 months of age was associated with six variables with a p-value < 0.2 in the univariate logistic regression analysis as outlined in Table 5-1, that were further included in the multivariate analysis. Maternal allergic disease was the only prenatal factor that remained significant as a predictor of high TEWL (OR: 1.80, CI 95%: 1.08-3.01)(p=0.025), Figure 5-1.

Figure 5-1 Prenatal (blue) and perinatal (orange) predictors for high TEWL (> 90th percentile) and atopic dermatitis (AD) at 3 months of age in 1150 infants, when using multivariate regression analysis.



* P ≤ 0.05
** P ≤ 0.01
*** P ≤ 0.001

Atopic dermatitis at 3 months of age was associated with 10 variables with a p-value <0.2 in the univariate logistic regression analysis as outlined in Table 5-1, that were further included in the multivariate analysis. Three predictors remained significant, of which two were prenatal factors,

namely multiparity (1 or more previous deliveries) (OR: 1.63, CI 95%: 1.03-2.57; p=0.037), and maternal allergic disease (OR: 1.61, CI 95%: 1.02-2.55; p=0.041), Figure 5-1.

5.2 Perinatal predictors for impaired skin barrier function and atopic dermatitis, including mode of delivery and a potential amniotic fluid microbiome. (Paper I, II and IV)

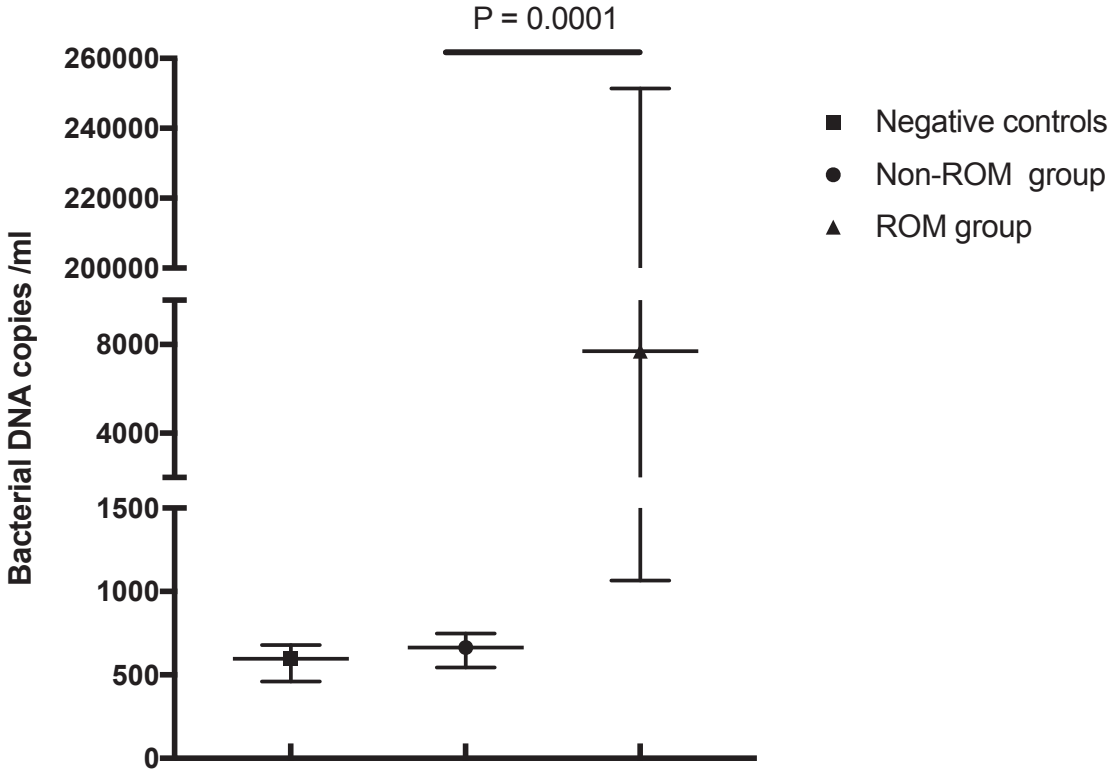
High TEWL (> 90th percentile/ > 11.3 g/m^2/h) was associated with six factors in univariate analyses as seen in Table 5-1, while in the multivariate analysis three variables remained significant, of which two were perinatal factors, namely female sex (OR: 0.61, CI 95%: 0.40-0.93; p=0.022), and birth during winter season (OR: 2.02, CI 95%: 1.31-3.14; p=0.002), Figure 5-1.

For AD, one of the three variables that remained significant in the multivariate analysis was a perinatal factor, namely elective CS (OR: 2.50, CI 95%: 1.19-5.25; p=0.016), Figure 5-1.

5.2.1 Investigating amniotic fluid for a potential microbiota

In the non-ROM group, the amniotic fluid had comparable concentration of bacterial DNA (16S rRNA gene copies/ml; ddPCR) (median (min-max))(664 (544-748)) to the negative controls (596 (461-679)). The concentration of bacterial DNA in the ROM group was significantly higher (7700 (1066-251430)) (p = 0.0001, by Mann-Whitney U-test) than in the non-ROM group and the negative controls, Figure 5-2. The difference between the non-ROM and ROM groups remained significant (p= 0.0001) also after excluding five women with clinical infection at the time of CS (median (min-max) of 1462 (1066-6743) 16S rRNA gene copies/ml).

Figure 5-2 Quantitative ddPCR of 10 amniotic fluid samples from caesarean sections with intact amntiotic membranes (non-ROM group) and 14 samples with prior rupture of amniotic membranes (ROM group)



In the non-ROM group and the negative controls, we could not detect any bacterial growth by culturing anaerobically and aerobically, or by performing PCR on the samples cultured in broth. In the ROM group, bacterial DNA were detected by PCR in 50 % of the anaerobically cultured samples and in 14.3% of the aerobically cultured samples. In 21.4 % of the ROM group samples grown anaerobically on agar, we detected bacterial colonies. These were identified (by Sanger sequencing) as bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections.

Illumina amplicon sequencing of the 16S rRNA gene revealed in five of the six amniotic fluid samples from the ROM group with >1000 16S rRNA copies/µl, species mainly belonging to bacterial genera that are part of a normal vaginal flora, and some associated with bacterial vaginosis and/or infections, as well as a few related to possible contamination. In the negative control samples from the operating room and the laboratory we found genera associated with reagent and laboratory contamination (146). As no bacterial microbiome was identified in the

amniotic fluid prior to delivery this could not be assessed in relation to skin barrier function or AD as outcomes.

5.3 Prevalence and predictors for dry skin in early infancy. (Paper I, III and IV)

Dry skin, in at least 1/11 anatomical skin locations was present in 59 % of the 3 months old infants. Dry skin only was observed in 540/1143 (47.2%) infants while 458/1143 (40 %) had unaffected skin and 145/1143 (13 %) infants had AD. Among the infants with dry skin only, the two most common locations were the cheeks in 329/529 (62 %) and extensors 258/530 (49 %). Dry skin was also observed in 138/144 (96 %) of the infants with AD; most commonly localized in the cheeks in 113/138 (82 %) and the extensors in 122/138 (88 %), Table 5-2.

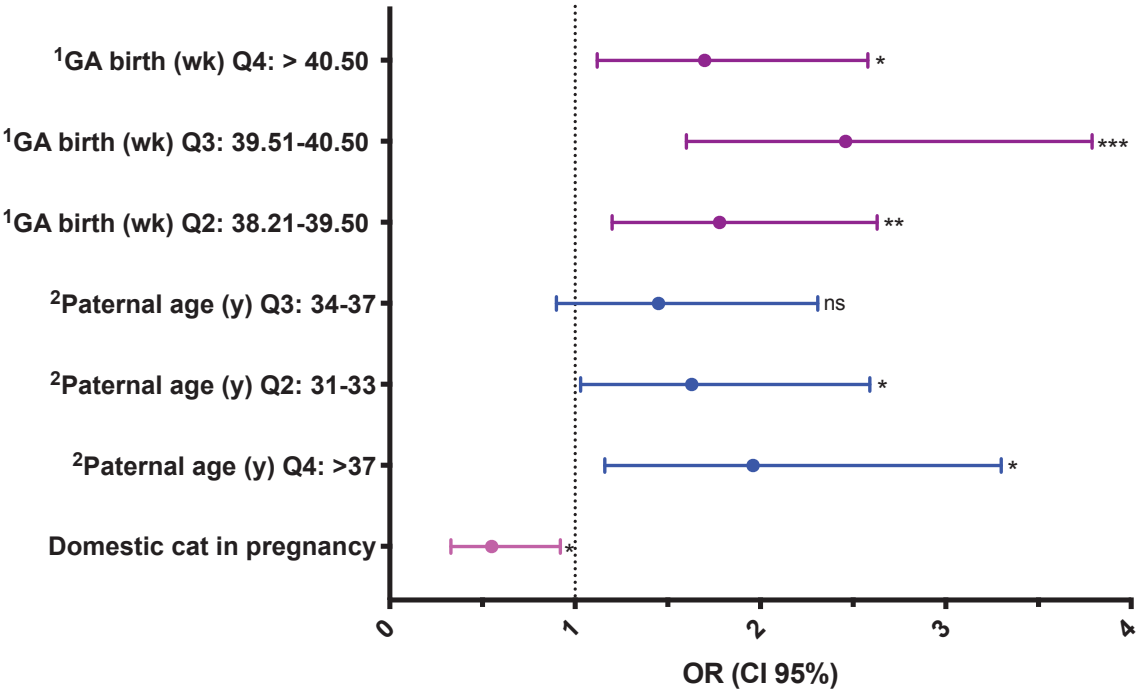
Table 5-2 The locations of observed dry skin in 3 months old infants are given for infants with and without AD. Data were available in 530/540 infants without atopic dermatitis (AD) and 138/145 infants with AD.

		Dry skin			No dry skin
		Mild	Moderate/Severe	Total	
Scalp	Dry skin only	57 (10.9)	2 (0.4)	59 (11.3)	463 (88.7)
	AD	24 (18.0)	14 (10.5)	38 (28.6)	95 (71.4)
Head and neck	Dry skin only	135 (25.8)	9 (1.7)	144 (27.5)	379 (72.5)
	AD	57 (41.6)	34 (24.8)	91 (66.4)	46 (33.6)
Cheeks	Dry skin only	314 (59.4)	15 (2.8)	329 (62.2)	200 (37.8)
	AD	85 (61.6)	28 (20.3)	113 (81.9)	25 (18.1)
Extensors arms and legs	Dry skin only	229 (43.2)	29 (5.5)	258 (48.7)	272 (51.3)
	AD	65 (47.1)	57 (41.3)	122 (88.4)	16 (11.6)
Trunk	Dry skin only	168 (31.8)	11 (2.1)	179 (33.9)	349 (66.1)
	AD	66 (48.2)	26 (19.0)	92 (67.2)	45 (32.8)
Flexors arms and legs	Dry skin only	83 (15.8)	6 (1.1)	89 (16.9)	437 (83.1)
	AD	54 (39.4)	23 (16.8)	77 (56.2)	60 (43.8)
Flexors elbows and knees	Dry skin only	29 (5.5)	1 (0.2)	30 (5.7)	493 (94.3)
	AD	42 (31.3)	10 (7.5)	52 (38.8)	81 (61.2)
Dorsal hands	Dry skin only	41 (7.9)	3 (0.6)	44 (8.4)	477 (91.6)
	AD	39 (29.3)	12 (9.0)	51 (38.3)	82 (61.7)
Palmar hands	Dry skin only	6 (1.2)	2 (0.4)	8 (1.5)	511 (98.5)
	AD	8 (6.0)	1 (0.8)	9 (6.8)	124 (93.2)
Feet	Dry skin only	148 (28.0)	16 (3.0)	164 (31.1)	364 (68.9)
	AD	44 (32.4)	18 (13.2)	62 (45.6)	74 (54.4)
Diaper area	Dry skin only	15 (2.9)	1 (0.2)	16 (3.1)	504 (96.9)
	AD	15 (11.2)	1 (0.7)	16 (11.9)	118 (88.1)

With dry skin as dependent variable in a univariate logistic regression analysis, 10 variables having a p-value <0.2, as seen in Table 5-1, were included in a multivariate analysis. The only variables that remained significant were GA at birth, paternal age and domestic cat in pregnancy,

Figure 5-3. Increasing paternal age was significantly associated with dry skin, especially in the highest quartile (> 37 years) with an OR of 1.96 (CI 95 %: 1.16-3.30; p=0.012). Increasing GA at birth was also significantly associated with dry skin, with infants in the third quartile having the highest OR (OR: 2.46, CI 95%: 1.60-3.79; p<0.0001). Domestic cat exposure during pregnancy was a significant protective factor for dry skin in the multivariate analysis (OR: 0.55, CI 95%: 0.33-0.92; p=0.023).

Figure 5-3 Predictors for dry skin.



ns	not significant
*	P ≤ 0.05
**	P ≤ 0.01
***	P ≤ 0.001

[1]Gestational age (GA) at birth in weeks divided in quartiles (Q) where first quartile is 35.00-38.20 weeks and used as reference value.
[2]Paternal age in years divided in quartiles (Q), where the first quartile of 21-30 years is used as reference value.

5.4 Dry skin; association with increased transepidermal water loss and predictor of atopic dermatitis. (Paper I and IV)

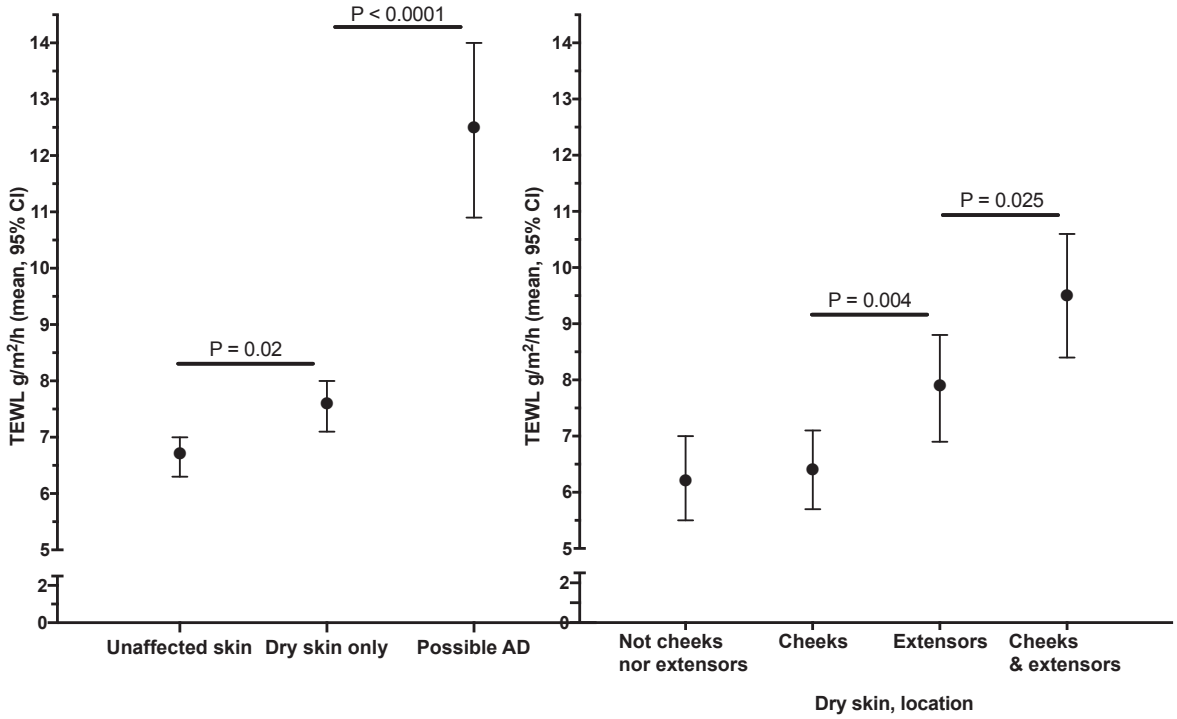
Compared to the mean (95% CI) TEWL (g/m^2/h) among infants with unaffected skin 6.7 (6.3, 7.0), TEWL was significantly higher among infants with dry skin only 7.6 (7.1, 8.0), while the

mean TEWL in infants with AD of 12.5 (10.9, 14.0) was significantly higher than both, Figure 5-4.

Compared to infants with unaffected skin, the mean (95% CI) TEWL was significantly higher among the 258 infants with dry skin extensors 7.9 (6.9, 8.8) (p=0.004) (Student's t-test). The mean (95% CI) TEWL in infants with dry skin extensors and cheeks of 9.5 (8.4, 10.6) significantly exceeded that of infants with dry skin extensors. Mean (95% CI) TEWL in those with unaffected skin, was similar for those with not dry skin cheeks nor extensors *6.2 (5.5, 7.0)* and dry skin cheeks 6.4 (5.7, 7.1), Figure 5-4.

Analyses of possible confounders for the association between skin dryness and TEWL resulted in gender, GA at birth, age at examination, humidity and room temperature. Only humidity and temperature were significantly associated with TEWL, with room air humidity observed to be significantly lower for measures in infants with unaffected skin compared to dry skin cheeks and extensors. However, the TEWL difference between these two categories remained significant after adjusting for humidity by using linear regression analysis ($p<0.001$), also the estimated TEWL difference between unaffected skin and dry skin cheeks and extensors changed only marginally.

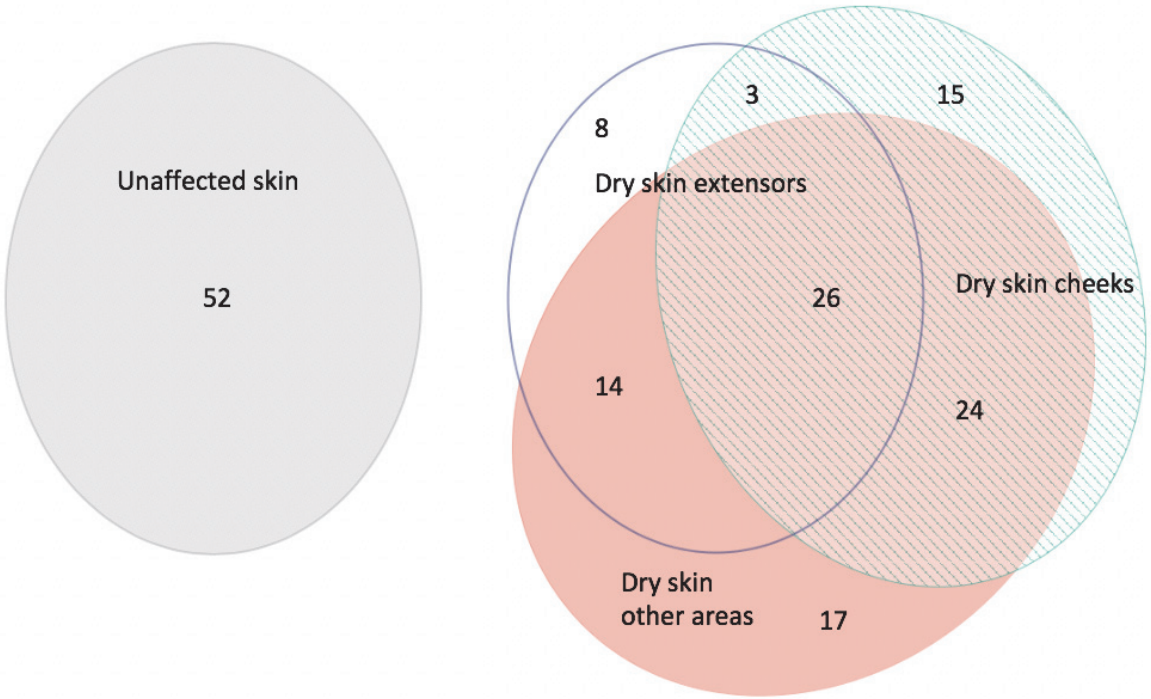
Figure 5-4 Skin barrier function, given as mean (95% confidence interval (CI) transepidermal water loss (TEWL) is shown for 1019 3-month old infants and further in 472 with dry skin only (no signs of eczema) that has been subgrouped into dry skin located anywhere but on the cheeks or extensors (*Not cheeks or extensors*, N=79), dry skin anywhere including cheeks, but not extensors *(cheeks,* N=161), dry skin anywhere including extensors, but not cheeks (*Extensors,* N=98 and dry skin including *Cheeks and extensors* (N=134). Reproduced with Creative Commons Attribution License (CC BY) Rehbinder & Winger et al. BJD 2019 DOI: 10.1111/bjd.17626



5.4.1 Dry skin or high TEWL at 3 months of age as predictors of AD at 6 months of age

Infants with dry skin only, at 3 months of age regardless of location had significantly more often AD at 6 months of age (21.7%) compared to the infants with unaffected skin (12.4%), Figure 5-5. Dry skin at 3 months significantly increased the risk of AD at 6 months (n=927), with an unadjusted OR (95% CI) of 1.96 (1.37, 2.80) (p<0.0001). The OR was marginally lower (OR 1.92 (1.21-3.05) (p=0.005)) after adjusting for relevant covariates (elective CS, GA at birth, multiparity, paternal age, maternal allergic disease, paternal allergic disease, paternal atopic dermatitis, alcohol consumption during pregnancy and domestic cat during pregnancy). Dry skin at the predilection sites of AD, either the cheeks and/or the extensors, at 3 months of age was similarly predictive for AD at 6 months of age with an OR (CI 95%) of 1.94 (1.20-3.15; p=0.007), adjusted for the same covariates. Dry skin at 3 months for the prediction of AD at 6 months of age had a sensitivity of 68% and a specificity of 48%.

Figure 5-5 The Euler diagram depicts the distribution of dry skin at 3 months in 159 infants who at 6 months had atopic dermatitis. Dry skin at 3 months, regardless of location was a significant predictor for AD at 6 months of age as well as for dry skin in the cheeks and/or the extensors specifically. Produced with courtesy of: Luana Micallef and Peter Rodgers (2014). eulerAPE: Drawing Area-proportional 3-Venn Diagrams Using Ellipses. PLoS ONE 9(7): e101717. doi:10.1371/journal.pone.0101717. http://www.eulerdiagrams.org/eulerAPE



The TEWL (g/m^2/h) in 3 months old infants was not significantly associated with AD at 6 months when analysed as a continuous variable using logistic regression. No significant associations were found using quartiles of TEWL with the first quartile as a reference. Although high TEWL (TEWL > 90th percentile) (N=82) compared to TEWL <90th percentile (N=750) was significantly associated with AD at 6 months of age (OR: 1.80, CI 95 %: 1.07-3.04)(p=0.028) in univarate analyses, the association did not remain statistically significant after adjusting for covariates.

No significant interaction was found between dry skin and TEWL at 3 months of age for AD at 6 months of age.

6 Discussion

6.1 Prenatal predictors for impaired skin barrier function or atopic dermatitis.

Maternal allergic disease was the only prenatal factor predicting impaired skin barrier function, given as high TEWL at 3 months of age. Maternal allergic disease also predicted AD at 3 months together with multiparity (previous deliveries).

In line with previous studies (16, 147) parental allergic disease, was a predictor of AD in our population, however it has not been reported previously as a predictor for increased TEWL in infancy. Multiparity being a predictor of AD at 3 months in our study is in contrast to one of the key arguments for the hygiene hypothesis where presence of older siblings has been shown to reduce the risk of AD (148). However, these findings are inconsistent, as severe AD has been found to be significantly associated with larger sibships (148), also supported by a strong association between having older siblings and *FLG* loss-of-function mutations (149) where having older siblings did not provide any protective effect of AD. Early onset AD in combination with skin barrier dysfunction often have a more persistent course as well as being one of the risk factors for the atopic march (115, 150-152). Our results imply that in early onset AD, a genetic predisposition to allergic disease may override certain environmental factors.

6.2 Perinatal predictors for impaired skin barrier function and atopic dermatitis, including mode of delivery and a potential amniotic fluid microbiome.

Birth during winter season was predictive of impaired skin barrier function, given as high TEWL, while female sex was found to be protective. For AD, elective CS was the only significant perinatal predictor found. We did not identify a unique amniotic fluid bacterial

microbiota, and the amniotic fluid was only colonized with bacteria with the start of labour with rupture of membranes in healthy term pregnancies.

Our findings of significantly higher TEWL in male infants are in line with a recent Japanese study (153), in contrast to an Indian study where TEWL in neonates was indistinguishable between males and females (154). Males often have an earlier onset of AD compared to females (28, 155), possibly relating this to a higher degree of impaired skin barrier function at an earlier age in males than in females.

Cold climate and low environmental humidity has been repeatedly associated with impaired skin barrier function (156-160), supporting our finding of higher TEWL in infants born during the fall and winter season. Exposure to a dry and cold Scandinavian winter climate could have led to depletion of filaggrin, and perhaps other skin barrier proteins as well as lipids, due to the reduced environmental humidity (37, 160). Onset of AD is more common during the winter season (28), and birth during fall and winter has been associated with increased risk of AD (158, 161, 162). This negative seasonal effect on the skin barrier could have other explanations than low humidity and cold air. Lower cumulative UV irradiation before and after birth, could reduce the production of filaggrin (158), which may also contribute to seasonal risk variations.

The initial microbial colonization and its composition early in life seems important for the development of the child´s immune system and further promoting health or disease (73-75). The timing of this colonization could therefore be helpful in further understanding the developmental origin of health and disease (DOHaD)(163). Older studies, only using culturing techniques, where amniotic fluid cultures were negative in term uncomplicated pregnancies with intact membranes (164-166), are being challenged by the use of 16S rRNA sequencing. Recently, in the amniotic fluid of 15 term uncomplicated pregnancies, a core set of bacterial phylotypes was

identified overlapping with the microbiota found in placenta and meconium (63). These findings and studies supporting a unique placental microbiome (61, 62) are questioning the "sterile womb" hypothesis. Our findings of no microbiota in amniotic fluid prior to the rupture of amniotic membranes are in line with the findings that the initial microbial colonization of the offspring starts during labour and is affected by mode of delivery, with vaginal and fecal microbes colonizing vaginally born newborns (69). And in CS delivered newborns skin microbes originating from the mother (69, 167) and the operating room (168) predominate. Studies investigating the newborn pioneer microbiota in uncomplicated term pregnancies also support that bacterial colonization does not start before labour (69, 165, 167, 169, 170).

The risk of contamination when performing highly sensitive sequencing studies is high, demonstrated by Lauder et al. (67), where their placental samples were identical to the negative controls, not only by a small number of DNA copies, but also through sequencing. Also, the bacterial DNA found in studies on low-microbial biomass samples have been criticized to not originate from live bacteria, but as a result from contamination or transport of dead microbial products brought by the blood stream (60, 68).

Molecular based studies on amniotic fluid that incorporate appropriate measures for reducing contamination and including negative controls have been essential for the deciphering of microbial invasion of the intrauterine cavity. However, bacterial cultivation should not be omitted, and are often strengthening these studies. We designed our study so that the sampling, aliquoting, and analysing processes were as "sterile" as possible, including negative controls at each step. The amniotic fluid that was sampled during elective CSs in the 10 subjects selected for the non-ROM group were all from the same operating room to reduce contamination variations. However, as demonstrated by our "sterile" controls it is nearly impossible to prevent contamination completely in a busy clinical setting.

Our findings that there are no live bacteria in amniotic fluid prior to labour, are in line with previous studies using only cultivation techniques (164-166, 171), studies using qPCR and sequencing techniques (169, 172, 173) as well as studies using both cultivation and 16S rDNA qPCR (174). As we did not identify a unique amniotic fluid microbiome, we therefore could not study this directly in relation to AD development. However, initial colonization of the infant is affected by amniotic membrane rupture and pregnancy complications (165, 169, 170, 173, 175, 176), leading to an earlier in utero microbial exposure, which may enhance a dysbiotic offspring microbiota and possibly lead to long-term offspring adverse health effects such as allergic disease (73-76).

The association between CS and offspring allergic disease has been extensively investigated with conflicting results (73, 78, 79, 177). However, it is less clear whether there is a difference between being exposed to an acute (emergency) or elective (planned) CS. To our knowledge, our study is the first reporting elective CS being a predictor of AD in early infancy. Interestingly, acute CS was not predictive of AD and as the majority of the elective CSs were prior to rupture of amniotic membranes it means that the initial colonization was most probably not a vaginal one. We therefore hypothesize that our findings of a lacking exposure to the vaginal flora in elective CSs (without rupture of amniotic membranes) may contribute to an offspring gut and skin microbiome dysbiosis associated with AD (178). A possibility of partially restoring a primary vaginal colonization in newborns delivered by CS have been implied in a pilot study by swabbing the infant with vaginal microbes from their mother immediately after the CS delivery (179). These are, however preliminary results and an ongoing larger RCT in Sweden, the Restoration of Microbiota in Neonates (RoMaNs), also including AD as an outcome, might answer more questions in the future.

6.3 The prevalence and predictors for dry skin in early infancy.

In 3 months old infants from a general population, 59 % had dry skin, most commonly observed on the cheeks and extensor surfaces of the extremities. Dry skin without the presence of AD was observed in 47 %. The predictors for dry skin in these infants were increasing GA at birth as well as increasing paternal age.

To our knowledge, this is the first study to report on the prevalence of dry skin in early infancy. However, similar findings have been observed previously in two other Swedish studies, one with two-year old children with AD and their controls (111), and one with eight-year old children through questionnaires on dry skin and AD (112). The dry skin prevalence reported in countries with a more temperate climate such as Germany (113), and India (114) were lower, however this was in a different population including young adults and older children.

The indicated higher prevalence of dry skin in children living in countries on the northern hemisphere may be due to lower air humidity and low temperatures in the winter compared to countries further south (156). This is in line with recent reviews suggesting that decreased humidity is associated with an increase in signs of dry skin, TEWL and flares of AD (156, 159). Another reason for the high number of infants with dry skin in our study may be related to our broad criteria; mild dry skin in at least one location. The German and Indian studies (113, 114), used the Hanifin and Rajka diagnostic criteria for diagnosing AD (33). Xerosis is one of the minor criteria in the Hanifin and Rajka criteria and is defined as the presence of generalized dry skin, but does not offer a detailed definition of dry skin. In the study by Böhme from 2000, the Hanifin and Rajka criterion for xerosis was specified to be dry skin in at least 20% of the body surface (111). Dry skin was defined as rough skin with fine scaling and no erythema, which is similar to our definition. None of these studies specified the location of dry skin.

In our study, the two most common areas of dry sin in infants without AD were the cheeks and extensors. This was also true for the infants with AD and perhaps reflecting the predilection sites of AD during the first year of life (32, 119). The cheeks and extensors are exposed areas subjected to environmental stress, which might impair the skin barrier, possibly leading to dry skin, and ultimately to AD. This supports the outside-inside hypothesis where external stimuli enters through an impaired skin barrier driving the Th2 inflammation, which further impairs the skin barrier and perhaps causing the onset of AD (37).

Increasing GA at birth being a predictor of dry skin is interesting, as increasing GA previously has been reported to be associated with AD (155, 180, 181), although the evidence have been somewhat conflicting. Prematurity with very low GA (<29 weeks) has especially been inversely associated with AD (162, 182). These studies speculate that the reasons for this association may be related to the child´s shorter exposure time to the maternal immune system and Th2 cytokines, lower levels of IgE and a different composition of early gut and skin microbiome in infants delivered at a lower GA compared to a higher GA (155, 180, 182). Pregnancy complications has not been found to be associated with AD (180), and although low birthweight has been inversely associated with AD (162), there is more evidence pointing to the length of GA in itself and not an increase in fetal growth that is a risk factor for AD (155, 182). In our cohort we included only neonates born after 35 weeks of gestation, and therefore we cannot investigate whether prematurity before 35 weeks is protective of dry skin at 3 months of age.

Older fathers more often having children with dry skin could reflect a possible age related increase in mutations (183). Although the maternal genetic influence seems to be greater than the paternal on offspring AD (147), there is scarce documentation on the effect of advanced paternal age on allergic disease and impaired skin barrier in the offspring.

6.4 Dry skin; associated with increased transepidermal water loss and predictor of atopic dermatitis.

Dry skin in 3 months old infants was significantly associated with reduced skin barrier function, indicated by significantly higher TEWL values than in infants with unaffected skin. The reduced skin barrier function appeared to be more pronounced in infants with dry skin affecting both cheeks and extensor areas. Dry skin at 3 months of age predicted AD at 6 months of age, while high TEWL did not.

Our sub-group analyses found higher mean TEWL when both cheeks and extensors were affected with dry skin, compared to dry skin involving the extensor surfaces alone. This may indicate a more extensively defective skin barrier, not only at the site of measurement, again indicating a possible increased risk of AD development. Aligning with our findings, a study of 88 3 months old infants suggested that clinically dry skin was associated with increased TEWL, even in the absence of AD (120).

In our study, TEWL was measured with an open-chamber system, which is affected by ambient temperature and humidity. The recommended level of humidity when measuring TEWL is approximately 40%, but up to 60% is accepted (145). Tight control of humidity was not possible as the investigations were performed throughout the year in settings resembling regular clinical practice. Furthermore, humidity during winter in northern climate can drop to levels below 20 % indoors. It was important to include all children attending the investigations throughout different seasons, including those that attended the 3 months investigation during the winter season. Importantly, the variation in humidity during TEWL measurements was found to be of limited clinical relevance, as all our results remained significant after adjusting for humidity. This is in line with previous findings (108).

The choice of anatomical measuring location may influence TEWL values in infants (106), young children (108) and adults (184) . We measured TEWL on the lateral upper arm, as this has previously been shown to be as good as, or possibly superior to, mid-volar forearm measures in differentiating skin barrier function related to AD (108). Considering the easy access to the lateral upper arm as well as being a predilection site of AD in infants, we chose this area for measurement.

Dry skin is well established as a cardinal sign of AD (16, 37, 109), and in our population we found dry skin in 96% of the 3 months old infants with AD. To our knowledge, no previous studies have reported on clinical dry skin in early infancy as a predictor for AD. The cheeks and extensors were overrepresented in infants with dry skin without AD at 3 months of age that later developed AD at 6 months of age, although not necessarily with a concurrent presence. Although high TEWL at 3 months did not predict AD at 6 months, it remains to be investigated whether TEWL can predict AD at later time-points (116-118) in our cohort. Also, we chose to include all infants where AD was suspected, not only those that fulfilled the UK Working Party criteria for AD (109), mainly due to a reduced ability to scratch at such an early age. This may have resulted in a more heterogeneous AD population, including those with very mild manifestations of the disease.

Eczema lesions often appears first on the cheeks in infancy, and a recent study found that cheeks were slower to mature than the skin of the nasal tip and elbow creases, and had lower levels of natural moisturizing factor (NMF) in a population of 188 infants (185). This indicates that AD starting on the cheeks can be due to a physiological skin barrier dysfunction restricted to a specific body location. Maternal allergic disease, male gender and birth season being one of predictors of high TEWL, could possibly enhance the onset of AD. Although dry skin was

associated with increased TEWL we did not find any interaction between dry skin and TEWL, perhaps justifying that the presence of dry skin could precede AD without increased TEWL. However, dry skin in early infancy had low sensitivity and specificity and cannot be used as a single predictive tool for such a heterogenous disease with a complex pathogenesis (16) and several proposed phenotypes (151, 152) as AD.

6.5 Strengths and limitations

The prospective design of the PreventADALL study involving a large number of participants from a general population is a major strength. We collected a substantial amount of data from questionnaires and clinical investigations including thorough skin examination performed by trained health care personnel including information on presence and distribution of dry skin as well as eczema. The multicentre study was limited to three different study sites with close collaboration ensuring standardisation of all skin scoring assessments and other data collection methods. Also, the high rate of infants attending the follow-up investigations is a strength of the study.

We chose a highly sensitive and accurate ddPCR quantification (142) allowing us to identify bacterial DNA at the single copy level, strengthening our finding of too low amount of DNA in the non-ROM group to identify a bacterial microbiota. With the less sensitive regular qPCR single copies of bacterial DNA cannot accurately be detected. Using qPCR is therefore less useful when analysing low bacterial content biological samples such as amniotic fluid, as demonstrated in a study where no 16S rRNA nor 18S rRNA was found in amniotic fluid from amniocentesis in 344 uncomplicated pregnancies at mid-gestation (186), and in another study from term-gestation a median 16S rRNA gene copy number of 0 from 20 samples (174).

A limitation to our study is that we did not include any objective measurement evaluating dry skin, such as corneometry, which has been reported to be correlated to TEWL in non-lesional skin in AD patient (187). Although, a standardized extensive dry skin examination can potentially be challenging even with trained medical health personnel, it is more available in a busy clinical setting compared to the use of biophysical measurements. The lacking data on *FLG* mutations and the short follow-period to only 6 months is a limitation when investigating impaired skin barrier and AD, which may lead to some limited information. Due to very low numbers of infants fulfilling the UK Working Party diagnostic criteria for AD until 6 months of age, we chose not to use this as an outcome in our study subjects. The clinical presence of eczema with exclusion of differential diagnosis to AD was considered to be possible AD, and at such an early age we have used it as a proxy for AD. This could have potentially caused an over-diagnosis of AD. The relatively high number of possible predictors for the outcomes dry skin and AD at 3 months of age included in the multivariate analysis, as well as the possible bias of missing data may introduce a risk of false positive results.

With the majority of the study participants being Nordic there can be a limitation to the generalizability of the study since the characteristics of AD differs among regions in the world (188). The small number of amniotic fluid samples could be a limitation, with a large variation in bacterial load among the samples in the ROM group, as well as a relatively large timespan from rupture of membranes until delivery. However, the lack of bacterial detection in the non-ROM group is consistent, and similar to the findings of negative controls and clearly different to the consistent positive bacterial findings (both by highly sensitive DNA quantifications and cultures) in the ROM group.

7 Main conclusions

7.1.1 To identify prenatal factors that predicts impaired skin barrier function or atopic dermatitis in early infancy. (Paper I and IV)

Maternal allergic disease significantly predicted high TEWL and AD at 3 months of age.

Multiparity was another significant prenatal predictor for AD.

7.1.2 To identify perinatal predictors, also exploring mode of delivery and a potential amniotic fluid microbiota, for impaired skin barrier function and atopic dermatitis in infancy. (Paper I, II and IV)

Significant perinatal predictors for high TEWL were birth during winter season and male sex, for AD it was elective CS. Amniotic fluid is sterile (no microbiota) in uncomplicated term pregnancies with intact amniotic membranes before the start of labour.

7.1.3 To determine the prevalence and predictors for dry skin in early infancy. (Paper I, III and IV)

In a general population of 3 months old infant, 59 % had dry skin regardless of AD and 47% without AD, with cheeks and extensor surfaces of the extremities most commonly affected. Significant predictors for dry skin in 3 months old infant were increasing GA at birth and increasing paternal age, while domestic cat during pregnancy was protective.

7.1.4 To determine if dry skin is associated with increased transepidermal water loss, and if both these factors predicts atopic dermatitis in infancy (Paper I and IV)

Mean TEWL was significantly higher in 3 months old infants with dry skin than in infant with unaffected skin. Concurrent presence of dry skin in cheeks and extensor areas impaired the skin barrier by further increasing TEWL, however being significantly lower than in infants with AD. Dry skin at 3 months of age predicted AD at 6 months of age, while high TEWL did not.

8 Clinical implications and future perspectives

The PreventADALL study included pregnant women and collected detailed information with a substantial amount of biological material already from fetal life. The prospective design of the study, together with all the data from around 2400 mother-child pairs that will be followed through childhood and adult life might help answering many questions regarding the early origins of immune related non-communicable diseases, and perhaps recognize new primary prevention strategies.

We believe that our findings that the offspring is not in contact with vaginal microbiota in elective CS and that it could increase the risk of AD adds to the arguments that an indication for an elective CS should considered conscientiously each and every time and should not be taken lightly by the doctor or the mother to be.

Our novel findings on significant predictors for impaired skin barrier estimated as high TEWL and dry skin maybe of value in deciphering the complex nature of AD and it´s different phenotypes, where some of them might be more barrier driven at the starting point. This was also shown through the distinct increase in AD risk in infants having dry skin prior to the first eczema lesions. By showing that dry skin in early infancy, and especially dry skin on cheeks and extensor surfaces of the extremities (predilection sites for infant-onset AD) was associated with higher TEWL than in infants without dry skin, we have demonstrated that dry skin is a sign of impaired skin barrier early in life and might be a way of selecting infant for primary prevention.

In addition to being an observational general population cohort study investigating the early origins of allergic diseases and other NCDs, PreventADALL is also a RCT with a factorial design investigating the effect of primary prevention of allergic diseases through both early skin care and early complementary food introduction. The PreventADALL study, also collaborates

widely and currently we are analysing data from the skin microbiota and gut microbiota during the first year of life which may give novel information on the pioneer microbiota in relation to AD. PreventADALLs epidemiological data collection together with the ample biobank gives endless possibilities and I hope to continue the work that I have started in this unique and inspiring study together with so many dedicated fellow researchers.

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Preventing Atopic Dermatitis and ALLergies in Children—the PreventADALL study

To the Editor

Reversing or aborting the increase in allergic and other immune-related noncommunicable diseases (NCDs) in the Western world, first observed for allergic rhinitis from the 1890s,[1] requires primary prevention strategies, probably on a general population level. The diseases are likely to be related to changes in lifestyle, environment, or both,[2] including reduced microbial diversity, increased use of xenobiotics in industrial and consumer products, exposure to tobacco or nicotinic products, and variations in diets and nutritional elements. While some primary allergy preventive strategies may be effective in high-risk children,[3] the relevance for preventive strategies on a population level is unclear.[4] We propose using allergic diseases as model diseases for understanding effects of modern lifestyle upon immune-related noncommunicable diseases (NCDs), with allergy manifestations already from the start of life. The Preventing Atopic Dermatitis and ALLergies in children (PreventADALL) study will provide new insight into early life prevention of NCDs. This letter briefly outlines why and how we will determine effects of a dual approach to preventing allergic disease development in early infancy, as well as provide a basis for identification of novel strategies for future prevention of NCDs.

Allergic diseases often start with atopic dermatitis (AD) or food allergy in early infancy, followed by wheeze or asthma and allergic rhinitis in childhood, and frequently occur as comorbidities. The reduced skin barrier in AD may predispose for food and other allergy development, suggesting that primary allergy prevention should start early and target barrier enhancement and the alimentary tract.[5] While no commonly accepted effective primary prevention is currently available, skin emollients have reduced AD in high-risk children[6] and peanut intake from infancy in children with severe AD and/or egg allergy reduced peanut allergy.[3] However, in a general population-based study of breast-fed infants,[7] food allergy was prevented only in children fully adherent to the protocol of regular intake of 6 food items from 3 months of age.

The PreventADALL study has 2 main objectives: primarily to determine whether primary prevention of allergic diseases is possible by simple and low-cost strategies and secondarily to assess early life factors and exposures, including intrauterine environment, microbiota, and xenobiotics, involved in the development of asthma and allergic diseases or other NCDs including cardiovascular diseases, obesity, and diabetes.

A general population-based mother-child birth cohort recruited at 18-week pregnancy will be assessed at follow-up investigations (Figure 1) into adulthood of the children in this international, multicenter study, including a 2 × 2 factorially designed, randomized clinical trial of 2 primary prevention interventions (skin care and early food introduction) in infancy. Based upon an estimated 22% relative risk reduction in AD, deemed clinically relevant, we recruited 2697 women (2701 pregnancies) from December 2014 through October 2016, with their last baby enrolled April 11, 2017 (Online Supplement). Based upon femur length[8] at the 18-week ultrasound investigation, mean (range) gestational age (GA) was 18.7 (15.7-22.7) weeks, among the 2149 women enrolled in Norway (Oslo University Hospital and Østfold Hospital Trust) and the 552 in Sweden (Karolinska Institutet, Stockholm) (Table 1). Most women (mean [range] age 32 [18-42]) were well educated, lived with their husbands (41.2%) or cohabiting partner (55.9%), as is common in Scandinavia (Table 1). With 88.2% (n = 2397) of all fetuses included at birth (52.7% boys), we largely met the targeted 2400 mother-child pairs, which is larger than the 1306 children in the Enquiring About Tolerance (EAT) study.[7] Mean (range) infant estimated GA was 39.2 (35.6-42.9) weeks (Figure S1), and 16.4% were delivered by Caesarian section rate, in line with national practice.[9] The mothers reported at least one (42%) or two (20.1%) doctor diagnosed allergic disease (Table 1).

To ensure a general, nonselected population, all *pregnant women* (GA 16-22 weeks) attending routine ultrasound screening at, or in collaboration with (in Sweden), the 3 participating hospitals were eligible, provided sufficient language skills. Women carrying more than 2 fetuses, fetuses with severe malformations or disease and infants born prior to 35.0 weeks of GA, were excluded.

All infants were randomized at birth to 1 of 4 similar sized groups: (1) no intervention; (2) skin care (oil-bath at least 5 days per week from 0.5 to 9 months of age); (3) consecutive introduction, between 3 and 4 months of age, of peanut, milk, wheat, and egg at least 4 days per week complementary to breastfeeding; or (4) both interventions. Weekly electronic diaries (2-26 weeks of age) recorded skin care, infant feeding, and symptoms of allergic diseases. Adverse events (0-12 months of age) elicited relevant investigations and treatment by direct access for the participants to the local pediatric department.

Data collection (Figure 1, Table S1) includes electronic questionnaires with information of health and disease in the mother, child, and family; lifestyle; environment; stress; quality of life; diet in the mother and offspring; clinical investigations; fetal and child anthropometrics; lung function; skin barrier; allergy; and blood pressure

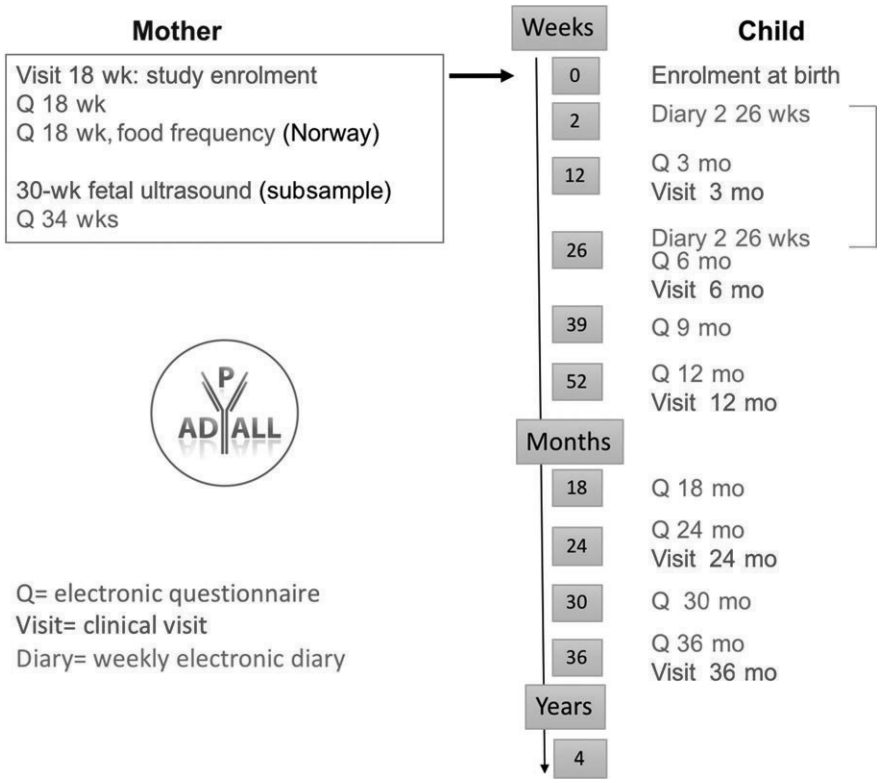

Figure 1. The PreventADALL study overview is shown for the enrolment and follow-up investigations for the first 4 years of the children. The study is planned for future follow-up studies from year 4 onward

measurements. *Biological sampling* includes blood (serum, DNA, RNA), urine, skin swabs and feces for microbiota, placental biopsies and swabs, amniotic fluid (if caesarean section), vernix caseosa, saliva, and breast milk.

The main intervention outcomes are AD and food allergy to intervention foods, assessed first at 12 and 36 months, respectively. Assessment of AD with validated international criteria is performed by a blinded assessor, and food allergy will be confirmed by food challenges, when appropriate. Secondary outcomes assessed annually include recurrent wheeze or asthma, allergic sensitization, allergy to other foods, anaphylaxis, and allergic rhinitis. Other NCDs will be defined in future phases of the study.

The study was approved by the Regional board for Medical Ethics in Oslo (2014/518) and Stockholm (2014/2242-31/4) and registered at clinicaltrial.gov *NCT02449850*.

We are unaware of studies other than the PreventADALL study testing whether primary prevention of allergy in early infancy is effective, based upon the dual allergen exposure hypothesis.[5] The high participant educational attainment reflects that of Scandinavian women and may influence identification of lifestyle factors that affect NCD development. The comprehensive data collected and careful phenotyping of participants will enable identification of personalized novel preventive strategies to related microbial diversity, diet, lifestyle, and gene-environment influence on allergic and other NCD development from fetal life.

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Patji Haakon Alnæs-Katjavivi (University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Lene Frost Andersen (Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway), Line Arntzen (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Hassan Arshad (University of Southampton, UK), Teresa Løvold Berents (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Department of Dermatology, Oslo University Hospital, Oslo, Norway), Ann Berglind (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Abelone Bidstrup (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Jessica Björk (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Magnus Borres (Akademiska Hospital Uppsala, Sweden), Randi

TABLE 1 The Table Shows the background characteristics of the 2701 PreventADALL participants, reported by the 2397 mother-child pairs and the 315 enrolled women who did not have their babies included in the final mother-child cohort. The GA was estimated from ultrasonic measures of femur length at enrollment (GA 16-22)

	Pregnancies registered at 18-w ultrasound (n = 2701)								
		Mother-child cohort							
		Mother (n = 2386)				Father (n = 2260)			
	Not in the mother-child cohort (n = 315)	Total	Oslo (n = 1530)	Østfold (n = 339)	Stockholm (n = 517)	Total	Oslo (n = 1473)	Østfold (n = 301)	Stockholm (n = 486)
Mean age (range) -year	31.8 (18-45)	32.4 (20-48)	32.8 (21-48)	30.6 (20-42)	32.5 (21-47)	34.6 (21-72)	34.9 (23-65)	33.2 (21-52)	34.8 (21-72)
Maternal body measurements									
Height, cm, median (min, max)	168 (147-186)	168 (147-187)	168 (147-186)	167 (152-187)	167 (151-185)				
Weight, kg, median (min, max)	69 (46-121)	68 (45-133)	68 (45-123)	70 (46-134)	69 (49-124)				
Body mass index (median, min, max)	24 (18-45)	24 (17-48)	24 (17-41)	25 (18-48)	25 (19-43)				
Gestational age at 18-w ultrasound	18.9 (16.6-21.8)	18.9 (15.1-22.5)	19.0 (15.4-22.5)	18.6 (15.2-22.2)	19.0 (16.0-21.4)				
Multiple pregnancies (total 4)	1	3							
Twin pregnancies (total 17)	6	11							
Education - no. (%)									
Preliminary school only (9/10 y)	2 (1.1)	16 (0.7)	3 (0.2)	5 (1.7)	8 (1.6)	26 (1.3)	8 (0.6)	10 (3.5)	8 (1.7)
High school only	18 (9.6)	221 (10.3)	66 (4.8)	65 (22.7)	90 (17.9)	388 (18.7)	142 (10.7)	119 (42.2)	127 (26.6)
Higher education <4 y	71 (37.8)	687 (31.9)	394 (28.9)	132 (46.2)	161 (32.0)	629 (30.3)	419 (31.7)	83 (29.4)	127 (26.6)
Higher education 4y or more	90 (47.9)	1167 (54.3)	862 (63.3)	76 (26.6)	229 (45.5)	946 (45.6)	685 (51.9)	62 (22.0)	199 (41.7)
PhD	7 (3.7)	60 (2.8)	38 (2.8)	7 (2.4)	15 (3.0)	70 (3.4)	53 (4.0)	4 (1.4)	13 (2.7)
Country of origin—no. (%)									
Norway	128 (68.1)	1434 (66.3)				1381 (65.4)			
Sweden	32 (17.0)	491 (22.7)				486 (23.0)			
Other Nordic	3 (1.6)	28 (1.3)				29 (1.4)			

(Continues)

TABLE 1 (Continued)

	Pregnancies registered at 18-w ultrasound (n = 2701)								
		Mother-child cohort							
		Mother (n = 2386)				Father (n = 2260)			
	Not in the mother-child cohort (n = 315)	Total	Oslo (n = 1530)	Østfold (n = 339)	Stockholm (n = 517)	Total	Oslo (n = 1473)	Østfold (n = 301)	Stockholm (n = 486)
Rest of the world	25 (13.3)	209 (9.7)				216 (10.2)			
Marital status—no. (%)									
Married	77 (41.0)	891 (41.2)	548 (40)	121 (41.9)	222 (44.1)				
Cohabitants	99 (52.7)	1214 (56.2)	786 (57.4)	159 (55.0)	269 (53.5)				
Single	5 (2.7)	39 (1.8)	27 (2.0)	3 (1.0)	9 (1.8)				
Divorced/ separated		1 (0.0)		1 (0.3)					
Other	7 (3.7)	17 (0.8)	9 (0.6)	5 (1.7)	3 (0.6)				
Previous pregnancies—no. (%)	103 (54.8)	1189 (55.0)	714 (52.1)	177 (38.8)	298 (59.2)				
Previous deliveries—no. (%)									
1	63 (61.2)	678 (57.0)	400 (56.0)	109 (61.6)	169 (56.7)				
2	10 (9.7)	160 (13.5)	81 (11.3)	30 (16.9)	49 (16.4)				
3		17 (1.4)	6 (0.8)	3 (1.7)	8 (2.7)				
4		5 (0.4)	2 (0.3)	2 (1.1)	1 (0.3)				
5 or more		2 (0.2)	1 (0.1)	1 (0.6)					
Living environment—no. (%)									
City, densely populated	77 (41.0)	839 (38.8)	689 (50.3)	25 (8.7)	125 (24.9)				
City, less densely populated	60 (31.9)	822 (38.0)	530 (38.7)	147 (50.9)	145 (28.8)				
Suburb	29 (15.4)	344 (15.9)	106 (7.7)	19 (6.6)	219 (43.5)				
Village	6 (3.2)	46 (2.1)	9 (0.7)	30 (10.4)	7 (1.4)				
Countryside, outside village	16 (8.5)	111 (5.1)	36 (2.6)	68 (23.5)	7 (1.4)				

(Continues)

TABLE 1 (Continued)

	Pregnancies registered at 18-w ultrasound (n = 2701)								
		Mother-child cohort							
		Mother (n = 2386)				Father (n = 2260)			
	Not in the mother-child cohort (n = 315)	Total	Oslo (n = 1530)	Østfold (n = 339)	Stockholm (n = 517)	Total	Oslo (n = 1473)	Østfold (n = 301)	Stockholm (n = 486)
Doctor diagnosed parental diseases—no. (%)									
Total no. of responders	188	2162	1370	289	503	2151	1401	280	470
Asthma	34 (18.1)	371 (17.2)	227 (16.6)	59 (20.4)	85 (16.9)	277 (12.9)	180 (12.8)	34 (12.1)	63 (13.4)
Atopic eczema	32 (17.0)	429 (19.8)	272 (19.9)	66 (22.8)	91 (18.1)	220 (10.2)	141 (10.1)	15 (5.4)	64 (13.6)
Allergic rhinitis	32 (17.0)	445 (20.6)	294 (21.5)	65 (22.5)	86 (17.1)	507 (23.6)	353 (25.2)	60 (21.4)	94 20.0)
Food allergy	29 (15.4)	280 (13.0)	179 (13.1)	37 (12.8)	64 (12.7)	196 (9.1)	140 (10.0)	14 (5.0)	42 (8.9)
Anaphylactic reaction	8 (4.3)	73 (3.4)	34 (2.5)	13 (4.5)	26 (5.2)	92 (4.3)	13 (1.0)	5 (1.8)	24 (5.1)
Urticaria	23 (12.2)	287 (13.2)	199 (14.6)	41 (14.2)	47 (9.3)				
Newborn babies N (% of total)	321	2397	1537	342	518				
Boys (%)		(52.7)	(52.9)	(50.1)	(54.0)				
Caesarian section (%)		391 (16.4)	237 (15.5)	62 (18.3)	92 (17.8)				
Gestational age at delivery, weeks	38.5 (33.2-42.6)	39.6 (35.1-43.0)	39.4 (35.1-42.9)	39.4 (35.1-42.6)	37.9 (35.0-42.0)				

Brøseth (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Kristine Wedum Davanger (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Vibeke Dyrseth (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; Diakonova University College, Oslo, Norway), Ingvild Essén (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway), Peter Gaustad (University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Alexandra Goldberg (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Malén Gudbrandsgard (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway), Sandra Götberg (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Susanne Halken, Ann Edløy Holstvoll (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Ragnhild Kinge (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Mari Kjendsli (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway), Nora Nilsson (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Monika Nordenbrand (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Sandra Olsson (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Céline Pattaroni (Service de Pneumologie, Department of Biology and Medicine, CHUV-UNIL, Lausanne, Switzerland), Alexis Rapin (École polytechnique fédérale de Lausanne, EPFL, Lausanne, Switzerland), Kajsa Sedergren (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Natasha Sedergren (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Sigrun Irene Skei (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Liv Julie Sørdal (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway), Ellen Tegnerud (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Lovisa Tolander (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Ann Kristin M. von Übisch (Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Angelica Johansen Winger (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway)

CONFLICT OF INTEREST

None of the authors have declared real or perceived conflict of interest for the present study, as outlined in the COI forms; however, Eva Maria Rehbinder has received honorary for presentations from Sanofi Genzyme and Omega Pharma.

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Karin C. Lødrup Carlsen[1,2]
Eva Maria Rehbinder[1,2,3]
Håvard O. Skjerven[1,2]
Monica Hauger Carlsen[4]
Thea Aspelund Fatnes[1]
Pål Fugelli[2]
Berit Granum[5]
Guttorm Haugen[2,6]
Gunilla Hedlin[7,8]
Christine Monceyron Jonassen[9,10]
Linn Landrø[3]
Jon Lunde[11]
Benjamin J Marsland[12]
Björn Nordlund[7,8]
Knut Rudi[10]
Katrine Sjøborg[13]
Cilla Söderhäll[8,14]
Anne Cathrine Staff[2,6]
Riyas Vettukattil[2]
Kai-Håkon Carlsen[1,2]
on behalf of the study group*
[1]*Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway*
[2]*Faculty of Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway*

[3]*Department of Dermatology, Oslo University Hospital, Oslo, Norway*
[4]*Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway*
[5]*Department of Toxicology and Risk Assessment, Norwegian Institute of Public Health, Oslo, Norway*
[6]*Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway*
[7]*Astrid Lindgren Children's* Hospital, Karolinska University Hospital, Stockholm, Sweden
[8]*Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden*
[9]*Genetic Unit, Centre for Laboratory Medicine, Østfold Hospital Trust, Kalnes, Norway*
[10]*Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway*
[11]*Department of Pediatrics, Østfold Hospital Trust, Kalnes, Norway*
[12]*Service de Pneumologie, Department of Biology and Medicine, CHUV-UNIL, Lausanne, Switzerland*
[13]*Department of Obstetrics and Gynaecology, Østfold Hospital Trust, Kalnes, Norway*
[14]*Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden*
Email: *k.c.l.carlsen@medisin.uio.no*

*PreventADALL study group members are listed in Appendix 1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

APPENDIX 1

ON BEHALF OF THE PREVENTADALL STUDY GROUP (ALPHABETICAL ORDER):

Anna Asarnoj, MD, PhD (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Petri Auvinen, Professor, PhD (Institute of Biotechnology, University of Helsinki, Helsinki, Finland), Karen Eline Stensby Bains, MD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Oda C. Lødrup Carlsen (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway), Kim Advocaat Endre, MD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Department of Dermatology, Oslo University Hospital, Oslo, Norway), Peder Annæus Granlund (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Hrefna Katrín Gudmundsdóttir, MD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Tari Haahtela, Professor, MD, PhD (Skin and Allergy Hospital, Helsinki University of Helsinki, Finland), Katarina Hilde, MD (University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway), Henrik Holmstrøm, Professor, MD, PhD (University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Department of Cardiothoracic Surgery, Oslo University Hospital, Oslo, Norway), Geir Håland, MD, PhD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; Department of Cardiothoracic Surgery, Oslo University Hospital, Oslo, Norway), Ina Kreyberg, MD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway.; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Caroline-Aleksi Olsson Mägi (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Live Solveig Nordhagen MSc (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Diakonova University College, Oslo, Norway), Unni C. Nygaard, PhD (Department of Toxicology and Risk Assessment, Norwegian Institute of Public Health, Oslo, Norway), Carina Madelen Schinagl, MD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway), Ingebjørg Skrindo, MD, PhD (Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway; Department of Ear, nose and throat, Akershus University Hospital, Lørenskog, Norway), Sigrid Sjelmo (Department of Pediatrics,

Østfold Hospital Trust, Kalnes, Norway), Sandra G. Tedner, MD (Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Department of Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden), Magdalena R. Værnesbranden, MD (University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway; Institute of Biotechnology, University of Helsinki, Helsinki, Finland), Johanna Wiik, MD (Institute of Biotechnology, University of Helsinki, Helsinki, Finland; Department of Obstetrics and Gynecology, Institute of Clinical Sciences, Sahlgrenska Academy, Gothenburg, Sweden)

II

OBSTETRICS

Is amniotic fluid of women with uncomplicated term pregnancies free of bacteria?

Eva Maria Rehbinder, MD; Karin C. Lødrup Carlsen, MD, PhD[1]; Anne Cathrine Staff, MD, PhD[1]; Inga Leena Angell, MSc; Linn Landrø, MD, PhD; Katarina Hilde, MD; Peter Gaustad, MD, PhD; Knut Rudi, PhD

BACKGROUND: The "sterile womb" paradigm is debated. Recent evidence suggests that the offspring's first microbial encounter is before birth in term uncomplicated pregnancies. The establishment of a healthy microbiota early in life might be crucial for reducing the burden of diseases later in life.

OBJECTIVE: We aimed to investigate the presence of a microbiota in sterilely collected amniotic fluid in uncomplicated pregnancies at term in the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study cohort.

STUDY DESIGN: Amniotic fluid was randomly sampled at cesarean deliveries in pregnant women in 1 out of 3 study sites included in the PreventADALL study. From 65 pregnancies at term, where amniotic fluid was successfully sampled, we selected 10 from elective (planned, without ongoing labor) cesarean deliveries with intact amniotic membranes and all 14 with prior rupture of membranes were included as positive controls. Amniotic fluid was analyzed by culture-independent and culture-dependent techniques.

RESULTS: The median (min-max) concentration of prokaryotic DNA (16S rRNA gene copies/mL; digital droplet polymerase chain reaction) was low for the group with intact membranes [664 (544—748)]—corresponding to the negative controls [596 (461—679)], while the rupture of amniotic membranes group had >10-fold higher levels [7700 (1066—251,430)] ($P = .0001$, by Mann-Whitney U test). Furthermore, bacteria were detected in 50% of the rupture of amniotic membranes samples by anaerobic culturing, while none of the intact membranes samples showed bacterial growth. Sanger sequencing of the rupture of amniotic membrane samples identified bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections.

CONCLUSION: We conclude that fetal development in uncomplicated pregnancies occurs in the absence of an amniotic fluid microbiota and that the offspring microbial colonization starts after uterine contractions and rupture of amniotic membrane.

Key words: amniotic fluid, bacteria, fetus, microbiome, microbiota, placenta, sterile

Introduction

The human microbiome discovery has developed quickly over the last decades with culture-independent techniques and unique microbial communities being identified in various body sites.[1,2] A diverse and well-balanced maternal and infant microbiome seems important for normal development of the child's immune system, and a dysbiotic maternal gut microbiome has been associated with offspring allergic disease development, as well as other immune-mediated diseases.[3—5] Identifying the timing of the initial microbial colonization of the offspring could therefore be helpful in further understanding the developmental origin of health and disease.[6]

It has recently been suggested, by the use of 16S rRNA sequencing, that amniotic fluid has a microbiome of its own in term uncomplicated pregnancies.[7] These findings are challenging earlier studies, where cultures from amniotic fluid were negative in term uncomplicated pregnancies with intact membranes.[8—10] The emerging evidence of a unique placental microbiome[11,12] are also questioning the "sterile womb" hypothesis.

Although sensitive molecular techniques are suggesting an intrauterine microbiota, the arguments for a sterile womb, including germ-free mice and contamination bias in molecular studies, are still strong.[13—15] However, the current evidence for a sterile intrauterine environment is inconclusive and to what extent, if, and how maternal microbiome influences the fetal immunological development and the shaping of the infant microbiome is not settled.[4,5]

The aim of our study was to investigate the presence of a microbiota in amniotic fluid in term uncomplicated pregnancies. We therefore combined sampling under strictly sterile and DNA-free conditions with highly sensitive techniques to determine the amniotic fluid bacterial load.

Materials and Methods

Study population

Within 22 months from December 2014, 2701 pregnant women were enrolled in the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study[16] in Norway and Sweden at the 18-week gestational age (GA) ultrasound screening.[16] Investigations included fetal ultrasound and maternal weight, length, and blood pressure on inclusion, with electronic questionnaires completed at 18- and 34-week GA to assess maternal health, family, sociodemographic, and lifestyle factors. The healthy newborn babies of at least GA 35 weeks were included for the mother-child cohort. All mothers consented to amniotic fluid sampling, in case of delivery by cesarean delivery at the Oslo University Hospital location, by signing the study consent form. From the PreventADALL cohort,[16] 65 women at Oslo University Hospital

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AJOG at a Glance

Why was this study conducted?
It is unclear if the amniotic fluid prior to delivery is sterile or not, the latter possibly influencing offspring health programming through in utero microbiota exposure.

Key findings
We found that prior to uterine contractions and rupture of amniotic membranes, amniotic fluid is sterile in uncomplicated term pregnancies.

What does this add to what is known?
This study resolves the uncertainty about a sterile intrauterine environment in uncomplicated pregnancies at term, due to stringent amniotic fluid sampling procedures, together with accurate and high-sensitivity microbiota analyses.

had amniotic fluid sampled during term cesarean delivery by dedicated health personnel in 3 different operating rooms. Out of these 65 women, 51 had intact amniotic membranes and 14 had prior rupture of amniotic membranes (ROM). For the no prior ROM group, we selected 10 amniotic fluid samples, all from elective term cesarean deliveries, none of these having started labor and all sampled in the same operating room. We included all 14 samples with prior ROM (ROM group) as positive controls for the non-ROM group (see Figure 1 for a detailed description on how the study population was selected). The study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2014/518) as well as registered at clinicaltrial.gov (NCT02449850).

Sampling

Amniotic fluid was collected in a sterile manner during elective (planned, with no ongoing labor) or acute (labor already started) cesarean delivery, after uterotomy, by aspiration of amniotic fluid through intact amniotic membranes using a sterile 19G needle and 10-mL syringe. The amniotic fluid samples were left at 4°C for maximum 24 hours and subsequently aliquoted into 1-2 sterile Cryotubes 4.5 mL SI 363452 (Millipore Sigma, Damstadt, Germany) and 0.5 mL into 1 sterile tube containing 1 mL Aimes medium (ESwab Copan 490CE; Thermo Fischer Scientific). These vials were stored at −80°C until further analysis. Negative controls were sampled from 2 different operating rooms using sterile containers with NaCl (9 mg/mL, 100 mL intravenous infusion; B. Braun), using the same sampling and aliquoting procedure as the amniotic fluid samples. In addition, 2 negative controls from the polymerase chain reaction (PCR) water used in the laboratory were included.

Initial handling and DNA extraction

Amniotic fluid (1 mL) was pulse centrifuged at 1200 rpm × 3 to remove large particles before it was centrifuged at 13,000 rpm for 10 minutes. We included negative controls in all steps, both sterile NaCl from the operating theater and PCR water from the laboratory. Pellet was washed twice in PBS suspended in 100 μL PBS, 50 μL was used for the DNA extraction, done manually by mag midi kit (LGC Genomics, United Kingdom) following the manufacturer's recommendations.

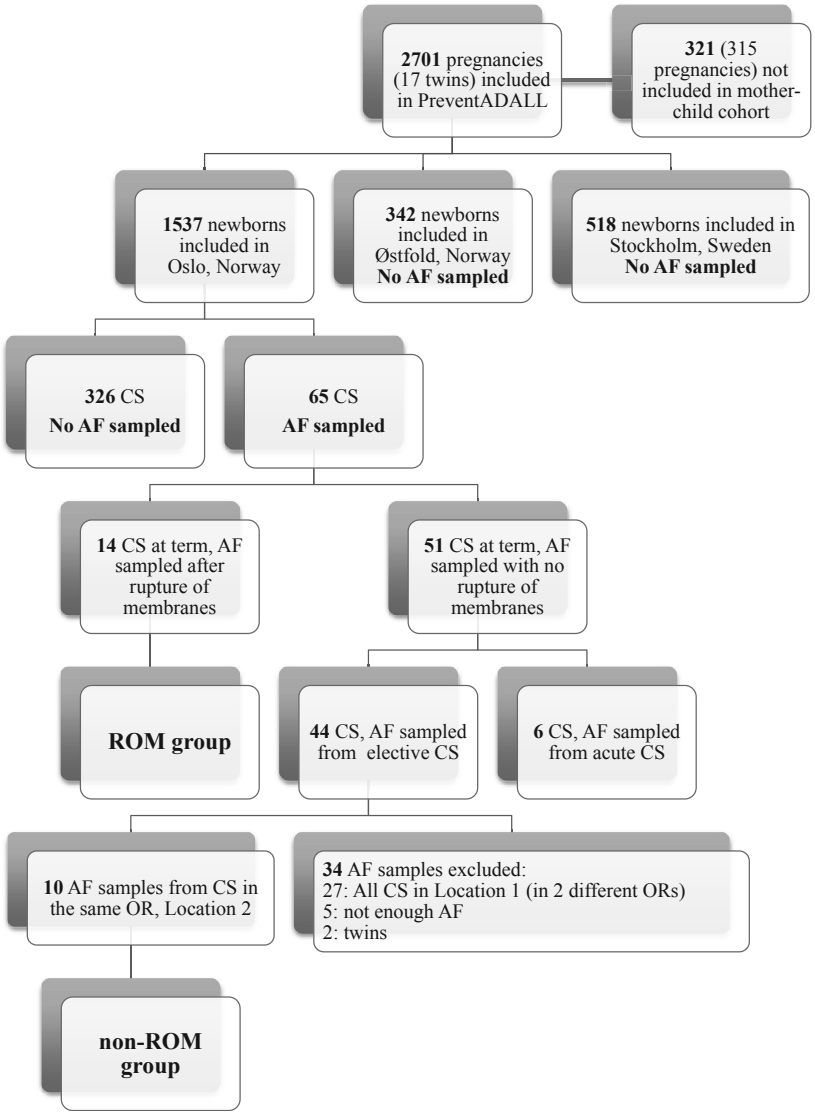
Quantification by digital droplet PCR

Quantification of prokaryotic 16S rRNA gene copies in the amniotic fluid samples was done using digital droplet PCR (ddPCR) (Bio-Rad, Hercules, CA).[17] Droplet generation, droplet transfer, and plate sealing was done according to the manufacturer's instructions. DNA was amplified by PCR using reaction mixes containing 1x QX 200 ddPCR EvaGreen Supermix (Bio-Rad), 0.2 μmol/L of each primers PRK341F (5'-CCTAC GGGRB GCASC AG-3') and PRK806R (5'-GGACT ACYVG GGTAT CTAAT-3') (Thermo Fisher Scientific),[18] and 2 μL DNA. Thermal cycles involved initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 45 seconds, <1 cycle at 4°C for 5 minutes and finally 1 cycle at 90°C for 5 minutes. All reactions were performed on a 2720 Thermal Cycler (Applied BioSystems, Waltham, MA). The droplets were quantified using software (Quantisoft; Bio-Rad). The baseline was set manually with a fluorescence threshold of 15,000 relative fluorescence units. Both the interassay and intraassay variability of ddPCR was validated by *Escherichia coli* spiking of non-ROM amniotic fluid (30,000 and 3000 colony-forming unit/mL) with 3 interassay replicates for each dilution, and duplicates analyses for each interassay replicate. In all cases the coefficient of variation was <15%, with the DNA recovery being ∼100%.

Culturing, DNA extraction, and PCR

In all, 150 μL of amniotic fluid in Aimes medium was suspended in 1350 μL of liquid brain heart infusion (BHI) medium, making a 10^{-1} dilution and further diluted to a 10^{-2} dilution, for both aerobic and anaerobic culturing. Tubes for anaerobic culturing were prepared in a closed jar using Oxoid AnaeroGen 3.5-L sachets (Thermo Fisher Scientific) for 48 hours; the closed jar and new sachets were used for the anaerobic culturing both in liquid BHI medium and on the BHI agars. The samples in liquid BHI medium were incubated at 37°C for 48 hours and 10 μL from each sample was plated out on BHI agar for aerobe (48 hours) and anaerobe (120 hours) incubation at 37°C. DNA was extracted manually by mag midi kit (LGC Genomics, United Kingdom) following the manufacturer's recommendations from all the cultures in liquid BHI 10^{-1} dilutions, as well as from the bacterial colonies found on the BHI plates after incubation. Amplification by PCR was performed on DNA from all the liquid culture samples, using 1xHotFirePol DNA polymerase ready to load (Solis BioDyne, Estonia), 0.2 μmol/L of the same PRK primers used in ddPCR, and 2 μL template DNA. Thermal cycles involved initial denaturation at 95°C for

FIGURE 1
Selection of study population for amniotic fluid analysis in the PreventADALL study



In the PreventADALL study, amniotic fluid (AF) was only sampled from cesarean delivery (CS) performed in Oslo, in location 1 (2 operating rooms [ORs]) and location 2 (1 OR). AF was randomly sampled in 65/326 CS (20%), where main indication for sampling was no prior rupture of membranes (ROM), but 14/65 samples were from CS with prior ROM in both locations.

Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

15 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C, and elongation at 72°C for 45 seconds. A final elongation at 72°C for 7 minutes was included.

Gel electrophoresis

The size of the PCR products was determined using gel electrophoresis with a 1.5% agarose (Sigma Aldrich). The electrophoresis ran at 80 V for 30 minutes. A 100-base pair DNA ladder (Solis BioDyne, Tartu, Estonia) was used as size marker for the DNA fragments. The fragments were visualized using the Molecular Imager Gel Doc XR Imaging system with Quantity One 1-D analysis software v.4.6.7 (Bio-Rad), using ultraviolet light.

Measuring DNA concentration by Qubit

DNA concentrations were measured on the Qubit fluorometer (Life Technologies, Waltham, MA), by using the double-stranded DNA high-sensitivity assay kit (Life Technologies). The measurements were done following the kit protocol, mixing 198 μL of working solution (Quant-iT reagent diluted 1:200 in Quant-iT buffer) with 2 μL sample. Calibration of the instrument was performed before the measurements as recommended by manufacturer.

Sanger sequencing

DNA of the isolates from culturing were amplified using 1xHotFirePol DNA polymerase ready to load (Solis BioDyne), 0.2 μmol/L of each of the primers, GA-map CoverAll primer pair (Genetic Analysis AS, Oslo, Norway), and 2 μL template DNA. Thermal conditions involved initial denaturation at 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. A final elongation at 72°C for 7 minutes was included. PCR products were purified using 0.8x AMPure XP beads (Beckman Coulter, Brea, CA) before measuring DNA concentration using a Qubit fluorometer (Life Technologies). GATC BioTech, Norway, sequenced the resulting purified PCR products.

Illumina sequencing

The taxonomic composition of the microbiota in the samples with a DNA concentration >1000 16S rRNA gene copies/μL was determined by sequencing the resulting amplicons from a 2-step PCR using the same primers as used in ddPCR. The 2 negative controls (1 from the hospital operating room and 1 from the laboratory) were also included. Amplification was performed in 25 μL volumes containing 1x HotFirePol blend master mix ready to load (Solis BioDyne), 0.2 μmol/L of both primers (Thermo Fisher Scientific), and 2 μL (0.4−60 ng) genomic DNA. First PCR was performed with initial denaturation at 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. A final elongation at 72°C for 7 minutes was included. Resulting amplicons were purified with AMPure XP beads (Beckman-Coulter), following the manufacturer's instructions. For attachment of dual indices and Illumina sequencing adapters, a second PCR was

performed with Illumina-modified primers following the same conditions as before, only with 10 cycles and an increased annealing step to 1 minute. Amplicon libraries were quantified by Qubit double-stranded DNA HS assay kit and normalized to a sequencing pool before purification by AMPure XP beads. Final library was quantified in a QX200 Droplet Digital PCR System (Bio-Rad) using primers targeting Illumina adaptors, following the manufacturer's recommendations. The library was loaded on a MiSeq platform (Illumina) following manufacturer's recommendations.

Analysis of Illumina data

Resulting sequences were analyzed using the open-source Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline,[19] implementing Ultrafast Sequence analysis (USEARCHs)[20] High-accuracy, high-throughput operational taxonomic unit (UPARSE-OTU) algorithm[21] for OTU clustering. OTUs were defined at 97% similarity and taxonomy was assigned based on >97% identity using the High quality ribosomal RNA (SILVA) databases.[22]

Statistical analysis

The nonparametric data (ddPCR results) were calculated using independent samples Mann-Whitney *U* test. The significance level was set to 5%. The statistical analysis including the descriptive statistics was performed in software (SPSS Statistics, Version 24; IBM Corp, Armonk, NY).

Results

Study population characteristics

From the 65 amniotic fluid samples, collected at cesarean delivery from the PreventADALL cohort,[16] we analyzed 10 with intact amniotic membranes (non-ROM group) and all 14 samples with prior ROM (ROM group). The women in both groups were similar in age, while GA and weight at birth was slightly higher in the ROM group, as shown in Table 1. None of the newborns had low Apgar score, and none needed intensive care. The median (min-max) time of ROM until cesarean delivery was 14 (2−36) hours in the ROM group.

TABLE 1
Baseline characteristics in group with intact amniotic membranes and rupture of amniotic membrane group

Characteristics	non-ROM n = 10	ROM N = 14
Mothers		
Age, y, mean (SD)	34.4 (3.6)	33.1 (3.6)
Pregnancy complications		
Clinical chorioamnionitis	0	4
GBS in urine	0	1
Antibiotics antepartum	0	5
Antibiotics intrapartum	0	14
Indications for CS		
Maternal request	6	
Heart disease mother	1	
2 Previous CS	1	
Breech and/or large for GA	1	1
Breech and fetal growth restriction	1	
Slow progression of birth		7
Fetal distress		2
Chorioamnionitis		4
ROM, h, median (min−max)	−	14 (2−36)
GA at CS, wk, mean (min−max)	39.1 (37.9−40.0)	40.5 (37.7−42.3)
Birthweight, g, mean (SD)	3548.6 (546.4)	3749.0 (578.7)

CS, cesarean delivery; *GA*, gestational age; *GBS*, group B streptococcus; *ROM*, rupture of amniotic membranes.
Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

Digital droplet PCR

The amniotic fluid in the non-ROM group contained very low numbers of bacterial DNA, with a median (min-max) of 664 (544−748) 16S rRNA gene copies/mL. This was comparable to our 4 negative controls (2 sterile NaCl samples from 2 different operating rooms and 2 sterile PCR water samples from the laboratory) where 596 (461−679) copies were detected. In contrast, the ROM group had significantly higher bacterial DNA levels of 7700 (1066−251,430) 16S rRNA gene copies/mL ($P = .0001$, by Mann-Whitney *U* test). The difference between non-ROM and ROM groups remained significant ($P = .0001$) also after exclusion of the 4 women who had a clinical infection and 1 with group B streptococcus in urine at cesarean delivery [median (min-max) of 1462 (1066−6743) 16S rRNA gene copies/mL]. In our samples we did not see any clear relation between time from ROM to cesarean delivery and/or clinical infection and bacterial DNA levels, as depicted in Table 2, however, the sample size in the ROM group is too small to study correlations.

Cultures and Sanger sequencing

No bacteria were detected from amniotic fluid in the non-ROM group, nor from the negative controls by culturing (anaerobically and aerobically) and PCR. In the ROM group, bacteria were detected in 50% by performing PCR on the samples cultured in broth under anaerobic conditions, and in 14.3% of the samples cultured in broth under aerobic conditions. In addition, bacterial colonies were detected in 21.4% of the samples grown anaerobically on agar (Tables 2 and 3). These colonies were

TABLE 2
Clinical information on 14 women with cesarean delivery with prior rupture of membranes and results from microbiological amniotic fluid analysis

ROM group	GA (wk + d) at ROM	ROM prior to start of labor	Spontaneous ROM or amniotomy	Regular contractions prior to CS	Time from ROM to cesarean delivery, h	Indication for cesarean delivery	Other information	ddPCR DNA copies/mL	Culture aerobic/ anaerobic	Sanger sequencing species (percentage represents identity to closest match in NCBI database)	Illumina 16S rRNA gene sequencing taxonomy present in ≥1%
1	42+2	Yes	Amniotomy	Yes	11	Slow progression	Meconium-stained amniotic fluid	45,066	Positive		*Lactobacillus* (69.5%) *Caulobacteraceae* (10.6%) *Sphingomonas* (1.5%) *Pseudomonas* (7.6%)
2	39+0	No	Spontaneous	No	2	Breech		1553	Negative		Not sequenced
3	41+6	Yes (PROM)	Spontaneous	Yes	6	Fetal distress	Induction with prostaglandins after external version from breech	6873	Negative		Not sequenced
4	38+2	Yes (PROM)	Spontaneous	Yes	36	Slow progression	GBS	1888	Negative		Not sequenced
5	39+4	Yes	Amniotomy	Yes	4	Slow progression	Pathologic CTG	46,893	Positive	*Streptococcus agalactiae* (99%) *Peptoniphilus harei* (99%) *Peptoniphilus asaccharolyticus* (99%)	*Bifidobacterium* (22.4%) *Olsenella* (38.6%) *Prevotella* (18.7%) *Aerococcus* (4.6%) *Lactobacillus* (6.2%) *Shuttleworthia* (1.2%) *Megasphaera* (1.3%) *Sneathia* (1.9%) *Caulobacteraceae* (1.0%)
6	37+5	Yes	Amniotomy	Yes	17	Slow progression and clinical chorioamnionitis	MCDA twins, induction with balloon catheter and amniotomy	1462	Positive		Not sequenced
7	40+4	Yes (PROM)	Spontaneous	No	31	Slow progression		67,077	Positive	*Lactobacillus reuteri* (98%) *L crispatus* (99%) *L vaginalis* (98%)	Inconclusive results

Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

(continued)

TABLE 2
Clinical information on 14 women with cesarean delivery with prior rupture of membranes and results from microbiological amniotic fluid analysis *(continued)*

ROM group	GA (wk + d) at ROM	ROM prior to start of labor	Spontaneous ROM or amniotomy	Regular contractions prior to CS	Time from ROM to cesarean delivery, h	Indication for cesarean delivery	Other information	ddPCR DNA copies/mL	Culture aerobic/ anaerobic	Sanger sequencing species (percentage represents identity to closest match in NCBI database)	Illumina 16S rRNA gene sequencing taxonomy present in ≥1%
8	41+1	No	Amniotomy	Yes	18	Slow progression	Induction with balloon catheter and prostaglandins	57,246	Positive	*Prevotella amnii* (99%) *Prevotella bivia* (99%)	*Bifidobacterium* (28.1%) *Olsenella* (8.4%) *Aerococcus* (50.5%) *Sneathia* (2.9%) *Caulobacteraceae* (1.0%)
9	40+5	No	Spontaneous	No	13	Slow progression and clinical chorioamnionitis	Induction with balloon catheter and prostaglandins	1275	Negative		Not sequenced
10	42+1	Yes	Amniotomy	Yes	9	Slow progression and clinical chorioamnionitis	Induction with prostaglandins and amniotomy	6743	Negative		Not sequenced
11	40+3	No	Spontaneous	Yes	22	Slow progression and clinical infection	Pathologic CTG	1066	Positive		Not sequenced
12	41+6	No	Amniotomy	No	20	Slow progression		251,430	Positive		*Sneathia* (98.3%)
13	40+0	No	Spontaneous	Yes	6	Slow progression and fetal distress	Breech	170,520	Negative		*Lactobacillus* (21.1%) *Caulobacteraceae* (27.7%) *Bradyrhizobium* (2.7%) *Sphingomonas* (5.1%) *Halomonas* (1.0%) *Pseudomonas* (17.8%)
14	41+1	Yes	Amniotomy	No	15	Slow progression	Induction with balloon catheter and amniotomy	8526	Negative		Not sequenced

CTG, cardiotocography; *ddPCR*, digital droplet polymerase chain reaction; *GA*, gestational age; *GBS*, group B streptococcus; *MCDA*, monochorionic diamniotic; *NCBI*, National Center for Biotechnology Information; *PROM*, premature rupture of membranes; *ROM*, rupture of amniotic membranes.

Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

TABLE 3
Results from digital droplet polymerase chain reaction, gel electrophoresis of polymerase chain reaction products from aerobic and anaerobic cultures, and Sanger sequencing

	ddPCR DNA copies/mL	GE aerobic (band)	GE anaerobic (band)	Aerobic colonies	Anaerobic colonies	Sanger sequencing species (percentage represents identity to closest match in NCBI database)
Non-ROM (n = 10)	Mean: 672 Median: 664 (544–748) SD 65.5	No	No	No	No	
Negative control operating room	679	No	No	No	No	
Negative control laboratory	461	No	No	No	No	
Positive control (*Escherichia coli*) ddPCR	32,190					
Negative control ddPCR	104					
ROM (n = 14)	Mean: 47,687 Median: 7700 (1066–251,430) SD 74,751					
1	45,066	No	Yes	No	No	
2	1553	No	No	No	No	
3	6873	No	No	No	No	
4	1888	No	No	No	No	
5	46,893	Yes	Yes	No	3 Colonies	*Streptococcus agalactiae* (99%) *Peptoniphilus harei* (99%) *Peptoniphilus asaccharolyticus* (99%)
6	1462	No	Yes	No	No	
7	67,077	No	Yes	No	2 Colonies	*Lactobacillus reuteri* (98%) *L crispatus* (99%) *L vaginalis* (98%)
8	57,246	No	Yes	No	1 Colony	*Prevotella amnii* (99%) *Prevotella bivia* (99%)
9	1275	No	No	No	No	
10	6743	No	No	No	No	
11	1066	No	Yes	No	No	
12	251,430	Yes	Yes	No	No	
13	170,520	No	No	No	No	
14	8526	No	No	No	No	
Negative control operating room	618	No	No	No	No	
Negative control laboratory	574	No	No	No	No	
Positive control (*Escherichia coli*) ddPCR	24,012					
Negative control ddPCR	244					

ddPCR, digital droplet polymerase chain reaction; *GE*, gel electrophoresis; *NCBI*, National Center for Biotechnology Information; *ROM*, rupture of amniotic membranes.
Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

TABLE 4
Illumina 16S rRNA gene sequencing taxonomy in rupture of amniotic membranes group and in negative controls

	Total	1	5	8	12	13	Negative control laboratory	Negative control OR
Taxonomy — genera	%	%	%	%	%	%	%	%
Bifidobacterium	8.4	0.0	22.4	28.1	0.0	0.0	0.0	0.0
Olsenella	7.8	0.0	38.6	8.4	0.0	0.0	0.0	0.0
Bacteroidales uncultured	0.3	0.2	0.0	0.0	0.0	0.1	1.4	0.4
Prevotella	3.2	0.0	18.7	0.3	0.0	0.0	0.0	0.0
Aerococcus	9.2	0.0	4.6	50.5	0.1	0.0	0.0	0.0
Lactobacillus	16.2	69.5	6.1	0.2	0.0	21.0	0.1	0.0
Lachnospiraceae	0.4	0.2	0.0	0.0	0.0	0.5	2.0	0.2
Shuttleworthia	0.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0
Megasphaera	0.2	0.0	1.3	0.0	0.0	0.0	0.0	0.0
Sneathia	17.2	0.0	1.9	2.9	98.3	0.0	0.0	0.0
Caulobacteraceae; other	14.6	10.6	1.0	2.1	0.4	27.7	46.0	65.9
Bradyrhizobium	1.8	0.8	0.1	0.5	0.1	2.7	6.3	3.9
Sphingomonas	2.0	1.5	0.2	0.9	0.2	5.1	4.1	4.2
Ralstonia	0.7	0.3	0.0	0.1	0.0	0.9	2.9	0.2
Delftia	0.3	0.1	0.0	0.1	0.0	0.4	1.1	0.3
Pseudoalteromonas	0.4	0.3	0.1	0.1	0.0	0.3	1.7	1.0
Halomonas	0.7	0.4	0.1	0.1	0.0	1.0	2.8	2.2
Pseudomonas	9.4	7.6	1.1	2.6	0.4	17.8	26.7	19.5
Stenotrophomonas	0.3	0.2	0.0	0.0	0.0	0.1	1.3	0.4
Ureaplasma	1.0	0.0	0.2	0.0	0.0	5.9	0.0	0.0
Other	1.9	1.7	2.3	1.1	0.5	2.4	3.6	1.8
Unassigned; other	3.8	6.6	0.1	2.0	0.0	14.1	0.0	0.0

OR, operating room.
Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

identified (by Sanger sequencing) as bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections, namely *Streptococcus agalactiae*, *Peptoniphilus harei*, *Peptoniphilus asaccharolyticus*, *Lactobacillus reuteri*, *Lactobacillus crispatus*, *Lactobacillus vaginalis*, *Prevotella amnii*, and *Prevotella bivia*, as seen in Table 2.

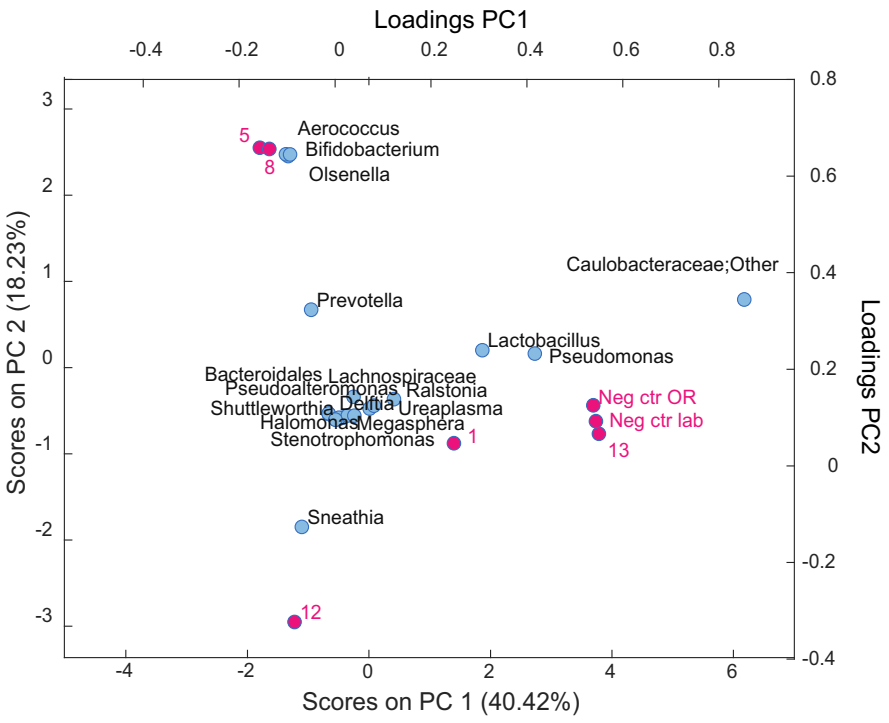
Illumina 16S rRNA gene sequencing

In 5 of the 6 amniotic fluid samples (with >1000 16S rRNA copies/μL) amplicon sequencing of the 16S rRNA gene revealed species belonging to bacterial genera that are part of a normal vaginal flora, namely *Bifidobacterium*, *Olsenella*, *Prevotella*, *Aerococcus*, *Lactobacillus*, *Shuttleworthia*, *Sneathia*, *Caulobacteraceae*, *Pseudomonas*, and *Ureaplasma*, of which some are known to contain species that are associated with bacterial vaginosis and/or infections, as well as possible contamination. In 2 negative controls (1 from operating room and 1 from the laboratory), we found genera associated with reagent and laboratory contamination, namely: *Caulobacteraceae*, *Pseudomonas*, *Sphingomonas*, *Bradyrhizobium*, *Ralstonia*, and *Stenotrophomonas*,[23] as seen in Table 4. Associations of microbiota with the samples analyzed are shown in a principal component plot; these analyses confirmed tight clustering of the negative controls and the relative large diversity in the ROM group (Figure 2).

Comment

Recently, the view that amniotic fluid does not have live bacterial communities present in uncomplicated term pregnancies was challenged by identifying an amniotic fluid microbiota (using 16S rRNA gene sequencing PCR) in 15 uncomplicated term pregnancies, finding a core set of bacterial phylotypes that was overlapping with the microbiota found in placenta and meconium.[7] Our

FIGURE 2
Associations of microbiota with samples analyzed in rupture of amniotic membranes (ROM) group



Taxonomic groups of bacteria were clustered based on principal component (PC) analysis, with corresponding scores for first 2 PC (blue circles) and explained variance (parentheses). Corresponding loadings for samples analyzed (red circles).

ctr, control; *lab*, laboratory; *neg*, negative; *OR*, operating room.

Rehbinder et al. Bacteria in amniotic fluid. Am J Obstet Gynecol 2018.

findings, however, support a sterile amniotic fluid until the start of labor, which are in line with previous studies using cultivation techniques,[8–10,24] as well as a study using both cultivation and 16S rDNA qPCR in term uncomplicated pregnancies.[25] Studies that demonstrate the pioneer microbiota in newborns are also supporting that fetal bacterial colonization in uncomplicated term pregnancies does not start before labor.[9,26–29] In newborns delivered by cesarean delivery, the initial colonization is predominately by skin microbes, not only originating from their mother,[26,27] but also from the operating room.[30] A recent study by Chu et al[28] found that cesarean delivery newborns from mothers having been in labor had similar initial colonization pattern to a vaginal delivery, with both vaginal and skin microbes present, compared to unlabored cesarean delivery infants, with predominantly skin microbes present.

We designed our study to minimize the source of possible contamination in the sampling, aliquoting, and analyzing process. In the 10 subjects selected for the non-ROM group, amniotic fluid was sampled during elective cesarean delivery, in the same operating room by the same health personnel, minimizing variations in case of contamination. As reflected by our sterile controls, avoiding all forms of minor contaminations in a clinical setting is nearly impossible. The bacterial DNA found in studies on low-microbial biomass samples has been criticized to not originate from live bacteria, but as a result of contamination or transport of dead microbial products brought by the bloodstream.[13,14] In a study by Lauder et al,[15] the placental samples were indistinguishable to the negative controls (both in the low number of DNA copies and by sequencing). It is likely that the fetus is exposed to maternal microbial components,[4] but if they have any role in promoting health or disease in the fetal and/or newborn life is unknown.

In the ROM group we found species that are known to be a part of the vaginal flora in women of reproductive age,[31] dominated by lactobacilli species, but we also found genera that can either be part of a normal vaginal flora or be associated with bacterial vaginosis, such as bifidobacteriae, prevotellae, aerococci, peptoniphili, streptococci, ureaplasma, and sneathiae. These findings support an ascending microbial colonization of the intrauterine cavity with term ROM,[24,28,32,33] helped by premature ROM and prolonged labor.[9,32,34] Previous studies also suggest that colonization depends upon the length of the labor and the number of vaginal examinations during labor.[9,29] However, in our study there were too few women with ROM to study potential correlations between the length of labor and bacterial load. In the ROM group samples, we also found bacterial genera that are associated with reagent and laboratory contamination,[23] namely *Caulobacteraceae* and *Pseudomonas*. These genera were also identified in our negative controls, and could therefore be accounted for as contamination, which emphasizes the need for appropriate controls when performing molecular-based studies.

Preterm deliveries and neonatal death are associated with microbial invasion of the intrauterine cavity both in those with preterm premature ROM and with intact membranes,[35] suggesting several routes of microbial spread; either ascending from the vagina or descending from other organs through the maternal bloodstream, from the peritoneal cavity via the fallopian tubes or due to prenatal intrauterine procedures. In several studies analyzing amniotic fluid with molecular techniques, from preterm deliveries, bacteria have been identified that would not have been found by the only use of culturing,[29,36,37] as is also demonstrated in the sequencing results of our study. In contrast to our study

where lactobacilli were dominating in the ROM group, they are rarely found in case of preterm microbial invasion of intrauterine cavity as the bacteria commonly found here are mostly associated with bacterial vaginosis, but periodontal pathogenic bacteria have also been identified.[29,36,37]

With molecular-based studies on amniotic fluid, if appropriate measures for avoiding contamination are considered, it has been possible to get a clearer picture of how microbial invasion of the intrauterine cavity occurs and which microbes are involved. With our study, we believe that we can settle that the first colonization of the fetus normally occurs during labor. If the baby is born by cesarean delivery in an uncomplicated term pregnancy without prior labor it will not be in contact with the vaginal microbiota, which in turn can negatively affect how the child's microbiota and immune system develops.[3–5] We therefore believe that our study adds to the arguments that an indication for an elective (planned) cesarean delivery should be carefully considered in each individual case and that it is not to be taken lightly. Interestingly, preliminary results of swabbing the infant with vaginal microbes from their mother immediately after cesarean delivery has implicated that the pioneer microbiota in these cesarean delivery–born infants resembles that of a vaginally born infant.[38]

Although the amount of DNA in the non-ROM group was too low to identify a bacterial microbiota, the highly sensitive and accurate ddPCR quantification[17] allowed us to identify bacterial DNA at the single copy level. Regular qPCR cannot accurately detect single copies of bacterial DNA, and would therefore be less useful due to the very low bacterial content in amniotic fluid, as shown in a recent study where no 16S rRNA nor 18S rRNA was found in amniotic fluid from amniocentesis in 344 asymptomatic women at midgestation,[39] and a median 16S rRNA gene copy number of 0 in 20 amniotic fluid samples from term gestation in another study.[25]

A limitation of our study is the small number of samples, with a heterogeneous bacterial load in the ROM group, as well as a relatively large time span from ROM until delivery. However, the lack of bacterial detection in the non-ROM group is consistent, and similar to the findings of negative controls and clearly different from the consistent positive bacterial findings (both by highly sensitive DNA quantifications and cultures) in the ROM group.

Despite our lack of identifying a unique amniotic fluid bacterial microbiota in our population of uncomplicated pregnancies, we cannot exclude the existence of a placental microbiota. The evidence of a placental microbiota is conflicting, nonetheless we hypothesize that in pregnancies with a dysfunctional placenta, such as in infections, fetal growth restriction, or preeclampsia, prenatal microbial intraamniotic invasion is possible. This is supported by findings of an altered placental microbiome in preterm births with and without chorioamnioinitis.[11,12,40–42] In a recent study by Doyle et al,[12] a placental microbiome was identified in 50% of the samples (by 16S rRNA sequencing), and specific bacterial communities were found to be associated with chorioamnionitis and low birthweight. These bacteria originated mostly from the vagina, which is in contrast to previous findings of placental microbiome resembling oral bacterial communities.[11] If these findings favor a healthy placental microbiome that could become dysbiotic, or if the bacterial colonization of the placenta only occurs in a diseased state, is still not clear.

We find it reasonable to assume, in the light of our findings, that previous publications of an amniotic fluid microbiome[7] may have been hampered by potential contamination, possibly combined with unrecognized placental dysfunction and/or uterine contractions with prior ROM. Initial colonization of the infant is affected by ROM.[9,28,29,32,33] We speculate that the long-term offspring adverse health effects seen in pregnancies with placental dysfunction[43] may partly be mediated through an early in utero microbial exposure.

We conclude that amniotic fluid is sterile in uncomplicated pregnancies with intact amniotic membranes at term. ■

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Author and article information

From the Faculty of Medicine, Institute of Clinical Medicine, University of Oslo, Oslo (Drs Rehbinder, Carlsen, Staff, Hilde, and Gaustad); Department of Pediatrics (Drs Rehbinder and Carlsen), Department of Dermatology (Drs Rehbinder and Landrø), and Division of Obstetrics and Gynecology (Dr Staff and Hilde), Oslo University Hospital, Oslo; Norwegian University of Life Sciences, Ås (Ms Angell and Dr Rudi); and Fürst Medical Laboratory, Oslo (Dr Gaustad), Norway.

[1]These authors contributed equally to this article.

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A full list of the members of the PreventADALL study group can be found in the supplementary information.

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Corresponding author: Eva Maria Rehbinder, MD. e.m.rehbinder@medisin.uio.no

Supplementary Material

PreventADALL study group:

Oslo: Karin C. Lødrup Carlsen (principal investigator), Håvard O. Skjerven (local principal investigator), Karen Eline Stensby Bains, Kai-Håkon Carlsen, Oda C. Lødrup Carlsen, Monica Hauger Carlsen, Kim Advocaat Endre, Thea Aspelund Fatnes, Pål Fugelli, Peder Annæus Granlund, Berit Granum, Hrefna Katrín Gudmundsdóttir, Guttorm Haugen, Katarina Hilde, Henrik Holmstrøm, Geir Håland, Ina Kreyberg, Linn Landrø, Petter Mowinckel, Live Solveig Nordhagen, Unni C. Nygaard, Eva Maria Rehbinder, Carina Madelen Schinagl, Ingebjørg Skrindo, Anne Cathrine Staff, Riyas Vettukattil

Østfold: Jon Lunde (local principal investigator), Christine Monceyron Jonassen, Knut Rudi, Katrine Sjøborg, Magdalena R. Værnesbranden, Johanna Wiik

Stockholm: Björn Nordlund (local principal investigator), Anna Asarnoj, Gunilla Hedlin, Cilla Söderhäll, Caroline-Aleksi Olsson Mägi, Sandra G. Tedner

Switzerland/Australia: Benjamin J. Marsland

Finland: Petri Auvinen, Tari Haahtela

III

Research letter

Dry skin and skin barrier in early infancy

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Dear Editor, Atopic dermatitis (AD) usually begins in infancy, commonly involving the cheeks and extensor surfaces of the extremities (hereafter, extensors). It is associated with a dysfunctional skin barrier, which can be measured as increased transepidermal water loss (TEWL) in both lesional and nonlesional skin in patients with AD.[1] Dry skin, a cardinal sign of AD, is associated with higher TEWL in adult patients with AD.[2] However, the documentation of the prevalence and manifestation of dry skin in infancy and its association to TEWL is limited. Therefore, we aimed to determine the prevalence of dry skin in early infancy and to assess if dry skin in general, or more specifically on the cheeks and extensors, was associated with a dysfunctional skin barrier.

From the population-based Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study[3] we found that 59% of the 1143 included 3-month old infants had dry skin, defined as roughness and visible scaling without erythema, in at least one of 11 predefined anatomical skin areas. Most infants (47%) had 'dry skin only', while 40% had 'unaffected skin' and 13% had 'possible AD' (of these, 96% had dry skin), defined as doctor-verified dermatitis, excluding differential diagnosis and including only a few infants fulfilling the diagnostic criteria for AD, as the majority were unable to itch at this early age. Among the 540 infants with 'dry skin only' the two most common locations were the cheeks in 62% and extensors in 49%. Dry skin was observed in 96% of the 144 infants with 'possible AD'; most commonly on the cheeks (82%) and the extensors (88%). Standardized TEWL examination, using an open-chamber DermaLab USB (Cortex, Hadslund, Denmark), was measured on the lateral upper arm as previously described,[4] and is presented as mean TEWL (g m^{-2} h) with 95% confidence interval (CI) for the 1019

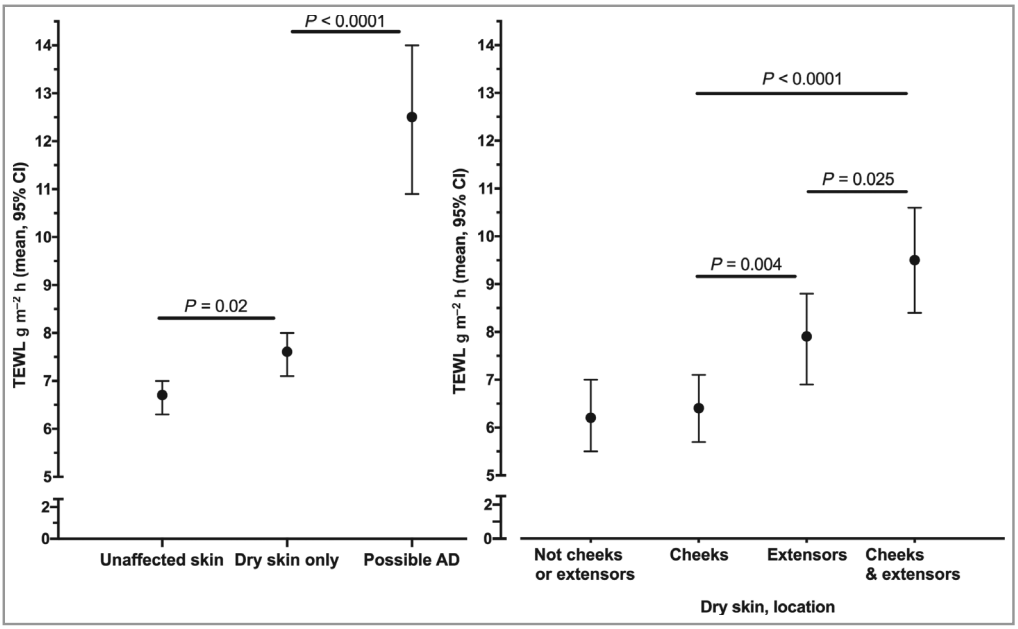

Fig 1. Skin barrier function, given as mean (95% CI) TEWL, measured on left lateral upper arm in 1-month old infants (n = 1019) in the PreventADALL study. (a) Mean TEWL, in 'dry skin only' (n = 483) was significantly higher than in 'unaffected skin' (n = 411, P = 0·02) and significantly lower than in 'possible AD' (n = 125, P < 0·0001). (b) Mean TEWL in infants with dry skin without AD located anywhere but 'not cheeks or extensors' (n = 79) was similar in infants with dry skin anywhere including 'cheeks, but not extensors' (n = 161), and significantly lower in those with dry skin including 'cheeks and extensors' (n = 134, P < 0·0001), as well as in those with dry skin anywhere including 'extensors, but not cheeks' (n = 98, P < 0·004). Mean TEWL in 'cheeks and extensors' was significantly higher than in 'extensors' (P = 0·025). AD, atopic dermatitis; CI, confidence interval; TEWL, transepidermal water loss

(89%) infants with available data. The TEWL was significantly higher among infants with 'dry skin only' (7·6; 7·1–8·0), compared with 'unaffected skin' (6·7; 6·3–7·0) and significantly lower than in infants with 'possible AD' (12·5; 10·9–14·0) (two-way ANOVA) (Fig. 1a). In the subgroup analysis (independent sample Student's *t*-test) shown in Figure 1b, TEWL was similar among infants with 'dry skin – cheeks' (6·4; 5·7–7·1) and 'unaffected skin' (6·7; 6·3–7·0), which was significantly lower than in infants with 'dry skin – extensors' (7·9; 6·9–8·8). The highest TEWL in infants without AD was found in infants with 'dry skin – extensors and cheeks' (9·5; 8·4–10·6), and it was significantly higher also compared with 'dry skin – extensors'. All our results remained significant after adjusting for possible confounders with linear regression analysis: sex, gestational age at birth, age at examination, room humidity and temperature. Statistical analyses were performed in IBM SPSS© v. 25 (Chicago, IL, U.S.A.).

To our knowledge, this is the first study to report on the prevalence of dry skin in early infancy, in a large general population-based study. However, a Swedish case–control study observed dry skin in 40% of 99 healthy 2-year-old children and in all children with AD.[5] The high prevalence of dry skin in children living in Nordic countries may be due to low temperatures in the winter and lower air humidity which is associated with an increase in signs of dry skin, TEWL and flares of AD.[6]

Tight control of room humidity when measuring TEWL in our study was not possible, as the investigations were performed in settings resembling regular clinical practice. Supported by previous findings,[4] all our results remained significant after adjusting for humidity, allowing us to include investigations throughout the different seasons.

The two most common areas of dry skin, the cheeks and extensors, are exposed to wear and tear from environmental factors, possibly impairing the skin barrier, which in turn can manifest as clinically dry skin, and ultimately as AD. This is supported by the outside-inside hypothesis where an initially impaired skin barrier leads to the entry of external stimuli that further drives the T-helper 2 inflammation causing the onset of AD.[7] Studies suggest that increased TEWL in infancy precedes AD development[4] and allergic sensitization[1] and that there is a regional and temporal immaturity in the skin barrier of infant cheeks.[8] Future follow-up investigations in the PreventADALL study[3] may demonstrate if our findings of impaired skin barrier in infants with dry skin, especially when present concurrently on the cheeks and extensor surfaces, may point to a potential role for dry skin examination when selecting children for primary prevention,[1] at risk for long-life allergic diseases.

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[1]*Oslo University Hospital and University of Oslo, Oslo, Norway*
[2]*Karolinska University Hospital and Karolinska Institutet, Stockholm, Sweden*
[3]*Østfold Hospital Trust, Kalnes, Norway*
E-mail: e.m.rehbinder@medisin.uio.no

E.M. Rehbinder[1]
A.J. Winger[1]
L. Landrø[1]
A. Asarnoj[2]
T.L. Berents[1]
K.H. Carlsen[1]
G. Hedlin[2]
C.M. Jonassen[3]
B. Nordlund[2]
L. Sandvik[1]
H.O. Skjerven[1]
C. Söderhäll[2]
R. Vettukattil[1]
K.C.L. Carlsen[1]
on behalf of the PreventADALL study group*

E.M.R. and A.J.W. contributed equally.

*P. Auvinen, K.E.S. Bains, M.H. Carlsen, O.C. Lødrup Carlsen, K.A. Endre, P.A. Granlund, B. Granum, H.K. Gudmundsdóttir, G. Haugen, K. Hilde, I. Kreyberg, C.A.O. Mägi, L.S. Nordhagen, U.C. Nygaard, K. Rudi, C.M. Saunders, I. Skrindo, K. Sjøborg, A.C. Staff, S.G. Tedner, M.R. Værnesbranden and J. Wiik.

Further author, institution and funding details are available directly from the corresponding author.

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Clinical Trial Registration
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IV

Predicting skin barrier dysfunction and atopic dermatitis in early infancy

Eva Maria Rehbinder, MD[1,2,3], Kim M. Advocaat Endre, MD[1,2,3], Karin C. Lødrup Carlsen, MD, PhD[1,2], Anna Asarnoj, MD, PhD[6,7,8], Karen Eline Stensby Bains, MD[1,2], Teresa Løvold Berents, MD, PhD[3], Kai-Håkon Carlsen, MD, PhD[1,2], Hrefna Katrín Gudmundsdóttir, MD[1,2], Guttorm Haugen, MD, PhD[2,9], Gunilla Hedlin, MD, PhD[6,7], Ina Kreyberg, MD[1,2], Live Solveig Nordhagen, MSc[1,2,15], Björn Nordlund, RN, PhD[6,7], Carina Madelen Saunders, MD[1,2] Leiv Sandvik, PhD[2,4], Håvard O. Skjerven, MD, PhD[1,2], Cilla Söderhäll, PhD[7], Anne Cathrine Staff, MD, PhD[2,9], Riyas Vettukattil, MBBS, PhD[1,2], Magdalena R. Værnesbranden, MD[2,5], Linn Landrø, MD, PhD[3]

On behalf of the study group:

Monica Hauger Carlsen, PhD[12], Oda C. Lødrup Carlsen[1], Peder Annæus Granlund[1,2], Berit Granum, PhD[10], Sandra Götberg[6,7], Katarina Hilde, MD[2,9], Christine Monceyron Jonassen, PhD[11,13], Unni C. Nygaard, PhD[10], Knut Rudi, Professor, PhD[13], Ingebjørg Skrindo, MD, PhD[1,16], Katrine Sjøborg, MD, PhD[5], Sandra G. Tedner, MD[6,7], Johanna Wiik, MD[5, 17] Angelica Johansen Winger, MD[1,2]

1: Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway.

2: University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway.

3. Department of Dermatology, Oslo University Hospital, Oslo, Norway

4: Oslo Centre for Biostatistics and Epidemiology, Oslo University Hospital, Oslo, Norway

5: Department of Obstetrics and Gynaecology, Østfold Hospital Trust, Kalnes, Norway.

6: Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden

7: Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

8: Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden

9: Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway

10: Department of Toxicology and Risk Assessment, Norwegian Institute of Public Health, Oslo, Norway

11: Genetic Unit, Centre for Laboratory Medicine, Østfold Hospital Trust, Kalnes, Norway.

12: Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway.

13: Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

14: Institute of Biotechnology, University of Helsinki, Helsinki, Finland

15: VID Specialized University, Oslo, Norway

16: Department of Ear, nose and throat, Akershus University Hospital, Lørenskog, Norway

17: Department of Obstetrics and Gynaecology, Institute of Clinical Sciences, Sahlgrenska Academy, Gothenburg, Sweden

Address of correspondence to:

Eva Maria Rehbinder

E-mail: e.m.rehbinder@medisin.uio.no

Phone nr: + 47 93842978

Department of Dermatology, Oslo University Hospital

Postboks 4950, Nydalen

NO-0424 Oslo

Norway

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Abstract

Background

Dry skin, associated with increased transepidermal water loss (TEWL), is found to precede atopic dermatitis (AD) in childhood.

Objective

We aimed to identify parental, prenatal and perinatal predictive factors of dry skin, high TEWL and AD at 3 months of age, and to determine if dry skin or high TEWL at 3 months can predict AD at 6 months.

Methods

From the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) prospective birth cohort study, we included 1150 mother-child pairs. Dry skin, TEWL and eczema were assessed at 3- and 6 months investigations. Eczema, used as a proxy for AD, was defined as the presence of eczematous lesions, excluding differential diagnoses to AD. High TEWL was defined as TEWL > 90th percentile, equalling 11.3 g/m^2/h. Potential predictive factors were recorded from electronic questionnaires at 18- and 34-week pregnancy and obstetric charts.

Results

Significant predictive factors ($p<0.05$) for dry skin at 3 months were delivery > 38 gestational weeks and paternal age > 37 years, for high TEWL; male sex, birth during winter season and maternal allergic disease, and for eczema; elective caesarean section, multiparity, and maternal allergic diseases. Dry skin without eczema at 3 months was predictive for eczema at 6 months, ($OR_{adjusted}$: 1.92, 95% CI: 1.21-3.05, p=0.005), while high TEWL at 3 months was not.

Conclusion

In early infancy, distinct parental and pregnancy-related factors were predictive for dry skin, high TEWL and AD. Dry skin at 3 months of age was predictive for AD three months later.

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Clinical implications: Recognizing dry skin in early infancy could be a way of selecting infants for primary prevention of atopic dermatitis.

Highlight box:

1. What is already known about this topic?

Skin barrier dysfunction, measured by increased transepidermal waterloss (TEWL) has been found to precede atopic dermatitis (AD). Dry skin, a cardinal sign of AD is associated with higher TEWL.

2. What does this article add to our knowledge?

The article reveals distinctive factors predictive for dry skin, high TEWL and AD at 3 months of age. Dry skin at 3 months was predictive for AD three months later.

3. How does this study impact current management guidelines?

Recognizing predictive factors for AD early in life, including the presence of dry skin, may help targeting infants for primary prevention of AD.

Key words: Dry skin, xerosis, skin barrier, atopic dermatitis, eczema, allergic diseases, atopy, TEWL

Abbreviations:
AD: atopic dermatitis

TEWL: transepidermal water loss

FLG: filaggrin

GA: gestational age

CS: Caesarean section

Introduction

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease that most often present during early childhood (1). The lifetime prevalence in industrialized countries is high, ranging from 15-20% (2). Dry skin, erythema and pruritus are hallmarks of the disease (1). Diagnosis of AD is made clinically, sometimes using validated diagnostic criteria (3, 4).

The pathophysiological aspect of AD involves complex interactions between skin barrier function, immune dysregulation and dysbiosis of the skin microbiota (1, 5). A dysfunctional skin barrier appears to be a key player in development of the disease (1, 6). The clinical presence of dry skin, a cardinal feature of AD (1, 3, 4), is indicative of an impaired skin barrier and correlates with elevated measures of transepidermal water loss (TEWL) (7, 8). Recent studies suggest that increased TEWL in early infancy may precede and even predict the development of AD (9-11). Infants with AD are at increased risk of developing food allergy, allergic rhinitis and asthma in line with the proposed atopic march (12, 13). These findings provide a rationale for early life skin-directed treatment to enhance the barrier function and possibly prevent AD (14-16).

The most prominent risk factors for development of AD are parental allergic disease and the presence of mutations in the gene encoding filaggrin (*FLG*) (1, 6, 17). The most consistent environmental risk factors are low UV-light exposure, dry climate, urban living, small family size, high parental education level and repeated treatment with antibiotics in early childhood (17, 18). In addition, the association between caesarean section and offspring allergic disease has been extensively investigated, however with conflicting results (19-21). Increased knowledge of predictive factors of skin barrier dysfunction and AD in infancy is warranted to provide targeted prevention strategies. Studies aiming to identify predictors of dry skin and

reduced skin barrier function measured by TEWL in early infancy have largely been lacking. We are not aware of previous studies investigating the presence and distribution of dry skin and later debut of AD in early infancy.

We recently showed in the PreventADALL cohort that 59% of 3-month old infants had dry skin, while of the 145 infants with eczema 96% had dry skin. Dry skin without eczema on age specific predilection sites of AD, cheeks and extensor surfaces of extremities were significantly associated with increased TEWL (8).

In the present study we hypothesized that dry skin or increased TEWL could predict AD in infancy. We aimed to identify factors that can predict dry skin, high TEWL and AD at 3 months of age. Further, we aimed to determine if dry skin, in general or on age specific predilection sites of AD, or high TEWL at 3 months of age could predict AD at 6 months of age.

Subjects and Methods

Study design

The present study included 1150 infants, attending the 3 months investigation, randomized to the two groups that did not receive skin care intervention from the general population based Preventing Atopic Dermatitis and Allergies (PreventADALL) study (22). The PreventADALL multicentre, prospective, 2x2 factorial, interventional birth-cohort study investigates the effect of primary prevention of allergic diseases by early skin care and early complementary food introduction.

Women were recruited during the routine 18-week gestational age (GA) ultrasound examination at Oslo University Hospital, Østfold Hospital Trust (Norway) and Karolinska

University Hospital (Stockholm, Sweden) between December 2014 and October 2016. Their infants, born at a GA of at least 35 weeks and without serious illnesses, were enrolled during the 1-2 first days of life. Infants attended follow-up visits at 3 and 6 months of age, with skin assessments performed by trained study personnel who were blinded to the randomization groups. Study information included comprehensive electronic questionnaires, weekly diaries, biological samples from mother and child, and clinical investigations. Study design, recruitment and inclusion criteria, as well as characteristics of the 2696 women and 2396 mother-child pairs have been described in detail in a previous paper (22).

Informed consent forms were signed by the mother at enrollment, and by both parents (when relevant) upon inclusion of the infant. The PreventADALL study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2014/518) and in Sweden (2014/2242-31/4), as well as registered at clinicaltrials.gov (*NCT02449850).*

Subjects

The 1150 infants had a mean GA of 39.3 weeks at birth and 46.2% were girls (Table I).
For the secondary aim, we included all 930 of the 1070 infants who also attended the 6-month follow-up visit, excluding infants with eczema at the 3-month investigation, as shown in Figure 1. Detailed information on dry skin location at 3 months and eczema at 6 months was available in 913 infants.

Health personnel were trained to examine the skin by visual inspection and palpation. Observations of dry skin, presented as scaling and roughness, were recorded for 11 predefined anatomical skin areas (23) in terms of no, mild, moderate or severe dry skin. Severity of dry skin was recorded in line with the principles of the Dry skin/Ichtyosis and Severity Index (DASI), but without their score of erythema (24). *Mild dryness* was categorized as barely

visible scaling and slight roughness when stroking the skin. *Moderate dryness* was categorized as clearly visible scaling with or without fissures, and roughness when stroking the skin. *Severe dryness* was categorized as abundant scaling and present fissures, as well as very rough skin when stroking the skin.

Eczema, used as a proxy for AD, was defined as the presence of eczematous lesions, verified by a medical doctor with the exclusion of differential diagnoses to AD.

TEWL measurements ($g/m^2/h$) were available in 1033 (89%) of the 3 months old infants, using an open chamber DermaLab USB (Cortex, Hadslund, Denmark). We included measurements performed in room temperature between 20 and 25°C only, in line with international recommendations (25), while accepting humidity within the whole range 6% - 73%, mean 29%, standard deviation (SD) 12.7. Parents were instructed not to bathe the infants or use any emollients within 24 hours prior to the examination. Three successive measurements were performed on the left upper lateral arm after 15 minutes of acclimatization where the child was only wearing diaper, keeping the room temperature as close to 22°C as possible, noting ambient temperature and humidity. Measurements were only performed on calm children and windows and doors were kept shut.

Potential predictive factors were chosen on the basis of previously described risk factors for allergic diseases, potential relevant pregnancy-related factors as well as baseline characteristics as outlined in Table 1.

Definitions and outcome

Unaffected skin was defined as no eczema and no dry skin. *Dry skin* included all infants with presence of dry skin on at least one location, regardless of eczema. *Dry skin only* was defined

as dry skin with no eczema and was further sub-categorized into dry skin on *Cheeks*, *Extensors* or *Both cheeks and extensors.*

The outcomes in the present study were *Dry skin* (any location of dry skin), *Eczema* and *High TEWL* (mean TEWL above 90th percentile) at 3 months of age and *Eczema* at 6 months of age.

Statistical analysis

Categorical variables are presented as numbers and percentages. Continuous variables are presented as means, SD and minimum (min) –maximum (max).

While the TEWL results did not display a perfect normal distribution, the deviation from normality was moderate, and we could therefore use parametric statistical methods for all our analyses. Independent sample t-test was used when comparing continuous variables, and chi-square test was used when comparing categorical variables.

Logistic regression analysis was used to investigate the associations between parental and pregnancy- related variables (Table I) and the outcome variables *Dry skin*, *Eczema* or *High TEWL.* We used univariate logistic regression analysis with a cut-off p-value of 0.2*,* followed by complete-case multivariate regression analysis. The continuous variables that were found to be significant in the univariate regression analysis were analysed as quartiles, with the lowest quartile as the reference value. If the strength of the association was higher in any quartile, we used the quartiles in the multivariate regression model. In each regression model the assumption underlying multivariate logistic regression analysis were checked and found to be adequately met.

In order to investigate the impact of dry skin and high TEWL at 3 months of age on eczema at 6 months of age, the following three regression models were performed: Model 1: Unadjusted. Model 2: The predictors from the multivariate logistic regression analyses at 3 months of age were used here. For dry skin we adjusted for the predictors found for dry skin and eczema, and for high TEWL we adjusted for the predictors found for high TEWL and eczema. Model 3: Variables from model 2 together with variables significantly associated with *Eczema* at 6 months from univariate logistic regression analysis (doctor diagnosed AD in father, alcohol consumption and domestic cat during pregnancy). Statistical significance level was set to 5%. All analyses were performed using IBM© SPSS© statistics version 25 (Chicago, IL, U.S.A.).

Results

Baseline characteristics

At 3 months of age 544 out of the 1150 infants investigated, had dry skin without eczema (dry skin only) and 145 had eczema. At 6 months of age 163 of the 930 infants that attended the follow-up had eczema, excluding the infants with eczema at 3 months. Out of 832 with valid TEWL measurements, 82 had high TEWL at 3 months. The clinical, socioeconomic, and demographic details of the study population are presented in Table I for the infants at 3 months of age and for the infants at 6 months are presented in Table EI in the online repository.

Predictive factors at 3 months of age

For *Dry skin*, GA and paternal age were statistical significant predictors in the multivariate analysis after including the 10 variables with a p-value <0.2 in the univariate logistic regression analysis (Figure 2a, Table E2a and E3a in the online repository). When analysed as continuous variables in univariate analyses, dry skin was significantly and positively associated with GA (OR: 1.16, CI 95%: 1.08-1.25; p<0.0001) and paternal age (OR: 1.05, CI 95%: 1.02-1.07; p=0.001). We analysed the predictive impact by categorising them into quartiles.

In multivariate analyses, compared to the lower quartile of GA (35.0-38.2), the highest OR (OR: 2.46, CI 95%: 1.60-3.79; p<0.0001) was found in the third quartile (GA 39.51 – 40.50), as shown in Figure 2a, Table E3a.

Similarly, for paternal age, the highest OR in multivariate analyses was found for the oldest age, with an OR: 1.96, CI 95%: 1.16-3.30; p=0.012 in the fourth compared to reference (lowest) quartile. Domestic cat exposure during pregnancy was a significant protective factor for dry skin in the multivariate analysis (OR: 0.55, CI 95%: 0.33-0.92; p=0.023).

For *High TEWL,* three variables were statistically significant in the multivariate analysis, namely female sex (OR: 0.61, CI 95%: 0.40-0.93; p=0.022), maternal allergic disease (OR: 1.80, CI 95%: 1.08-3.01; p=0.025) and birth during winter season (OR: 2.02, CI 95%: 1.31-3.14; p=0.002) (Figure 2b, Table E2b and E3b in the online repository), after including the six variables with a p-value < 0.2 in the univariate logistic regression analysis.

For *Eczema,* three variables were statistically significant in the multivariate analysis, namely elective caesarean section (OR: 2.50, CI 95%: 1.19-5.25; p=0.016), multiparity (one or more previous deliveries) (OR: 1.63, CI 95%: 1.03-2.57; p=0.037) and maternal allergic disease (OR: 1.61, CI 95%: 1.02-2.55; p=0.041) (Figure 2c, Table E2c and E3c in the online repository), after including 10 variables with a p-value <0.2 in the univariate logistic regression analysis. Paternal allergic disease was statistically significant in the univariate analysis (OR: 1.46, CI 95%: 1.01-2.13; p=0.046), as well as birthweight in the fourth quartile > 3.9 kg (OR: 1.89, CI 95%: 1.14-3.13; p=0.014) compared to reference (lowest quartile).

Dry skin or _High TEWL_ and _Eczema_ at 6 months of age

Infants who at 3 months of age had *Dry skin only,* regardless of location were significantly more often diagnosed with *Eczema* at 6 months of age (21.7%) compared to the infants with *Unaffected skin* (12.4%) (Figure 3), giving an unadjusted OR (95% CI) of 1.96 (1.37-2.80)

(p<0.0001). *Dry skin* at 3 months increased the risk of *Eczema* at 6 months by an OR (CI 95%) of 1.92 (1.21-3.05) (p=0.005) in the multivariate analysis adjusting for elective caesarean section, GA at birth, multiparity, paternal age, maternal allergic disease, paternal allergic disease, paternal atopic dermatitis, alcohol consumption during pregnancy and domestic cat during pregnancy. Similar risk was observed using dry skin in the cheeks and/or the extensors, OR (CI 95%) of 1.94 (1.20-3.15; p=0.007), adjusted for the same nine variables. The prediction of *Eczema* 6 months of age with *Dry skin* at 3 months of age had a sensitivity of 68% and a specificity of 48%.

Mean TEWL ($g/m^2/h$) in 3 month-old infants was not significantly associated with *Eczema* at 6 months as a continuous variable or by quartiles in univariate or multivariate analysis. *High TEWL* was significantly associated with *Eczema* at 6 months of age compared to mean TEWL <90^{th} percentile (N=750) (OR: 1.80, CI 95 %: 1.07-3.04; p=0.028) in univariate analysis, but did not remain statistically significant after adjustment for relevant factors outlined in Table E3 in the online repository.

Discussion

In the present population-based prospective mother-child cohort we found increased paternal age and GA at birth to be predictive of dry skin at 3 months of age, and maternal allergic disease, male sex and birth season were predictive for high TEWL (> 90^{th} percentile). For eczema at 3 months the predictors were elective caesarean section, at least one previous delivery, and maternal allergic disease. Dry skin at 3 months of age predicted AD at 6 months of age.

Our finding of increased GA as well as paternal age as predictors for dry skin has to our knowledge not previously been assessed. As dry skin is a main feature of AD, our findings are

supported by reports of increasing GA being associated with AD (26-28). The highest risk for dry skin was found among our children with the highest GA at birth, in line with reports of inverse associations between prematurity (GA<29 weeks) and AD (29, 30). These findings may be explained by shorter exposure time to the maternal immune system and Th2 cytokines, lower levels of IgE and a different composition of early gut and skin microbiome (26, 28, 29). Post-term neonatal skin having less vernix may experience longer direct exposure to amniotic fluid, which can disrupt the stratum corneum lipid bilayer (31, 32), and promote post-term skin dryness and higher TEWL values. Pregnancy length may thus be implicated in the skin integrity (28, 29). Our finding of advanced paternal age, especially above 37 years, being a predictor for dry skin, is to our knowledge novel, and could reflect a possible age related increase in mutations (33).

The protective effect of female sex on high TEWL is supported by previous findings that males have an earlier onset of AD compared to females (28, 34). Similarly to our study, a recent Japanese study found significantly higher TEWL in male infants (35). In contrast, TEWL in neonates was indistinguishable between males and females in an Indian study (36). Our findings that infants born during fall and winter season had higher TEWL at 3 months of age than those born during spring or summer is supported by reports that birth during fall and winter has been associated with increased risk of AD (30, 37, 38). These findings may be explained by cold climate and low environmental humidity that have been associated with impaired skin barrier function (18, 37, 39-41). Exposure to a dry and cold winter climate may lead to depletion of filaggrin and other skin barrier proteins as well as lipids (18, 42) and by lower cumulative UV irradiation before and after birth (37).

Our finding that multiparity was a predictor of AD at 3 months is in contrast to one of the key arguments for the hygiene hypothesis where having older siblings reduces the risk of AD (43),

but more in agreement with a study showing that the risk of AD was not reduced by having older siblings (44). In that study a higher prevalence of eczema in children carrying *FLG* mutations was found if they had older siblings (44), supported by larger sibships increasing the risk of severe AD (43). Parental allergic disease, a well-known risk factor for offspring AD (1, 17), was also a predictor of AD in our population. In our cohort, elective caesarean section was predictive of eczema at 3 months, while acute caesarean section was not. To our knowledge, this is the first study reporting on elective caesarean section being a predictor of AD in early infancy. The vast majority of the elective caesarean sections were prior to rupture of amniotic membranes and we hypothesize that a lacking exposure to the vaginal flora in elective caesarean sections (without rupture of amniotic membranes) (45) may contribute to an offspring gut and skin microbiome dysbiosis associated with AD (5). Our results may imply that onset of AD by 3 months of age, may be dominated by a genetic predisposition to allergic disease, but may be modified by mode of delivery and exposure to maternal vaginal flora.

Dry skin, but not TEWL at 3 months being a predictor of AD at 6 months, has to our knowledge not previously been reported. There are no direct comparable studies, nonetheless dry skin is a cardinal sign of AD (1, 8, 42, 46), and we (8) and others (47) have demonstrated that infants with dry skin have increased TEWL. In the present study the risk of AD at 6 months was particularly noticeable with dry skin on the cheeks and/or on the extensor surfaces of extremities at 3 months of age. Eczema of the cheeks is often the first manifestation of AD, and a recent Irish study by McAleer et al. (48) demonstrated that in 188 infants the skin of the cheeks were slower to mature than the skin of the nasal tip and elbow creases, and had lower levels of natural moisturizing factor. This indicates that early-onset AD may be due to a physiological skin barrier dysfunction restricted to a specific body location, possibly enhanced by factors such as male sex, birth season, and various environmental factors.

Although high TEWL at 3 months did not predict eczema at 6 months after adjusting for potential confounders, it remains to be investigated whether TEWL can predict AD at later time-points (9-11) in our cohort. The presence of clinically dry skin could precede AD without increased TEWL. Although our findings support the outside-inside hypothesis of AD (42), dry skin at 3 months as a predictor of AD at 6 months has low sensitivity and specificity and cannot be used as a single predictive tool for such a heterogeneous disease as AD (49, 50). In line with the concept of the atopic march (12, 13), or the association between dry skin and asthma in adults (51) early identification of dry skin may be useful as screening for targeted primary prevention provided that skin barrier enhancement is effective in reducing AD.

The strengths of our study include a large prospective cohort study from a general population, with high follow-up rate and stringent skin assessment by trained personnel as well as TEWL measurements, and parental risk factors prospectively recorded during pregnancy. The majority of the study participants originate from Nordic countries, which may to some extent limit the generalizability (52). Our study had several limitations including, infants only born from 35 week of GA, genetic analysis including *FLG* mutations were not available, and we could not use the UK Working Party criteria for AD (4) at this age, mainly due to difficulties in evaluating the infants sensation of itch. The relatively high number of possible predictors for the 3-month outcomes included in the analysis together with possible bias of missing data introduces a risk of false positive results. This must be taken into account when interpreting the results.

In conclusion, at 3 months of age, increasing paternal age and gestational age at birth were predictive for dry skin. Maternal allergic disease, male sex and winter birth season were predictive for high TEWL, while for eczema the predictors were elective caesarean section, at least one previous delivery, and maternal allergic disease. Dry skin at 3 months of age,

predicting AD at 6 months of age, may represent a factor in targeting infants for primary prevention of AD and possibly also food allergy and asthma.

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Table 1. Baseline characteristics for pregnancy variables in 1150 infants attending the 3-month investigation, where 'Unaffected skin' are infants without dry skin and eczema is defined as the presence of eczematous lesions, excluding differential diagnosis to atopic dermatitis (AD). Table 1a display parental variables, while Table 1b display prenatal and perinatal variables as well as variables related to the 3-month investigation.

Table 1a

Characteristics	Unaffected skin (N=461)	Dry skin (N=683) (139 with eczema)	Dry skin only (N=544)	Eczema (N=145)	Total (N=1150)
Age mother (years), mean, (SD, min-max)(N=1150)	32.1 (4.1, 21.0-48.0)	32.9 (4.1, 21.0-47.0)	32.8 (4.1, 21.0-47.0)	33.2 (4.2, 22.0-43.0)	32.6 (4.1, 21.0-48.0)
Age father (years), mean, (SD, min-max)(N=983)	34.0 (5.0, 21.0-53.0)	35.3 (5.4, 21.0-72.0)	35.2 (5.4, 21.0-72.0)	35.3 (5.5, 23.0-55.0)	34.8 (5.3, 21.0-72.0)
Mother Nordic origin N (%)(N=1046)	405 (93.8)	545 (89.5)	433 (89.3)	117 (90.7)	955 (91.3)
Father Nordic origin N (%)(N=1026)	386 (90.8)	525 (88.1)	419 (88.6)	111 (86.7)	916 (89.3)
Education mother, > 4 years of University, N (%)(N=1040)	239 (55.5)	371 (61.4)	299 (62.2)	73 (57.0)	611 (58.8)
Education co-parent, > 4 years of University, N (%)(N=1001)	201 (48.8)	294 (50.3)	237 (51.0)	59 (47.6)	497 (49.7)
Family income N (%)(N=1032)*					
Low	69 (16.2)	82 (13.6)	67 (14.0)	17 (13.4)	153 (14.8)
Middle	318 (74.6)	431 (71.7)	345 (72.0)	88 (69.3)	751 (72.8)
High	39 (9.2)	88 (14.6	67 (14.0)	22 (17.3)	128 (12.4)
Single mother N (%)(N= 1038)	6 (1.4)	11 (1.8)	8 (1.6)	3 (2.4)	17 (1.6)
BMI, mother at 18 weeks of pregnancy, mean, (SD, min-max)(N=1132)	24.7 (3.7, 17.2-39.7)	24.8 (3.7, 18.4-41.4)	24.8 (3.6, 18.4-39.5)	25.2 (4.0, 19.4-41.4)	24.8 (3.7, 17.2-41.4)
≥ 1 previous parity N (%)(N=1046)	161 (37.3)	264 (43.3)	199 (41.0)	70 (54.3)	430 (41.1)
Allergic disease mother, N (%) (N=1046)	261 (60.4)	408 (67.0)	318 (65.6)	94 (72.9)	673 (64.3)
Allergic disease father, N (%) (N=1048)	217 (51.1)	304 (49.1)	228 (46.4)	77 (58.3)	522 (49.8)
Atopic dermatitis mother, doctor diagnosed N (%)(N=1046)	83 (19.2)	132 (21.7)	101 (20.8)	32 (24.8)	216 (20.7)
Atopic dermatitis father, doctor diagnosed N (%)(N=1048)	48 (11.3)	67 (10.8)	46 (9.4)	22 (16.7)	116 (11.1)
Asthma mother, doctor diagnosed N (%)(N=1046)	79 (18.3)	106 (17.4)	84 (17.3)	24 (18.6)	187 (17.9)
Asthma father, doctor diagnosed N (%)(N=1048)	64 (15.1)	79 (12.8)	61 (12.4)	19 (14.4)	144 (13.7)
Allergic rhinitis mother, doctor diagnosed N (%)(N=1046)	77 (17.8)	142 (23.3)	115 (23.7)	29 (22.5)	221 (21.1)
Allergic rhinitis father, doctor diagnosed N (%)(N=1048)	93 (21.9)	149 (24.1)	114 (23.2)	36 (27.3)	243 (23.2)
Food allergy mother, doctor diagnosed N (%)(N=1046)	56 (13.0)	81 (13.3)	67 (13.8)	14 (10.9)	137 (13.1)
Food allergy father, doctor diagnosed N (%)(N=1048)	34 (8.0)	59 (9.5)	48 (9.8)	12 (9.1)	94 (9.0)

Table 1b

Characteristics	Unaffected skin (N=461)	Dry skin (N=683) (139 with eczema)	Dry skin only (N=544)	Eczema (N=145)	Total (N=1150)
Lifestyle during pregnancy					
Alcohol intake N (%)(N=914)	22 (5.1)	42 (7.7)	29 (6.0)	13 (10.1)	64 (7.0)
Tobacco use in general N (%)(N=1128)	54 (11.8)	66 (9.9)	54 (10.2)	13 (9.2)	121 (10.7)
Smoking N (%)(N=1128)	24 (5.3)	26 (3.9)	19 (3.6)	8 (5.7)	51 (4.5)
Snus use N (%)(N=1128)	34 (7.5)	42 (6.3)	37 (7.0)	5 (3.5)	76 (6.7)
Live rural N (%)(N=1046)	40 (9.3)	50 (8.2)	43 (8.9)	7 (5.4)	90 (8.6)
Exposure to humidity/mould N (%)(N=984)	51 (12.5)	83 (14.6)	69 (15.3)	16 (13.0)	136 (13.8)
Pets in general N (%)(N=1046)	116 (26.9)	133 (21.8)	105 (21.6)	29 (22.5)	250 (23.9)
Cat, no dog N (%)(N=1046)	48 (11.1)	41 (6.7)	30 (6.2)	12 (9.3)	90 (8.6)
Dog, no cat N (%)(N=1046)	53 (14.0)	70 (11.5)	59 (12.2)	11 (8.5)	123 (11.8)
Cat and dog N (%)(N=1046)	6 (1.4)	10 (2.0)	8 (1.6)	2 (1.6)	15 (1.4)
Pets except cat and dog N %)(N=1046)	9 (2.1)	12 (2.0)	8 (1.6)	4 (3.1)	22 (2.1)
Caesarean section, N (%)(N=1137)	69 (15.2)	106 (15.6)	80 (14.8)	27 (18.8)	176 (15.5)
Elective N (%)(N=1137)	22 (4.9)	42 (6.2)	30 (5.6)	12 (8.3)	64 (5.6)
Acute N (%)(N=1137)	47 (10.4)	64 (9.4)	50 (9.3)	15 (10.4)	112 (9.9)
Gestational age at birth (weeks), mean (SD, min-max) (N=1128)	39.1 (1.8, 35.0-42.9)	39.5 (1.6, 35.1-42.9)	39.6 (1.6, 35.1-42.9)	39.5 (1.6, 35.2-42.2)	39.3 (1.7, 35.0-42.9)
Female sex N (%) (N=1146)	221 (48.1)	307 (45.1)	251 (46.3)	58 (40.0)	530 (46.2)
Birth weight (kg), mean, (SD, min-max) (N=1114)	3.5 (0.5, 1.9-5.1)	3.6 (0.5, 2.1-5.0)	3.6 (0.5, 2.1-4.9)	3.7 (0.5, 2.6-5.0)	3.6 (0.5, 1.9-5.1)
Born during winter season (October – March) N (%)(N=1146)	238 (51.9)	392 (57.6)	306 (56.5)	87 (60.0)	631 (55.1)
3-month investigation					
Age (days), mean (SD, min-max) (N=1145)	94 (9.4, 55-150)	93 (7.6, 69-134)	93 (7.9, 69-134)	94 (6.4, 83-112)	93 (8.4, 55-150)
Weight (kg), mean, (SD, min-max) (N=1118)	6.2 (0.8, 4.4-9.3)	6.3 (0.8, 4.2-8.9)	6.3 (0.8, 4.2-8.7)	6.3 (0.7, 4.4-8.9)	6.3 (0.8, 4.2-9.3)
Length (cm), mean, (SD, min-max) (N=1125)	61.7 (2.4, 54.0-70.9)	62.0 (2.3, 51.0-69.5)	62.0 (2.3, 51.0-69.5)	62.1 (2.2, 56.8-68.5)	61.9 (2.3, 51.0-70.9)
TEWL ($g/m^2/h$) mean, (SD, min-max) (N=1026)	6.7 (3.5, 1.3-32.6)	8.5 (6.3 (1.6-46.2)	7.6 (5.3, 1.6-46.2)	12.4 (8.9, 3.3-45.2)	7.8 (5.5, 1.3-46.2)

Figure legend

Figure 1. Outline of children in the present study are based upon the source population of the Preventing Atopic Dermatitis and ALLergies in children (PreventADALL) with 2701 pregnancies included, resulting in a birth-cohort of 2396 mother-child pairs.

Figure 2. Significant predictors ($p < 0.05$) for dry skin (2a), TEWL > 90th percentile (11.3 $g/m^2/h$) (2b) and eczema (2c) at 3 months of age in 1150 infants, when using multivariate regression analysis shown as odds ratio and confidence intervals.

2a Pregnancy variables with cut-off p-value of < 0.2 for predicting dry skin used in the multivariate analysis were: GA at birth, birth weight, multiparity, domestic cat exposure, maternal age, paternal age, maternal allergic disease, maternal education, family income and birth season.

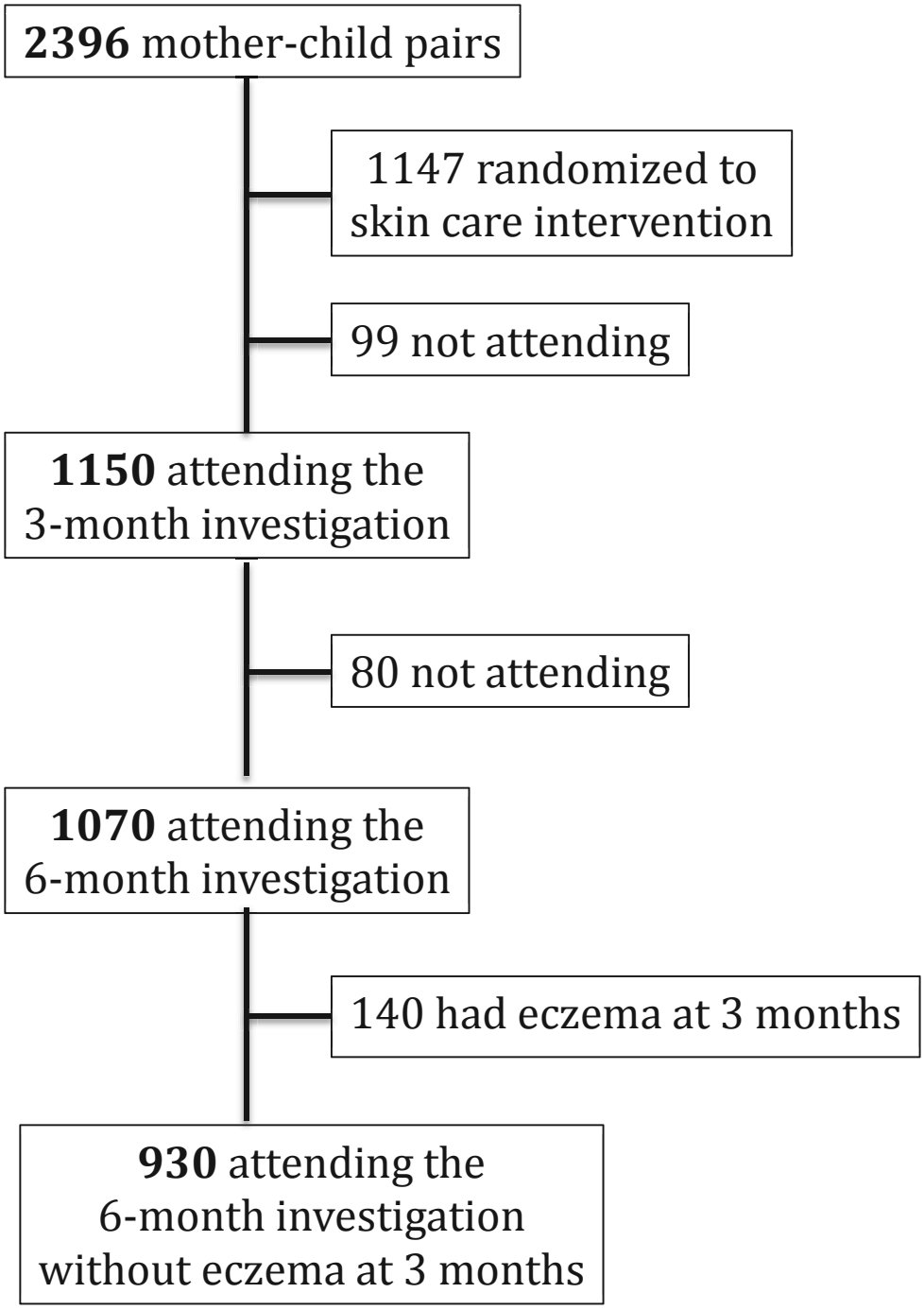
2b Pregnancy variables with cut-off p-value of < 0.2 for predicting TEWL > 90th percentile (11.3 $g/m^2/h$) used in the multivariate analysis were: female sex, birth weight, maternal allergic disease, maternal atopic dermatitis, and birth season.

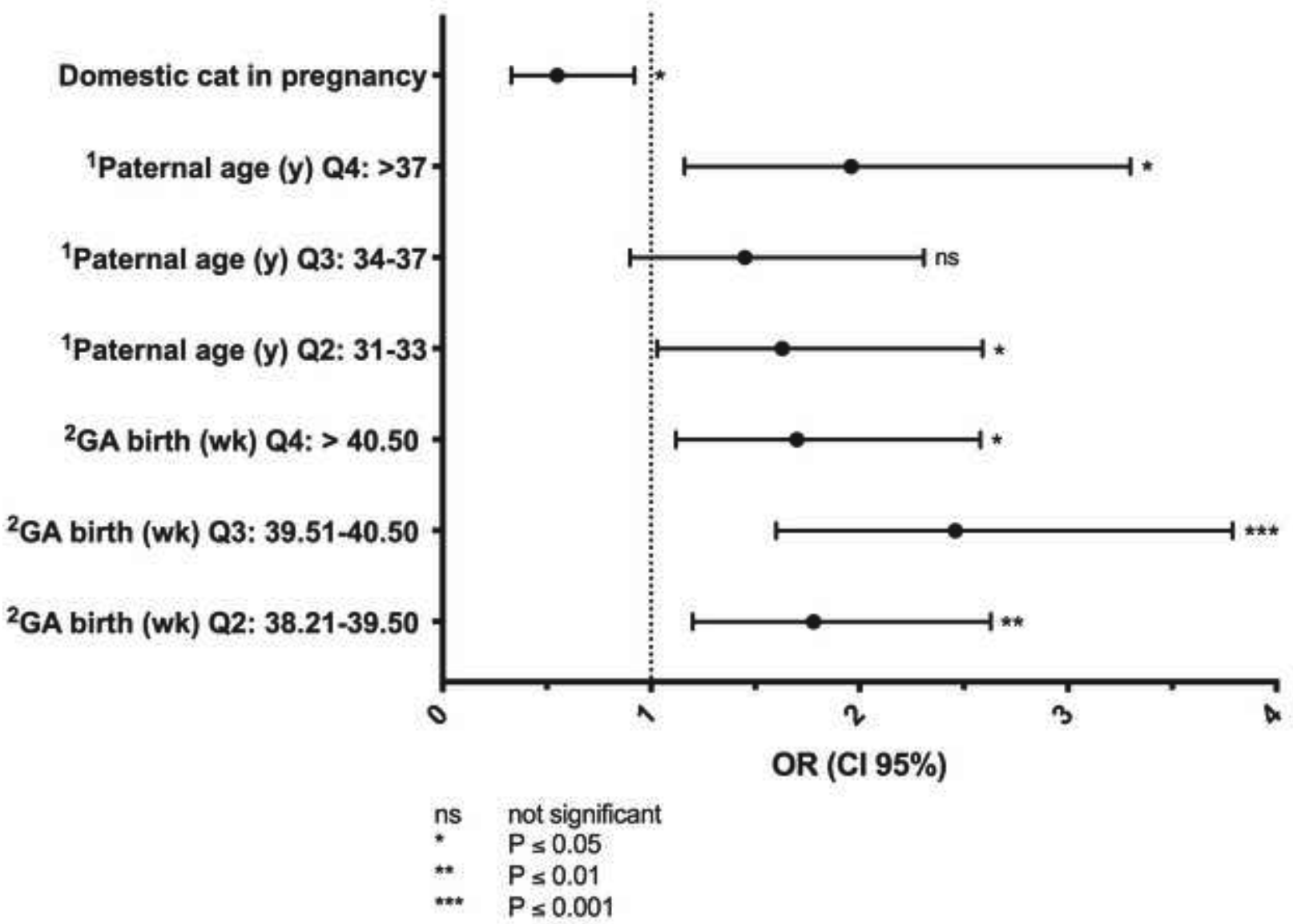
2c Pregnancy variables with cut-off p-value of < 0.2 for predicting eczema, defined as the presence of eczematous lesions, excluding differential diagnosis to atopic dermatitis, used in the multivariate analysis were: female sex, birth weight, multiparity, elective caesarean section (CS), maternal age, maternal allergic disease, paternal allergic disease, snus during pregnancy, rural living and family income.

Figure 3. The Euler diagram depicts the distribution of dry skin at 3 months in 159 infants who at 6 months presented with eczema, used as a proxy for atopic dermatitis. Dry skin at 3 months, regardless of location was a significant predictor for atopic dermatitis at 6 months of age with an OR (CI 95%) of 1.92 (1.21-3.05) (p=0.005), and OR (CI 95%) of 1.94 (1.20-3.15; p=0.007) for dry skin in the cheeks and/or the extensors specifically at 3 months.

Footnote for Figure 3:
Produced with courtesy of: Luana Micallef and Peter Rodgers (2014). eulerAPE: Drawing Area-proportional 3-Venn Diagrams Using Ellipses. PLoS ONE 9(7): e101717. doi:10.1371/journal.pone.0101717. http://www.eulerdiagrams.org/eulerAPE

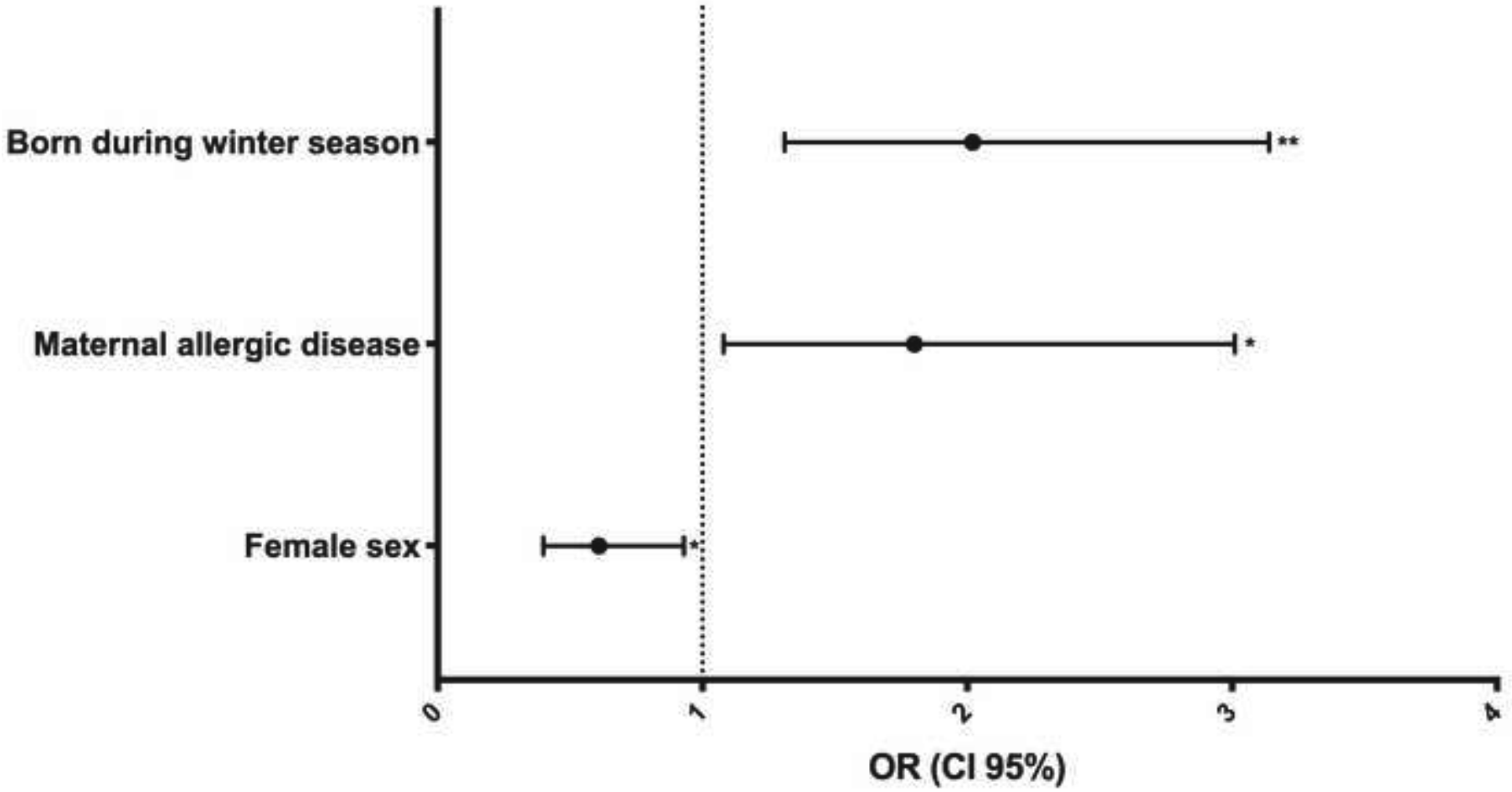
Figure No. 1 - Unmarked



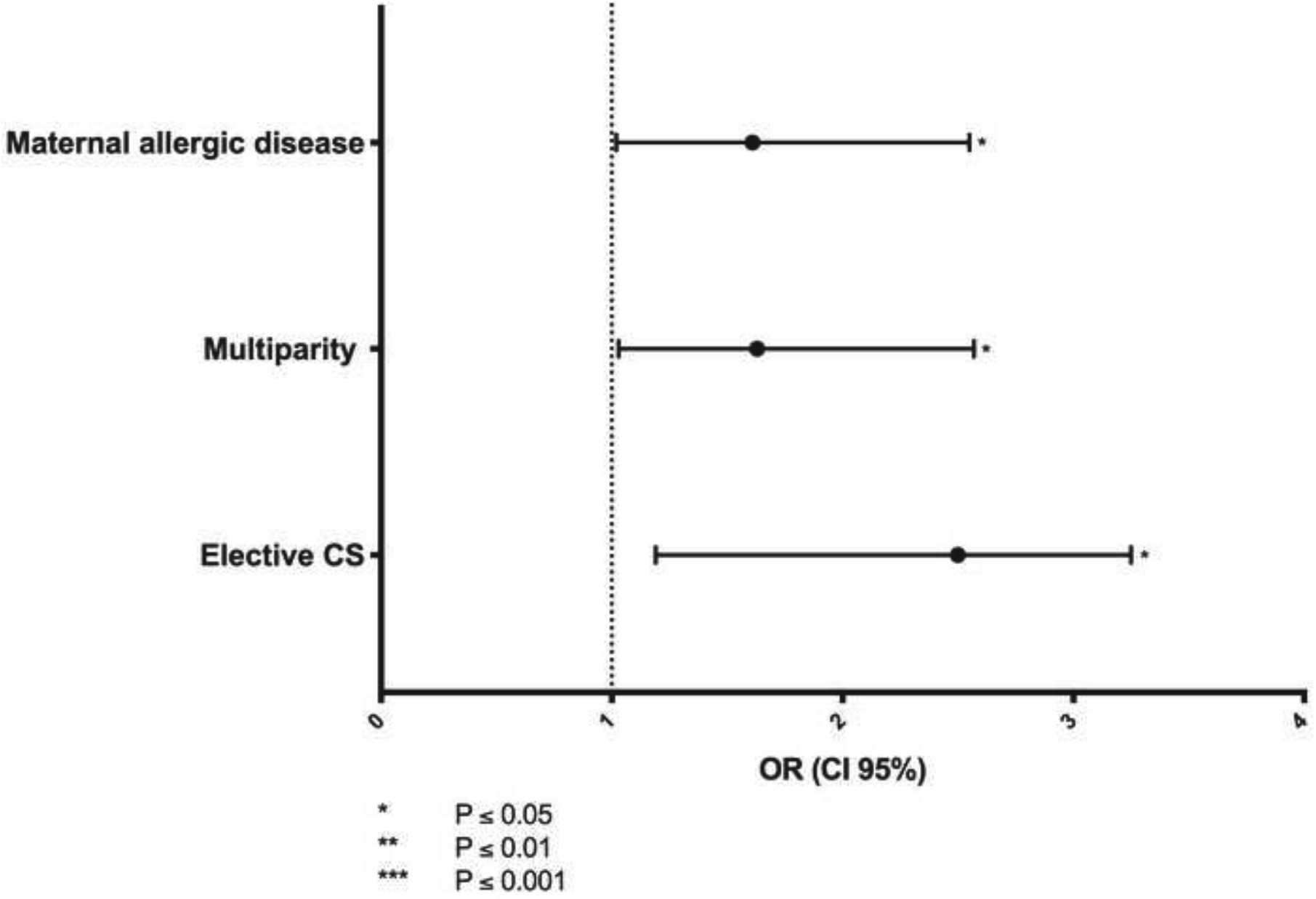


[1]Paternal age in years divided in quartiles (Q), where the first quartile of 21-30 years is used as reference value.

[2]Gestational age (GA) at birth in weeks divided in quartiles (Q) where first quartile is 35.00-38.20 weeks and used as reference value.



* P ≤ 0.05
** P ≤ 0.01
*** P ≤ 0.001



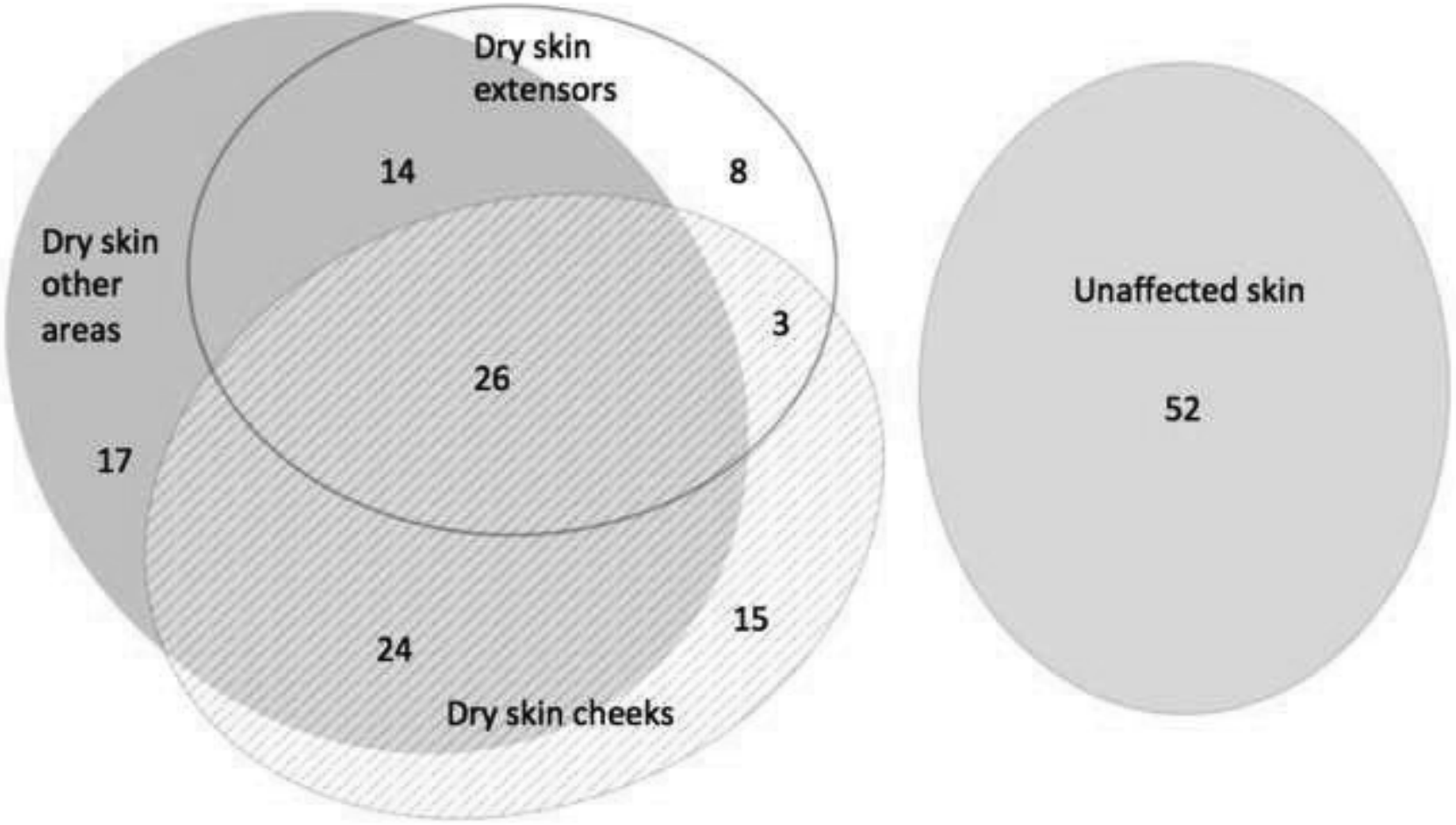


Table E1. Baseline characteristics in 930 infants attending 6-month investigation, grouped in to No eczema and Eczema, defined as the presences of eczematous lesions, excluding differential diagnosis to AD. Those with eczema at the 3-month investigation have been excluded.

Characteristics	No eczema 6 months (N=767)	Eczema 6 months (N=163)	Total (N=930)
Age mother (years), mean, (SD, min-max)(N=927)	32.6 (4.1, 21.0-47.0)	32.3 (3.7, 25.0-42.0)	32.5 (4.1, 21.0-47.0)
Age father (years), mean, (SD, min-max)(N=804)	34.8 (5.3, 21.0-72.0)	34.7 (5.1, 25.0-51.0)	34.8 (5.3, 21.0-72.0)
Mother Nordic origin N (%)(N=854)	648 (91.8)	135 (91.2)	783 (91.7)
Father Nordic origin N (%)(N=837)	621 (89.6)	128 (88.9)	749 (89.5)
Education mother, > 4 years of University, N (%)(N=849)	409 (58.3)	97 (65,5)	506 (59.6)
Education co-parent, > 4 years of University, N (%)(N=817)	344 (50.7)	68 (49.3)	412 (50.4)
Family income N (%)(N=842)			
Low	105 (15.1)	18 (12.2)	123 (14.6)
Middle	510 (73.4)	110 (74.8)	620 (73.6)
High	80 (11.5)	19 (12.9)	99 (11.8)
BMI, mother at 18 weeks of pregnancy, mean, (SD, min-max)(N=918)	24.8 (3.7, 18.3-39.5)	24.5 (3.2, 17.2-36.1)	24.8 (3.6, 17.2-39.5)
≥ 1 previous parity N (%)(N=854)	286 (40.5)	49 (33.1)	335 (39.2)
Allergic disease mother, N (%) (N=854)	449 (63.6)	94 (63.5)	543 (63.6)
Allergic disease father, N (%) (N=853)	334 (47.6)	82 (54.3)	416 (48.8)
Atopic dermatitis mother, doctor diagnosed N (%)(N=854)	141 (20.0)	28 (18.9)	169 (19.8)
Atopic dermatitis father, doctor diagnosed N (%)(N=774)	65 (10.1)	22 (16.5)	87 (11.2)
Asthma mother, doctor diagnosed N (%)(N=854)	123 (17.4)	28 (18.9)	151 (17.7)
Asthma father, doctor diagnosed N (%)(N=826)	96 (14.2)	22 (14.9)	118 (14.3)
Allergic rhinitis mother, doctor diagnosed N (%)(N=778)	150 (23.3)	26 (19.5)	176 (22.6)
Allergic rhinitis father, doctor diagnosed N (%)(N=781)	157 (24.3)	41 (30.6)	198 (25.4)
Food allergy mother, doctor diagnosed N (%)(N=808)	99 (14.8)	17 (12.2)	116 (14.4)
Food allergy father, doctor diagnosed N (%)(N=812)	60 (8.9)	15 (10.6)	75 (9.2)
Lifestyle during pregnancy			
Alcohol intake N (%)(N=774)	33 (5.4)	15 (11.3)	48 (6.5)
Tobacco use in general N (%)(N=915)	78 (10.4)	15 (9.3)	93 (10.2)
Smoking N (%)(N=915)	33 (4.4)	3 (1.9)	36 (3.9)
Snus use N (%)(N=915)	51 (6.8)	12 (7.4)	63 (6.9)
Live rural N (%)(N=854)	67 (9.5)	13 (8.8)	80 (9.4)
Exposure to humidity/mould N (%)(N=806)	87 (13.1)	27 (19.0)	114 (14.1)
Pets in general N (%)(N=854)	180 (25.5)	27 (18.2)	207 (24.2)
Cat, no dog N (%)(N=854)	69 (9.8)	5 (3.4)	74 (8.7)
Dog, no cat N (%)(N=854)	86 (12.2)	17 (11.5)	103 (12.1)
Cat and dog N (%)(N=854)	12 (1.7)	2 (1.4)	14 (1.6)
Pets except cat and dog N %)(N=854)	13 (1.8)	3 (2.0)	16 (1.9)
Caesarean section, N (%)(N=918)	104 (13.7)	27 (18.0)	133 (14.4)
Elective N (%)(N=918)	33 (4.4)	12 (7.5)	45 (4.9)
Acute N (%)(N=918)	71 (9.4)	17 (10.6)	88 (9.6)
Gestational age at birth (weeks), mean (SD, min-max) (N=913)	39.3 (1.7, 35.0-42.9)	39.4 (1.6, 35.2-42.9)	39.3 (1.7, 35.0-42.9)
Female sex N (%) (N=927)	370 (48.2)	70 (43.2)	440 (47.5)
Birth weight (kg), mean, (SD, min-max) (N=897)	3.6 (0.5, 1.9-4.9)	3.6 (0.5, 2.2-5.1)	3.5 (0.5, 1.9-5.1)
Born during winter season (October – March) N (%)(N=927)	429 (56.1)	84 (51.9)	513 (55.3)
6-month investigation			
Age (days), mean (SD, min-max) (N=927)	190 (13.5, 146-248)	189 (11.7, 155-224)	190 (13.2, 146-248)
Weight (kg), mean, (SD, min-max) (N=907)	8.1 (1.0, 5,3-11,9)	8.1 (1.0, 5.2-12.3)	8.1 (1.0, 5.2-12.3)
Length (cm), mean, (SD, min-max) (N=913)	68.5 (2.6, 52.0-82.3)	68.6 (2.7, 62,3-77.0)	68.5 (2.7, 52.0-82.7)

E2 a Results of univariate analysis for dry skin as dependent variable presented as complete case analysis showing N (%) of individuals included in the analysis with OR (CI 95%) and p-value.

Pregnancy variables		N (%) of 1150 included in analysis (complete cases for dry skin as outcome)	OR (CI 95%)	p-value
Maternal age (years)	Q1 (21 – 29)	1150 (100%)	Ref.	
	Q2 (30 – 32)		1.20 (0.86-1.65)	0.28
	Q3 (33 – 35)		1.66 (1.17-2.35)	0.004
	Q4 (>35)		1.81 (1.27-2.56)	0.001
Paternal age (years)	Q1 (21 – 30)	983 (85.5%)	Ref.	
	Q2 (31 – 33)		1.55 (1.06-2.26)	0.024
	Q3 (34 – 37)		1.53 (1.06-2.20)	0.023
	Q4 (> 37)		2.04 (1.40-2.97)	<0.0001
Education mother, > 4 years of University		1040 (90.4%)	1.30 (1.01-1.67)	0.039
Education co-parent, > 4 years of University		1001 (87%)	1.06 (0.82-1.36)	0.649
Family income	Low	1032 (89.7%)	Ref.	
	Middle		1.17 (0.82-1.65)	0.388)
	High		1.91 (1.17-3.11)	0.010
BMI, mother at 18 weeks of pregnancy		1132 (98.4%)	1.01 (0.98-1.04)	0.641
≥ 1 previous parity		1046 (91%)	1.25 (0.97-1.61)	0.082
Allergic disease mother		1046 (91%)	1.32 (1.02-1.70)	0.035
Allergic disease father		1023 (89%)	0.93 (0.72-1.19)	0.549
Atopic dermatitis mother, doctor diagnosed		1046 (91%)	1.16 (0.86-1.58)	0.334
Atopic dermatitis father, doctor diagnosed		954 (83%)	0.92 (0.62-1.37)	0.695
Asthma mother, doctor diagnosed		1046 (91%)	0.93 (0.67-1.28)	0.638
Asthma father, doctor diagnosed		1014 (88.2%)	0.83 (0.58-1.18)	0.291
Allergic rhinitis mother, doctor diagnosed		952 (82.8%)	1.48 (1.08-2.02)	0.014
Allergic rhinitis father, doctor diagnosed		957 (83.2%)	1.16 (0.86-1.56)	0.342
Food allergy mother, doctor diagnosed		975 (84.8%)	1.07 (0.74-1.54)	0.724
Food allergy father, doctor diagnosed		990 (86.1%)	1.20 (0.76-1.86)	0.411
Alcohol intake		914 (79.5%)	1.33 (0.78-2.27)	
Smoking		1128 (98.1%)	0.71 (0.40-1.24)	0.228
Snus use		1128 (98.1%)	0.84 (0.53-1.35)	0.478
Rural living		1046 (91%)	0.89 (0.57-1.37)	0.592
Exposure to humidity/mould		984 (85.6%)	1.16 (0.80-1.68)	0.430
Pets (no pets as ref.)		1046 (91%)		
Cat, no dog			0.56 (0.36-0.87)	0.01
Dog, no cat			0.89 (0.61-1.30)	0.544
Cat and dog			1.12 (0.40-3.11)	0.827
Pets except cat and dog			0.90 (0.37-2.15)	0.807
Caesarean section (vaginal as ref.)	Elective	1137 (98.9%)	1.29 (0.76-2.20)	0.344
	Acute		0.90 (0.61-1.34)	0.903
Birth GA (weeks)	Q1 (35.00 – 38.20)	1088 (94.6%)	Ref.	
	Q2 (38.21 – 39.50)		1.87 (1.33-2.63)	<0.0001
	Q3 (39.51 – 40.50)		2.50 (1.75-3.60)	<0.0001
	Q4 (> 40.50)		1.84 (1.32-2.60)	<0.0001
Female sex		1146 (99.7%)	0.89 (0.70-1.13)	0.338
Birth weight (kg)	Q1 (1.50 – 3.30)	1099 (95.6%)	Ref.	
	Q2 (3.31 – 3.60)		1.22 (0.87-1.71)	0.255
	Q3 (3.61 – 3.90)		1.28 (0.91-1.79)	0.159
	Q4 (> 3.90)		1.65 (1.17-2.33)	0.005
Born during winter season (October – March)		1146 (99.7%)	1.28 (1.01-1.63)	0.040

E2 b Results of univariate analysis for high TEWL as dependent variable presented as complete case analysis showing N (%) of individuals included in the analysis with OR (CI 95%) and p-value.

Pregnancy variables		N (%) of 1033 included in analysis (complete cases for high TEWL as outcome)	OR (CI 95%)	p-value
Maternal age (years)	Q1 (21 – 29)	1024 (99.1)	Ref.	
	Q2 (30 – 32)		1.14 (0.68-1.90)	0.621
	Q3 (33 – 35)		1.09 (0.63-1.86)	0.766
	Q4 (>35)		1.02 (0.59-1.75)	0.958
Paternal age (years)	Q1 (21 – 30)	876 (84.8%)	Ref.	
	Q2 (31 – 33)		0.78 (0.43-1.42)	0.415
	Q3 (34 – 37)		0.73 (0.41-1.30)	0.290
	Q4 (> 37)		0.97 (0.55-1.71)	0.919
Education mother, > 4 years of University		925 (89.5%)	1.15 (0.77-1.71)	0.508
Education co-parent, > 4 years of University		892 (86.4%)	1.03 (0.69-1.52)	0.900
Family income	Low	919 (89.0%)	Ref.	
	Middle		0.90 (0.51-1.57)	0.701
	High		1.45 (0.72-2.93)	0.298
BMI, mother at 18 weeks of pregnancy		1007 (97.5%)	1.02 (0.97-1.07)	0.392
≥ 1 previous parity		931 (90.1%)	1.09 (0.73-1.61)	0.683
Allergic disease mother		931 (90.1%)	1.88 (1.20-2.94)	0.006
Allergic disease father		907 (87.8%)	1.25 (0.85-1.84)	0.260
Atopic dermatitis mother, doctor diagnosed		931 (90.1%)	1.58 (1.01-2.47)	0.046
Atopic dermatitis father, doctor diagnosed		840 (81.3%)	1.41 (0.81-2.45)	0.221
Asthma mother, doctor diagnosed		931 (90.1%)	1.79 (1.14-2.82)	0.012
Asthma father, doctor diagnosed		899 (87%)	0.77 (0.43-1.40)	0.391
Allergic rhinitis mother, doctor diagnosed		853 (82.6%)	1.24 (0.77-1.99)	0.372
Allergic rhinitis father, doctor diagnosed		849 (82.2%)	1.40 (0.91-2.15)	0.131
Food allergy mother, doctor diagnosed		866 (83.8%)	1.67 (0.99-2.81)	0.055
Food allergy father, doctor diagnosed		876 (84.8%)	0.78 (0.38-1.61)	0.504
Alcohol intake		811 (78.5%)	1.55 (0.76-3.18)	0.231
Smoking		1004 (97.2%)	1.28 (0.56-2.92)	0.564
Snus use		1004 (97.2%)	1.17 (0.58-2.36)	0.653
Rural living		931 (90.1%)	1.27 (0.65-2.49)	0.483
Exposure to humidity/mould		874 (84.6%)	1.00 (0.56-1.78)	0.986
Pets (no pets as ref.)		931 (90.1%)		
Cat, no dog			0.96 (0.46-1.99)	0.911
Dog, no cat			1.40 (0.80-2.47)	0.240
Cat and dog			1.05 (0.24-4.70)	0.949
Pets except cat and dog			1.23 (0.35-4.25)	0.749
Caesarean section (vaginal as ref.)	Elective	1014 (98.2%)	1.12 (0.52-2.44)	0.768
	Acute		0.99 (0.53-1.82)	0.965
Birth GA (weeks)	Q1 (35.00 – 38.20)	969 (93.8%)	Ref.	
	Q2 (38.21 – 39.50)		1.05 (0.60-1.83)	0.868
	Q3 (39.51 – 40.50)		1.20 (0.69-2.09)	0.524
	Q4 (> 40.50)		1.24 (0.72-2.11)	0.438
Female sex		1020 (98.7%)	0.64 (0.44-0.94)	0.021
Birth weight (kg)	Q1 (1.50 – 3.30)	979 (94.8)	Ref.	
	Q2 (3.31 – 3.60)		0.92 (0.52-1.63)	0.771
	Q3 (3.61 – 3.90)		1.35 (0.79-2.30)	0.268
	Q4 (> 3.90)		1.54 (0.92-2.59)	0.103
Born during winter season (October – March)		1020 (98.7%)	1.90 (1.27-2.82)	0.002

E2 c Results of univariate analysis for eczema as dependent variable presented as complete case analysis showing N (%) of individuals included in the analysis with OR (CI 95%) and p-value.

Pregnancy variables		N (%) of 1150 included in analysis (complete cases for AD as outcome)	OR (CI 95%)	p-value
Maternal age (years)	Q1 (21 – 29)	1150 (100%)	Ref.	
	Q2 (30 – 32)		1.07 (0.63-1.85)	0.796
	Q3 (33 – 35)		1.62 (0.95-2.75)	0.074
	Q4 (>35)		1.80 (1.07-3.04)	0.028
Paternal age (years)	Q1 (21 – 30)	983 (85.5%)	Ref.	
	Q2 (31 – 33)		0.78 (0.42-1.47)	0.445
	Q3 (34 – 37)		1.42 (0.82-2.47)	0.207
	Q4 (> 37)		1.25 (0.71-2.20)	0.448
Education mother, > 4 years of University		1040 (90.4%)	0.92 (0.64-1.34)	0.673
Education co-parent, > 4 years of University		1001 (87.0%)	0.91 (0.62-1.32)	0.622
Family income	Low	1032 (89.7%)	Ref.	
	Middle		1.06 (0.61-1.84)	0.831
	High		1.66 (0.84-3.28)	0.145
BMI, mother at 18 weeks of pregnancy (continuous)		1116 (97.0%)	1.04 (0.00-1.09)	0.117
BMI, mother normal (BMI 18-24.9)			Ref.	
BMI, mother overweight (BMI 25-29.9)			1.23 (0.83-1.81)	0.307
BMI, mother obese (BMI ≥ 30)			1.25 (0.68-2.29)	0.483
≥ 1 previous parity		1046 (91%)	1.84 (1.27-2.67)	0.001
Allergic disease mother		1046 (91%)	1.57 (1.04-2.36)	0.032
Allergic disease father		1023 (89%)	1.46 (1.01-2.13)	0.046
Atopic dermatitis mother, doctor diagnosed		1046 (91%)	1.31 (0.85-2.02)	0.214
Atopic dermatitis father, doctor diagnosed		954 (83%)	1.75 (1.05-2.91)	0.032
Asthma mother, doctor diagnosed		1046 (91%)	1.06 (0.66-1.70)	0.818
Asthma father, doctor diagnosed		1014 (88.2%)	1.04 (0.62-1.75)	0.885
Allergic rhinitis mother, doctor diagnosed		952 (82.8%)	1.15 (0.73-1.80)	0.549
Allergic rhinitis father, doctor diagnosed		957 (83.2%)	1.34 (0.88-2.04)	0.174
Food allergy mother, doctor diagnosed		975 (84.8%)	0.87 (0.48-1.57)	0.643
Food allergy father, doctor diagnosed		990 (86.1%)	1.08 (0.57-2.04)	0.815
Alcohol intake		914 (79.5%)	1.79 (0.94-3.4)	0.076
Smoking		1128 (98.1%)	1.32 (0.61-2.87)	0.483
Snus use		1128 (98.1%)	0.474 (0.10-1.20)	0.114
Rural living		1046 (91%)	0.58 (0.26-1.28)	0.174
Exposure to humidity/mould		984 (95.6%)	0.92 (0.53-1.61)	0.780
Pets (no pets as ref.)		1046 (91%)		
Cat, no dog			1.07 (0.56-2.04)	0.687
Dog, no cat			0.68 (0.36-1.31)	0.254
Cat and dog			0.99 (0.22-4.44)	0.994
Pets except cat and dog			1.64 (0.54-4.97)	0.383
Caesarean section (vaginal as ref.)	Elective	1137 (98.9%)	1.67 (0.86-3.21)	0.128
	Acute		1.12 (0.63-1.99)	0.710
Birth GA (weeks)	Q1 (35.00 – 38.20)	1088 (94.6%)	Ref.	
	Q2 (38.21 – 39.50)		1.16 (0.69-1.94)	0.585
	Q3 (39.51 – 40.50)		1.16 (0.68-1.98)	0.590
	Q4 (> 40.50)		1.34 (0.81-2.22)	0.259
Female sex		1146 (99.7%)	0.75 (0.52-1.01)	0.107
Birth weight (kg)	Q1 (1.50 – 3.30)	1099 (95.6%)	Ref.	
	Q2 (3.31 – 3.60)		1.18 (0.68-2.03)	0.559
	Q3 (3.61 – 3.90)		1.34 (0.78-2.27)	0.280
	Q4 (> 3.90)		1.89 (1.14-3.13)	0.014
Born during winter season (October – March)		1146 (99.7%)	1.26 (0.88-1.80)	0.201

Table E3
Multivariate complete case logistic regression, where dependent variables were Dry skin (Table E3a), High TEWL (TEWL > 90th percentile (11.3 g/m^2/h)) (Table E3b) and 'Eczema' (Table E3c) in 1150 3 month-old infants.

GA: Gestational age
OR: Odds Ratio
CI: Confidence interval
Q: Quartile

E3a *Dry skin*

Pregnancy variables	**N=879 OR (95 % CI)**	**P-value**
Birth GA (weeks)		
Q1 (35.00 – 38.20)		Ref.
Q2 (38.21 – 39.50)	1.78 (1.20-2.67)	0.005
Q3 (39.51 – 40.50)	2.46 (1.60-3.79)	<0.0001
Q4 (> 40.50)	1.70 (1.12-2.58)	0.013
Birth weight (kg)		
Q1 (1.50 – 3.30)		Ref.
Q2 (3.31 – 3.60)	1.03 (0.69-1.53)	0.883
Q3 (3.61 – 3.90)	1.00 (0.66-1.52)	0.987
Q4 (> 3.90)	1.36 (0.89-2.08)	0.163
Multipara	1.02 (0.75-1.41)	0.882
Domestic cat exposure	0.554 (0.33-0.92)	0.023
Maternal age (years)		
Q1 (21 – 29		Ref.
Q2 (30 – 32)	0.84 (0.61-1.44)	0.769
Q3 (33 – 35)	1.36 (0.83-2.22)	0.747
Q4 (>35)	1.10 (0.63-1.90)	0.747
Paternal age (years)		
Q1 (21 – 30)		Ref.
Q2 (31 – 33)	1.63 (1.03-2.59)	0.037
Q3 (34 – 37)	1.45 (0.90-2.31)	0.124
Q4 (> 37)	1.96 (1.16-3.30)	0.012
Maternal allergic disease	1.28 (0.95-1.712)	0.106
Maternal education > 4 years University	1.10 (0.81-1.49)	0.565
Family income		
Low		Ref.
Middle	0.93 (0.61-1.44)	0.754
High	1.34 (0.73-2.46)	0.351
Born during winter season	1.29 (0.97-1.72)	0.076

E3b *High TEWL*

Pregnancy variables	N=888 OR (95 % CI)	P-value
Female sex	0.61 (0.40-0.93)	0.022
Birth weight (kg)		
Q1 (1.50 – 3.30)	Ref.	
Q2 (3.31 – 3.60)	0.95 (0.52-1.76)	0.879
Q3 (3.61 – 3.90)	1.26 (0.70-2.27)	0.445
Q4 (> 3.90)	1.33 (0.74-2.38)	0.337
Maternal any allergic disease	1.80 (1.08-3.01)	0.025
Maternal atopic dermatitis	1.29 (0.78-2.12)	0.321
Maternal asthma	1.34 (0.18-2.23)	0.256
Born during winter season	2.02 (1.31-3.14)	0.002

E3c *Eczema*

Pregnancy variables	N=893 OR (95%CI)	p-value
Sex (females)	0.83 (0.54-1.26)	0.380
Birth weight (kg)		
Q1 (1.50 – 3.30)		Ref.
Q2 (3.31 – 3.60)	1.17 (0.62-2.22)	0.632
Q3 (3.61 – 3.90)	1.50 (0.80-2.78)	0.203
Q4 (> 3.90)	1.77 (0.97-3.25)	0.065
Elective caesarean section	2.50 (1.19-5.25)	0.016
Multiparity	1.63 (1.03-2.57)	0.037
Maternal age (years)		
Q1 (21 – 29)		Ref.
Q2 (30 – 32)	0.90 (0.47-1.74)	0.757
Q3 (33 – 35)	1.41 (0.73-2.75)	0.311
Q4 (>35)	1.65 (0.85-3.22)	0.143
Maternal allergic disease	1.61 (1.02-2.55)	0.041
Paternal allergic disease	1.41 (0.93-2.14)	0.105
Snus during pregnancy	0.43 (0.15-1.24)	0.120
Rural living	0.48 (0.20-1.15)	0.101
Family income		
Low		Ref.
Middle	0.91 (0.47-1.75)	0.777
High	1.14 (0.51-2.54)	0.755