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'Barrier dysfunction in Atopic newBorns studY' (BABY): a birth cohort study protocol

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Keywords:	atopic dermatitis, birth cohort, Raman, skin barrier, preterm, TEWL





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‘Barrier dysfunction in Atopic newBorns studY’ (BABY): a birth cohort study protocol

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Conflicts of interest

None declared

Author contributions

TG, LS and JPT designed the study, created the study protocol, and obtained approval of the study design. ST, CBM and CE contributed to revision and refinement of the study design. TG, AH, MRR, NHR, MHK and JPT were responsible for data collection. TG, AH, MRR, LS and JPT drafted the manuscript. All authors critically revised the manuscript. All authors supervised the study.

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ABSTRACT

Introduction:

The skin barrier development in premature and mature newborns has been scarcely studied but may be important for the risk of atopic dermatitis (AD).

Methods and analysis:

The BABY Cohort is a prospective birth cohort study of 150 preterm and 300 term children. Skin barrier function is assessed by transepidermal water loss. Biomolecules important for skin barrier function and immune response are investigated by Raman-spectroscopy and stratum corneum (SC) and microbiome sampling. Clinical examinations are done and DNA from buccal swabs is collected for genetic analyses. Thymus size is assessed by ultrasound examination. Information on pregnancy, delivery and parental exposures and diseases are collected and structured telephone interviews are conducted at 18 and 24 months to assess exogenous exposures in the child and onset of AD. Hanifin and Rajka criteria as well as The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis are used to diagnose AD. Severity of AD is assessed using the Eczema Area and Severity Index (EASI) and Patient Oriented Eczema Measure (POEM).

Ethics and dissemination:

The study is approved by the local medical ethics committee (H-16042289 and H-16042294). Outcomes will be presented at national and international conferences and in peer-reviewed publications.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- ‘BABY Cohort’ is a Danish prospective birth cohort study that examines skin barrier functions and risk factors for atopic dermatitis.
- Comprehensive and repeated measurements of skin barrier function and factors affecting immune and antimicrobial barrier in preterm and term newborns from the general population.
- Being strictly non-invasive, no blood measurements are done.

INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing, inflammatory skin disease, characterized by dry and itchy skin that affects up to 20% of children in Northern Europe.[1] About 60-80% develop the disease in their first two years of life, and children with early onset are at increased risk of having severe and persistent disease.[2, 3] The risk of AD is increased in children of parents with atopic disorders such as AD, asthma and allergic rhinitis.[4-6]

Genetic and environmental risk factors contribute to the development of AD through skin barrier dysfunction and immune dysregulation.[2] While loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as the strongest genetic risk factor for AD,[7] genome wide association studies have only identified a relatively small proportion of genetic risk variants.[8] The inflammation in AD is characterized by overexpression of Th2 cytokines, including IL-4 and IL-13.[9] that together with IL-1 may lead to increased secretion of thymic stromal lymphopoietin (TSLP), decreased epidermal antimicrobial peptides and filaggrin levels, in turn worsening skin inflammation and epidermal barrier functions.[10] Changes in the skin microbiome is also associated with worsening

of AD, showing reduced bacterial diversity[11] and increased colonization with *Staphylococcus aureus* (*S. aureus*).[12]

While several environmental risk factors have been identified, e.g. winter birth and exposure to hard domestic water, this has not yet led to prophylactic solutions.[13] Interestingly, the risk of AD is decreased in premature newborns and infants undergoing heart surgery, which often includes partial or total thymectomy, perhaps due to a lower number of circulating T cells and an inappropriate immune response to antigens encountered in the skin.[14, 15]

There is a need for birth cohort studies that closely examines the skin of newborns at several time points to identify infants at risk of developing AD early in life. The BABY Cohort is a prospective birth cohort study that investigates early skin barrier development in preterm and term newborns to identify early prognostic skin barrier changes for development of AD.

OBJECTIVES

Primary objective:

To identify predictors of AD in early childhood.

Secondary objectives:

To closely describe the normal skin barrier development including immune activity and skin microbiome in preterm and term newborns during the first years of life.

METHODS AND ANALYSIS

Study population and setting

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4 127 The BABY Cohort is an ongoing prospective birth cohort study recruiting 150 preterm and 300 term
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6 128 newborn infants. Recruitment began in August 2017. Parents of eligible children are recruited at the
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9 129 maternity and neonatal wards at Rigshospitalet, Copenhagen, and Nordsjællands Hospital, Hillerød,
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11 130 in Denmark. Children eligible for enrolment are preterm newborns (GA below 37+0) excluding
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13 131 preterm newborns with severe congenital abnormality and healthy term singleton newborns (GA
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15 37+0 to 41+6) excluding mature newborns receiving antenatal steroids for fetal lung maturation.
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18 133 Children with parents unable to communicate in Danish are excluded.
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23 135 **Cohort design**

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25 136 All study procedures are summarised in Figure 1 and 2, and each component of the visit is detailed
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27 137 below. Preterm children are scheduled for two study visits: during the first 31 days of life and
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30 138 approximately two months after their scheduled due date (Figure 1). Term children are scheduled for
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32 139 four study visits: during the first 3 days of life and approximately at 2, 6 and 12 months of age (Figure
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34 140 2). If a child develops AD during the first 2 years of life, an additional follow-up visit is performed.
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37 141 All parents participate in a structured telephone interview when the child is 18 and 24 months old.
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41 143 **Baseline interview**

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43 144 During the first study visit, parents are interviewed to obtain information about the pregnancy and
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46 145 birth, including the type of delivery and maternal intrapartum antibiotic treatment. Furthermore,
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48 146 information about gestational age at birth, weight, height and head circumference, 1- and 5- minutes
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50 147 APGAR scores and medical treatment at the neonatal ward is obtained.
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55 149 **Study interview**
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At every study visit, we obtain detailed information about the child's health, vaccination status, method of feeding, admittance to hospital, medical treatments, bathing habits and skin care.

Parental questionnaires

Parents complete an online questionnaire on family structure, residential situation, pet exposure, occupation, maternal exposures during pregnancy, smoking and drinking habits, history about current and previous skin diseases and atopic diseases in the family.

Telephone interviews

At 18 and 24 months, parents participates in a structured telephone interview about the child's health, vaccination status, method of feeding, admittance to hospital, medical treatments, bathing habits, skin care, ultraviolet exposures and AD assessment according to the U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis.[16, 17] If AD is diagnosed during the telephone interview an extra study visit in the clinic is scheduled.

Anthropometric measures

At the first visit, birth information on height, weight and head circumference is retrieved from the birth record. At all follow-up visits, anthropometric measurements are made. A digital weight scale is used to record weight in kg without clothing and diaper. Height and head circumference are measured in cm using a flexible non-elastic measuring tape.

Skin barrier measurements

Transepidermal water loss

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4 173 During all study visits, transepidermal water loss (TEWL) is assessed using a portable closed
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6 174 condenser-chamber device (Aquaflux model AF200, Biox Systems Ltd, UK).[18] TEWL is measured
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9 175 three times on the same skin area located on the central part of the volar forearm. No preference is
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11 176 given to the left or right arm but depends on how the baby is positioned.
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16 178 *Natural moisturizing factors*
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18 179 Using a custom build device, the level of natural moisturizing factors (NMF) is measured on the
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20 180 thenar region using confocal Raman spectroscopy (RiverD International B.V., Rotterdam, The
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23 181 Netherlands).[19] Three values are recorded at all study visits, except the first study visit for the
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25 182 premature children. The thenar region of the child’s hand is placed on the device for approximately
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27 183 60 seconds. Scattered light is sent towards the skin surface, exiting the molecules in the skin. Each
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30 184 molecule represents a specific spectrum of light, and the specific composition of molecules is thereby
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32 185 represented in the returned spectrum of light.[20] Again, the most accessible hand is measured, in
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34 186 turn depending on the child’s posture at the time of examination.
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39 188 *Superficial stratum corneum (SC) sampling*
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41 189 During all study visits, SC is collected by tape stripping. Eight consecutive tape stripping discs (22
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43 190 mm) D-squame, CuDerm, Dallas, Texas) are applied on the skin followed by standardized pressure
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46 191 applied by a D-squame pressure application pen for 5 seconds. Tapes are stored at -80° C immediately
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48 192 after sampling. Preterm infants have SC collected from the skin between the shoulder blades, and at
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50 193 two months of age from the cheek as well. Term infants have SC collected from cheek skin and the
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53 194 dorsal surface of the hand. No preference is given to the left or right sides but depends on the
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55 195 positioning of the child. If a child develops AD, SC is collected from the dorsal surface of the hand
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and from a lesional skin site, preferably from the cheek, otherwise from a skin site with the most severe AD. SC will be examined for NMF, proteins, cytokines, lipids and morphology.

Clinical skin assessment

A complete examination of the skin is performed at each study visit. Size, number and location of both congenital and acquired naevi are registered. The palm of the hand is photographed to assess skin hyperlinearity at 2 months of age and in case the child develops AD.

Atopic dermatitis assessment

The skin is evaluated for signs of AD at each study visit. A diagnosis of AD is initially given by a physician and is subsequently diagnosed clinically using to the diagnostic criteria of Hanifin and Rajka except for IgE-levels and subcapsular cataract.[21] AD severity is assessed using the Eczema Area and Severity Index (EASI).[22] During all following visits, AD severity is assessed using EASI and Patient Oriented Eczema Measure (POEM) tool[17] and treatment for AD is recorded. As mentioned, during the structured telephone interviews, AD is diagnosed using The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis.[16]

Genetics

Buccal swabs (Isohelix, Harrietsham, U.K.) are used to collect DNA to screen for the most common *FLG* mutations in Northern European populations (R501X, 2282del4 and R2447X)[23] and for single nucleotide polymorphisms. For both analyses, cheek mucosa is rubbed for 60 seconds with a swab and stored at -80° C until analysis.

Skin swaps

During all study visits, a bacterial swab is collected from the cheek skin (ESwab Collection and Transport System Copan Italia, Brescia, Italy) and cultured for bacterial growth by routine methodology at the Department of Microbiology, Herlev and Gentofte Hospital, Denmark. Only samples positive for β -Hemolytic Streptococci isolates (groups A, B, C, G) or *S. aureus* have antimicrobial susceptibility testing performed and are subsequently stored at -80° C for future analyses. In preterm children, skin microbiome is collected from the lumbar area of the back at first visit and from cheek and lumbar area at two months of age. Skin microbiome samples (Isohelix, Harrietsham, U.K.) are collected from cheek and dorsal surface of the hand in term children. If a child develops AD, skin microbiome is also collected from a lesional skin site, preferably from the cheek otherwise from the most severe AD lesion. All samples are immediately stored at -80° C until analysis.

Ultrasound

During all study visits, ultrasound examination is performed to visualize the thymus gland and measure its size. The thymus index is defined as the multiplication of the two measurements and represents an estimate of the thymic volume.[24] The largest transverse diameter of the thymus is measured in a horizontal scan plane and the area of the largest lobe is measured in a sagittal scan plane. Both measurements are performed twice. The best measurement in both planes is selected. Measurements are performed with a transportable LOGIQ V2 ultrasound system with a 2-5.5 MHz C4-RS transducer (GE Healthcare, Milwaukee, WI).

Study settings

At each visit, air humidity, outdoor and indoor temperature is registered.

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Sample size estimation

The sample size calculation was based on a Dutch study showing a decrease in the filaggrin breakdown product 2-pyrrolidone-5-carboxylic acid (PCA) as a biomarker for the *FLG* genotype.[25] The lowest value of PCA was found in homozygote and compound *FLG* heterozygote mutation carriers (mean \pm SEM 0.18 ± 0.04 mmol g⁻¹), increasing to 0.50 ± 0.07 in heterozygote mutation carriers and 1.64 ± 0.11 mmol g⁻¹ protein in wild type.[25] In our cohort, we hypothesized a 5% change of NMF in children developing AD compared to children without AD. With a 5% two-sided significance level and a power of 80%, we calculated a sample size of 112 premature children and 223 in term children. Because of the high risk of loss-to-follow up during the two-year follow-up period, we estimated a sample size of 150 premature children and 300 term children would be needed.

Data management

Study data are collected and entered directly into an online REDCap (Research Electronic Data Capture) database hosted at the Capital Region of Denmark.

Patient and public involvement

Patients and the public were not involved in the design of the study. All participants will be acknowledged and thanked for their contribution in future publications.

STRENGTHS AND LIMITATIONS

The major strength of this birth cohort study is the extensive and repeated skin barrier measurements beginning right after birth. We will examine the skin barrier with multiple methodologies including Raman spectroscopy, TEWL and SC biomarkers. We will collect DNA and bacteria for genetic and skin microbiome analyses at several time points increasing the chance of finding a pathogenic role.

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4 268 We will include both preterm and term newborns allowing us to study the immature skin barrier and
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6 269 thymus in a large subset of children. We will use internationally accepted definitions to diagnose AD
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9 270 and assess severity.[21, 22] Collectively, the BABY cohort will cover a wide range of parameters
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11 271 with potential importance for the development of AD. Furthermore, we already now plan for future
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13 272 follow-up studies on skin barrier functions, AD and allergic diseases in this birth cohort.
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18 274 A potential limitation of the BABY Cohort is that all term children are recruited from Copenhagen
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20 275 only, possibly limiting the generalizability of the study to more rural areas. While we will register
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22 276 ambient room conditions including air humidity and indoor and outside temperature, seasonal and
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24 277 climatic variations will affect TEWL measurements. Children receiving incubator therapy have all
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26 278 measurements made directly in the incubator and the ambient conditions are recorded. As the study
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28 279 is strictly non-invasive, we will not make any blood measurements, and can therefore not assess the
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30 280 possible role of systemic inflammation. A concern in cohort studies is that participants may be lost
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32 281 to follow up. This is especially a concern for the premature children with many potential
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34 282 comorbidities who are recruited from Rigshospitalet; a highly specialized department responsible for
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36 283 treatment of all extremely premature children in eastern Denmark. To keep track of the included
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38 284 families, we gather contact information of both parents and contact them prior to follow-up visits.
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40 285 However, in case a family withdraws from the study, the date and reason for withdrawal will be
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50 288 **ETHICS AND DISSEMINATION**

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52 289 The study is approved by the local ethics committee (H-16042289 and H-16042294) and the local
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54 290 data protection agency (ID-no.: HGH-3017-040, I-suite no.:05578). The BABY Cohort is conducted
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in accordance with the Declaration of Helsinki. All relevant study results will be presented in peer-reviewed publications and presented at national and international conferences.

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Figure 1 - Scheduled investigations for preterm children in the BABY Cohort



Figure 2 - Scheduled investigations for term children in the BABY Cohort



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BMJ Open

'Barrier dysfunction in Atopic newBorns studY' (BABY): protocol of a Danish prospective birth cohort study

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**‘Barrier dysfunction in Atopic newBorns studY’ (BABY): protocol of
a Danish prospective birth cohort study**

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Atopic dermatitis, birth cohort, preterm, Raman, skin barrier, TEWL.

Conflicts of interest

None declared

Author contributions

TG, LS and JPT designed the study, created the study protocol, and obtained approval of the study design. ST, CBM, CE, IJ and SK contributed to revision and refinement of the study design. TG, AH, MRR, NHR, MHK and JPT were responsible for data collection. TG, AH, MRR, LS and JPT drafted the manuscript. All authors critically revised the manuscript. All authors supervised the study.

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ABSTRACT

Introduction:

Skin barrier development and dysfunction in premature and mature newborns is important for the risk of atopic dermatitis (AD).

Methods and analysis:

BABY Cohort is a prospective birth cohort study of 150 preterm children (gestational age (GA) below 37+0) and 300 term children (GA 37+0 to 41+6). Skin barrier is assessed through transepidermal water loss, tape stripping, Raman-spectroscopy and microbiome sampling. Clinical examinations are done and DNA from buccal swabs is collected for genetic analyses. Thymus size is assessed by ultrasound examination. Information on pregnancy, delivery and parental exposures and diseases are collected and structured telephone interviews are conducted at 18 and 24 months to assess exogenous exposures in the child and onset of AD. Hanifin and Rajka criteria as well as The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis are used to diagnose AD. Severity of AD is assessed using the Eczema Area and Severity Index (EASI) and Patient Oriented Eczema Measure (POEM).

Ethics and dissemination:

The study is approved by the scientific Ethical Committee of the Capital Region (H-16042289 and H-16042294).

Outcomes will be presented at national and international conferences and in peer-reviewed publications.

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78 **STRENGTHS AND LIMITATIONS OF THIS STUDY**

- 79 • This is a Danish prospective birth cohort study assessing skin barrier functions and risk factors
- 80 for atopic dermatitis.
- 81 • The study includes both preterm and term newborns from the general population.
- 82 • Repeated and comprehensive measurements of skin barrier will be performed at several time
- 83 points.
- 84 • A limitation is the lack of blood measurements as this study is strictly non-invasive.

86 **INTRODUCTION**

87 Atopic dermatitis (AD) is a chronic and relapsing, inflammatory skin disease, characterized by dry

88 and itchy skin that affects up to 20% of children in Northern Europe.[1] About 60-80% develop the

89 disease in their first two years of life, and children with early onset are at increased risk of having

90 severe and persistent disease.[2, 3] The risk of AD is increased in children of parents with atopic

91 disorders such as AD, asthma and allergic rhinitis.[4-6]

93 Genetic and environmental risk factors contribute to the development of AD through skin barrier

94 dysfunction and immune dysregulation.[2] While loss-of-function mutations in the filaggrin gene

95 (*FLG*) have been identified as the strongest genetic risk factor for AD,[7] genome wide association

96 studies have only identified a relatively small proportion of the genetic risk effect.[8] The

97 inflammation in AD is characterized by overexpression of Th2 cytokines, including IL-4 and IL-

98 13.[9] that together with IL-1 may lead to increased secretion of thymic stromal lymphopoietin

99 (TSLP), decreased epidermal antimicrobial peptides and filaggrin levels, in turn worsening skin

100 inflammation and epidermal barrier functions.[10] Changes in the skin microbiome is also associated

with worsening of AD, showing reduced bacterial diversity[11] and increased colonization with *Staphylococcus aureus* (*S. aureus*).[12]

While several environmental risk factors have been identified, e.g. winter birth and exposure to hard domestic water, this has not yet led to prophylactic solutions.[13] Interestingly, the risk of AD is decreased in premature newborns and infants undergoing heart surgery, which often includes partial or total thymectomy, perhaps due their reduced number of total lymphocytes and circulation T-cells resulting in an inappropriate immune response to antigens encountered in the skin.[14-16]

There is a need for birth cohort studies that closely examine the skin of newborns at several time points to identify infants at risk of developing AD early in life. The BABY Cohort is a prospective birth cohort study that investigates early skin barrier development in preterm and term newborns to identify early prognostic skin barrier changes for development of AD.

OBJECTIVES

Primary objective:

To identify early predictors of AD during the first two years of life.

Secondary objectives:

To closely describe the normal skin barrier development including immune activity and skin microbiome in preterm and term newborns during the first two years of life.

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METHODS AND ANALYSIS

Study population and setting

The BABY Cohort is an ongoing prospective and observational birth cohort study recruiting 150 preterm and 300 term newborn infants. Recruitment began in August 2017. Parents of eligible children are recruited at the maternity and neonatal wards at Rigshospitalet, Copenhagen, and Nordsjællands Hospital, Hillerød, in Denmark. Children eligible for enrolment are preterm newborns (GA below 37+0) excluding preterm newborns with severe congenital abnormality and healthy term singleton newborns (GA 37+0 to 41+6) excluding mature newborns receiving antenatal steroids for fetal lung maturation. Children with parents unable to communicate in Danish are excluded. Children are included independently of their hereditary risk for AD

Cohort design

All study procedures are summarised in Figure 1 and 2, and each component of the visit is detailed below. Preterm children are scheduled for two study visits: during the first 31 days of life and approximately two months after their scheduled due date (Figure 1). Term children are scheduled for four study visits: during the first 3 days of life and approximately at 2, 6 and 12 months of age (Figure 2). If a child develops AD during the first 2 years of life, an additional follow-up visit is performed. Overall, all children are recruited and examined as soon as possible after their delivery. Very immature born children often receive intensive medical care, and we wait until the child is stable until we perform the examinations. For all study visits the time of the study visit is registered, to be able to adjust for any effects that occur due to age differences. All parents participate in a structured telephone interview when the child is 18 and 24 months old. All study visits are conducted by trained medical doctors.

Baseline interview

During the first study visit, parents are interviewed to obtain information about the pregnancy and birth, including the type of delivery and maternal intrapartum antibiotic treatment. Furthermore, information about gestational age at birth, weight, height and head circumference, 1- and 5- minutes APGAR scores and medical treatment at the neonatal ward is obtained.

Study interview

At every study visit, we obtain detailed information about the child's health, vaccination status, method of feeding, admittance to hospital, medical treatments, bathing habits and skin care.

Parental questionnaires

Parents complete an online questionnaire on family structure, residential situation, pet exposure, occupation, maternal exposures during pregnancy, smoking and drinking habits, history about current and previous skin diseases and atopic diseases in the family.

Telephone interviews

At 18 and 24 months, parents participate in a structured telephone interview about the child's health, vaccination status, method of feeding, admittance to hospital, medical treatments, bathing habits, skin care, ultraviolet exposures and AD assessment according to the U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis, with parental assessment of visible flexural dermatitis in the elbows or knees. [17] If AD is diagnosed during the telephone interview an extra study visit in the clinic is scheduled.

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Anthropometric measures

At the first visit, birth information on height, weight and head circumference is retrieved from the birth record. At all follow-up visits, anthropometric measurements are made. A digital weight scale is used to record weight in kg without clothing and diaper. Height and head circumference are measured in cm using a flexible non-elastic measuring tape.

Skin barrier measurements

Transepidermal water loss

During all study visits, transepidermal water loss (TEWL) is assessed using a portable closed condenser-chamber device (Aquaflux model AF200, Biox Systems Ltd, UK).[18] TEWL is measured three times on the same skin area located on the central part of the volar forearm. No preference is given to the left or right arm but depends on how the baby is positioned.

Natural moisturizing factors

Using a custom build device, the level of natural moisturizing factors (NMF) is measured on the thenar region using confocal Raman spectroscopy (RiverD International B.V., Rotterdam, The Netherlands).[19-21] Three values are recorded at all study visits, except the first study visit for the premature children. The thenar region of the child’s hand is placed on the device for approximately 60 seconds. Scattered light is sent towards the skin surface, exiting the molecules in the skin. Each molecule represents a specific spectrum of light, and the specific composition of molecules is thereby represented in the returned spectrum of light.[20] Again, the most accessible hand is measured, in turn depending on the child’s posture at the time of examination.

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Superficial stratum corneum (SC) sampling

During all study visits, SC is collected by tape stripping as previously described.[22, 23] Eight consecutive tape stripping discs (22 mm) D-squame, CuDerm, Dallas, Texas) are applied on the skin followed by standardized pressure applied by a D-squame pressure application pen for 5 seconds and gently removed with tweezers. Tapes are stored at -80° C immediately after sampling. Preterm infants have SC collected from the skin between the shoulder blades, and at two months of age from the cheek as well. Term infants have SC collected from cheek skin and the dorsal surface of the hand. No preference is given to the left or right sides but depends on the positioning of the child. If a child develops AD, SC is collected from the dorsal surface of the hand and from a lesional skin site, preferably from the cheek, otherwise from a skin site with the most severe AD. SC samples will be analyzed for biomarkers of the immune response by multiplex immuno-assays, NMF using a liquid chromatography previously described by Kezic et al. [22] and corneocyte surface morphology by atomic force microscopy. [24]

Clinical skin assessment

A complete examination of the skin is performed at each study visit. Size, number and location of both congenital and acquired naevi are registered. The palm of the hand is photographed to assess skin hyperlinearity at 2 months of age and in case the child develops AD.

Atopic dermatitis assessment

The skin is evaluated for signs of AD at each study visit. A diagnosis of AD is initially given by a physician and is subsequently diagnosed clinically using to the diagnostic criteria of Hanifin and Rajka except for IgE-levels and subcapsular cataract.[25] AD severity is assessed using the Eczema Area and Severity Index (EASI).[26] During all following visits, AD severity is assessed using EASI

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6 222 mentioned, during the structured telephone interviews, AD is diagnosed using The U.K. Working
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14 225 **Genetics**

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16 226 Buccal swabs (Isohelix, Harrietsham, U.K.) are used to collect DNA to screen for the most common
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18 227 *FLG* mutations in Northern European populations (R501X, 2282del4 and R2447X)[28] and for single
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20 228 nucleotide polymorphisms. For both analyses, cheek mucosa is rubbed for 60 seconds with a swab
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23 229 and stored at -80° C until analysis.
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27 231 **Skin swabs**

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29 232 During all study visits, a bacterial swab is collected from the cheek skin (ESwab Collection and
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32 233 Transport System Copan Italia, Brescia, Italy) and cultured for bacterial growth by routine
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34 234 methodology at the Department of Microbiology, Herlev and Gentofte Hospital, Denmark. Only
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36 235 samples positive for β -Hemolytic Streptococci isolates (groups A, B, C, G) or *S. aureus* have
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39 236 antimicrobial susceptibility testing performed and are subsequently stored at -80° C for future
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41 237 analyses. In preterm children, skin microbiome is collected from the lumbar area of the back at first
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43 238 visit and from cheek and lumbar area at two months of age. Skin microbiome samples (Isohelix,
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46 239 Harrietsham, U.K.) are collected from cheek and dorsal surface of the hand in term children. If a child
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48 240 develops AD, skin microbiome is also collected from a lesional skin site, preferably from the cheek
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245 **Ultrasound**

246 During all study visits, ultrasound examination is performed to visualize the thymus gland and
247 measure its size. The thymus index is defined as the multiplication of the two measurements and
248 represents an estimate of the thymic volume.[29] The largest transverse diameter of the thymus is
249 measured in a horizontal scan plane and the area of the largest lobe is measured in a sagittal scan
250 plane. Both measurements are performed twice. The best measurement in both planes is selected.
251 Measurements are performed with a transportable LOGIQ V2 ultrasound system with a 2-5.5 MHz
252 C4-RS transducer (GE Healthcare, Milwaukee, WI).

254 **Study settings**

255 At each visit, air humidity, outdoor and indoor temperature is registered.

257 **Data management**

258 Study data are collected and entered directly into an online REDCap (Research Electronic Data
259 Capture) database hosted at the Capital Region of Denmark.

261 **Patient and public involvement**

262 Patients and the public were not involved in the design of the study. All participants will be
263 acknowledged and thanked for their contribution in future publications.

265 **STRENGTHS AND LIMITATIONS**

266 The major strength of this birth cohort study is the extensive and repeated skin barrier measurements
267 beginning shortly after birth. We will examine the skin barrier with multiple methodologies including
268 Raman spectroscopy, TEWL and SC biomarkers. We will collect DNA and bacteria for genetic and

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4 269 skin microbiome analyses at several time points increasing the chance of finding a pathogenic role.
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7 270 We will include both preterm and term newborns allowing us to study the immature skin barrier and
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9 271 thymus in a large subset of children. We will use internationally accepted definitions to diagnose AD
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11 272 and assess severity.[25, 26] Collectively, the BABY cohort will cover a wide range of parameters
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13 273 with potential importance for the development of AD. Furthermore, we already now plan for future
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15 274 follow-up studies on skin barrier functions, AD and allergic diseases in this birth cohort.
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20 276 A potential limitation of the BABY Cohort is that all term children are recruited from Copenhagen
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22 277 only, possibly limiting the generalizability of the study to more rural areas. While we will register
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24 278 ambient room conditions including air humidity and indoor and outside temperature, seasonal and
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26 279 climatic variations will affect TEWL measurements. Children receiving incubator therapy have all
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28 280 measurements made directly in the incubator and the ambient conditions are recorded. As the study
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30 281 is strictly non-invasive, we will not make any blood measurements, and can therefore not assess the
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32 282 possible role of systemic inflammation. A concern in cohort studies is that participants may be lost
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34 283 to follow up. This is especially a concern for the premature children with many potential
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36 284 comorbidities who are recruited from Rigshospitalet; a highly specialized department responsible for
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38 285 treatment of all extremely premature children in eastern Denmark. To keep track of the included
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40 286 families, we gather contact information of both parents and contact them prior to follow-up visits.
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42 287 However, in case a family withdraws from the study, the date and reason for withdrawal will be
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ETHICS AND DISSEMINATION

The study is approved by the scientific Ethical Committee of the Capital Region (H-16042289 and H-16042294) and the local data protection agency (ID-no.: HGH-3017-040, I-suite no.:05578). Both parents or guardians will give written informed consent prior to entry to the study.

The BABY Cohort is conducted in accordance with the Declaration of Helsinki. All relevant study results will be presented in peer-reviewed publications and presented at national and international conferences.

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377 Figure legend 1:

378 Scheduled investigations for preterm children in the BABY Cohort

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380 Figure legend 2:

381 Scheduled investigations for term children in the BABY Cohort

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Figure 1 - Scheduled investigations for preterm children in the BABY Cohort



Figure 2 - Scheduled investigations for term children in the BABY Cohort



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'Barrier dysfunction in Atopic newBorns studY' (BABY): protocol of a Danish prospective birth cohort study

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**‘Barrier dysfunction in Atopic newBorns studY’ (BABY): protocol of
a Danish prospective birth cohort study**

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44 TG, LS and JPT designed the study, created the study protocol, and obtained approval of the study
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47 drafted the manuscript. All authors critically revised the manuscript. All authors supervised the
48 study.

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63 **ABSTRACT**

64 Introduction:

65 Skin barrier development and dysfunction in premature and mature newborns is important for the
66 risk of atopic dermatitis (AD).

67 Methods and analysis:

68 BABY Cohort is a prospective birth cohort study of 150 preterm children (gestational age (GA)
69 below 37+0) and 300 term children (GA 37+0 to 41+6). Skin barrier is assessed through
70 transepidermal water loss, tape stripping, Raman-spectroscopy and microbiome sampling. Clinical
71 examinations are done and DNA from buccal swabs is collected for genetic analyses. Thymus size
72 is assessed by ultrasound examination. Information on pregnancy, delivery and parental exposures
73 and diseases are collected and structured telephone interviews are conducted at 18 and 24 months to
74 assess exogenous exposures in the child and onset of AD. Hanifin and Rajka criteria as well as The
75 U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis are used to diagnose AD. Severity
76 of AD is assessed using the Eczema Area and Severity Index (EASI) and Patient Oriented Eczema
77 Measure (POEM).

78 Ethics and dissemination:

79 The study is approved by the scientific Ethical Committee of the Capital Region (H-16042289 and
80 H-16042294).

81 Outcomes will be presented at national and international conferences and in peer-reviewed
82 publications.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is a Danish prospective birth cohort study assessing skin barrier functions and risk factors for atopic dermatitis.
- The study includes both preterm and term newborns from the general population.
- Repeated and comprehensive measurements of skin barrier will be performed at several time points.
- A limitation is the lack of blood measurements as this study is strictly non-invasive.

INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing, inflammatory skin disease, characterized by dry and itchy skin that affects up to 20% of children in Northern Europe.[1] About 60-80% develop the disease in their first two years of life, and children with early onset are at increased risk of having severe and persistent disease.[2, 3] The risk of AD is increased in children of parents with atopic disorders such as AD, asthma and allergic rhinitis.[4-6]

Genetic and environmental risk factors contribute to the development of AD through skin barrier dysfunction and immune dysregulation.[2] While loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as the strongest genetic risk factor for AD,[7] genome wide association studies have only identified a relatively small proportion of the genetic risk effect.[8] The inflammation in AD is characterized by overexpression of Th2 cytokines, including IL-4 and IL-13.[9] that together with IL-1 may lead to increased secretion of thymic stromal lymphopoietin (TSLP), decreased epidermal antimicrobial peptides and filaggrin levels, in turn worsening skin inflammation and epidermal barrier functions.[10] Changes in the skin microbiome is also associated

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4 109 with worsening of AD, showing reduced bacterial diversity[11] and increased colonization with
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6 110 *Staphylococcus aureus* (*S. aureus*).[12]
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11 112 While several environmental risk factors have been identified, e.g. winter birth and exposure to hard
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13 113 domestic water, this has not yet led to prophylactic solutions.[13] Interestingly, the risk of AD is
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15 114 decreased in premature newborns and infants undergoing heart surgery, which often includes partial
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18 115 or total thymectomy, perhaps due their reduced number of total lymphocytes and circulation T-cells
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20 116 resulting in an inappropriate immune response to antigens encountered in the skin.[14-16]
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25 118 There is a need for birth cohort studies that closely examine the skin of newborns at several time
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27 119 points to identify infants at risk of developing AD early in life. The BABY Cohort is a prospective
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30 120 birth cohort study that investigates early skin barrier development in preterm and term newborns to
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32 121 identify early prognostic skin barrier changes for development of AD.
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36 123 **OBJECTIVES**
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39 124 Primary objective:
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41 125 To identify early predictors of AD during the first two years of life.
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45 127 Secondary objectives:
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48 128 To closely describe the normal skin barrier development including immune activity and skin
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50 129 microbiome in preterm and term newborns during the first two years of life.
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METHODS AND ANALYSIS

Study population and setting

The BABY Cohort is an ongoing prospective and observational birth cohort study recruiting 150 preterm and 300 term newborn infants. Recruitment began in August 2017. Parents of eligible children are recruited at the maternity and neonatal wards at Rigshospitalet, Copenhagen, and Nordsjællands Hospital, Hillerød, in Denmark. Children eligible for enrolment are preterm newborns (GA below 37+0) excluding preterm newborns with severe congenital abnormality or conditions affecting their life expectancy and fullterm healthy term singleton newborns (GA 37+0 to 41+6) excluding mature newborns receiving antenatal steroids for fetal lung maturation. Children with parents unable to communicate in Danish are excluded, since it is not possible to use (for practical and financial reasons) interpreters right after birth given that we have to be very flexible and recruit at odd hours. Children are included independently of their hereditary risk for AD

Cohort design

All study procedures are summarised in Figure 1 and 2, and each component of the visit is detailed below. Preterm children are scheduled for two study visits: during the first 31 days of life and approximately two months after their scheduled due date (Figure 1). Term children are scheduled for four study visits: during the first 3 days of life and approximately at 2, 6 and 12 months of age (Figure 2). Many premature born children continue to have many hospital visits after their discharge, and many of the families lives far away from the hospital, i.e. other parts of Denmark. Therefore, preterm children are only scheduled to participate in one follow-up visit. We can therefore only make certain comparisons across the two groups.

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4 156 If a child develops AD during the first 2 years of life, an additional follow-up visit is performed.
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7 157 Overall, all children are recruited and examined as soon as possible after their delivery. Very
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9 158 immature born children often receive intensive medical care, and we wait until the child is stable until
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11 159 we perform the examinations.
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16 161 For all study visits the time of the study visit is registered, to be able to adjust for any effects that
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18 162 occur due to age differences. All parents participate in a structured telephone interview when the child
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20 163 is 18 and 24 months old. All study visits are conducted by trained medical doctors.
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25 165 **Baseline interview**

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27 166 During the first study visit, parents are interviewed to obtain information about the pregnancy and
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29 167 birth, including the type of delivery and maternal intrapartum antibiotic treatment. Furthermore,
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31 168 information about gestational age at birth, weight, height and head circumference, 1- and 5- minutes
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33 169 APGAR scores and medical treatment at the neonatal ward is obtained.
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39 171 **Study interview**

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41 172 At every study visit, we obtain detailed information about the child's health, vaccination status,
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43 173 method of feeding, admittance to hospital, medical treatments, bathing habits and skin care.
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48 175 **Parental questionnaires**

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50 176 Parents complete an online questionnaire on family structure, residential situation, pet exposure,
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52 177 occupation, maternal exposures during pregnancy, smoking and drinking habits, history about current
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54 178 and previous skin diseases and atopic diseases in the family.
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Telephone interviews

At 18 and 24 months, parents participate in a structured telephone interview about the child's health, vaccination status, method of feeding, admittance to hospital, medical treatments, bathing habits, skin care, ultraviolet exposures and AD assessment using a modification to the U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis, with parental assessment of visible flexural dermatitis in the elbows or knees.[17] If AD is diagnosed during the telephone interview an extra study visit in the clinic is scheduled.

Anthropometric measures

At the first visit, birth information on height, weight and head circumference is retrieved from the birth record. At all follow-up visits, anthropometric measurements are made. A digital weight scale is used to record weight in kg without clothing and diaper. Height and head circumference are measured in cm using a flexible non-elastic measuring tape.

Skin barrier measurements

Transepidermal water loss

During all study visits, transepidermal water loss (TEWL) is assessed using a portable closed condenser-chamber device (Aquaflux model AF200, Biox Systems Ltd, UK).[18] TEWL is measured three times on the same skin area located on the central part of the volar forearm. No preference is given to the left or right arm but depends on how the baby is positioned.

Natural moisturizing factors

Using a custom build device, the level of natural moisturizing factors (NMF) is measured on the thenar region using confocal Raman spectroscopy (RiverD International B.V., Rotterdam, The

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4 204 Netherlands).[19-21] Three values are recorded at all study visits, except the first study visit for the
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6 205 premature children. The thenar region of the child's hand is placed on the device for approximately
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9 206 60 seconds. Scattered light is sent towards the skin surface, exiting the molecules in the skin. Each
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11 207 molecule represents a specific spectrum of light, and the specific composition of molecules is thereby
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13 208 represented in the returned spectrum of light.[20] Again, the most accessible hand is measured, in
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16 209 turn depending on the child's posture at the time of examination.
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20 211 *Superficial stratum corneum (SC) sampling*
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23 212 During all study visits, SC is collected by tape stripping as previously described.[22, 23] Eight
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25 213 consecutive tape stripping discs (22 mm) D-squame, CuDerm, Dallas, Texas) are applied on the skin
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27 214 followed by standardized pressure applied by a D-squame pressure application pen for 5 seconds and
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30 215 gently removed with tweezers. Tapes are stored at -80° C immediately after sampling. Preterm infants
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32 216 have SC collected from the skin between the shoulder blades, and at two months of age from the
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34 217 cheek as well. Term infants have SC collected from cheek skin and the dorsal surface of the hand. No
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36 218 preference is given to the left or right sides but depends on the positioning of the child. If a child
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39 219 develops AD, SC is collected from the dorsal surface of the hand and from a lesional skin site,
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41 220 preferably from the cheek, otherwise from a skin site with the most severe AD. SC samples will be
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43 221 analyzed for biomarkers of the immune response by multiplex immuno-assays, NMF using a liquid
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46 222 chromatography previously described by Kezic et al. [22] and corneocyte surface morphology by
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48 223 atomic force microscopy. [24]
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52 225 **Clinical skin assessment**
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55 226 A complete examination of the skin is performed at each study visit to describe the normal skin barrier
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Size, number and location of both congenital and acquired naevi are registered. Studies and meta-analysis have shown that the number of nevi is inverse with AD. However, we are not aware of prospective data collection. The palm of the hand is photographed to assess skin hyperlinearity at 2 months of age and in case the child develops AD.

Atopic dermatitis assessment

The skin is evaluated for signs of AD at each study visit. A diagnosis of AD is initially given by a physician and is subsequently diagnosed clinically using to the diagnostic criteria of Hanifin and Rajka except for IgE-levels and subcapsular cataract.[25] AD severity is assessed using the Eczema Area and Severity Index (EASI).[26] During all following visits, AD severity is assessed using EASI and Patient Oriented Eczema Measure (POEM) tool[27] and treatment for AD is recorded. As mentioned, during the structured telephone interviews, AD is diagnosed using The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis.[17]

Genetics

Buccal swabs (Isohelix, Harrietsham, U.K.) are used to collect DNA to screen for the most common *FLG* mutations in Northern European populations (R501X, 2282del4 and R2447X)[28] by TaqMan genotyping assay, a routine analysis in our Biochemical department, and for single nucleotide polymorphisms. For both analyses, cheek mucosa is rubbed for 60 seconds with a swab and stored at -80° C until analysis.

Skin swabs

During all study visits, a bacterial swab is collected from the cheek skin (ESwab Collection and Transport System Copan Italia, Brescia, Italy) and cultured for bacterial growth by routine

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4 252 methodology at the Department of Microbiology, Herlev and Gentofte Hospital, Denmark. Only
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6 253 samples positive for β -Hemolytic Streptococci isolates (groups A, B, C, G) or *S. aureus* have
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9 254 antimicrobial susceptibility testing performed and are subsequently stored at -80° C for future
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11 255 analyses. In preterm children, skin microbiome is collected from the lumbar area of the back at first
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13 256 visit and from cheek and lumbar area at two months of age. Skin microbiome samples (Isohelix,
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15 Harrietsham, U.K.) are collected from cheek and dorsal surface of the hand in term children. If a child
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18 258 develops AD, skin microbiome is also collected from a lesional skin site, preferably from the cheek
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20 259 otherwise from the most severe AD lesion. Skin swabs are rubbed on the skin for 60 seconds and are
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23 260 immediately stored at -80° C until analysis.
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27 262 **Ultrasound**

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29 263 During all study visits, ultrasound examination is performed to visualize the thymus gland and
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31
32 264 measure its size. The thymus index is defined as the multiplication of the two measurements and
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34 265 represents an estimate of the thymic volume.[29] The largest transverse diameter of the thymus is
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36 266 measured in a horizontal scan plane and the area of the largest lobe is measured in a sagittal scan
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39 267 plane. Both measurements are performed twice. The best images with a full visualization of the gland
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41 268 are selected by a trained radiologist. Measurements are performed with a transportable LOGIQ V2
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43 269 ultrasound system with a 2-5.5 MHz C4-RS transducer (GE Healthcare, Milwaukee, WI).
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48 271 **Study settings**

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50 272 At each visit, air humidity, outdoor and indoor temperature is registered.
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55 274 **Sample size estimation**
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The sample size calculation is based on including preterm and mature children in a 1:2 ratio. The power calculation was based on an expected prevalence of AD in 20 % of the cohort population. Based on previous knowledge, where adult controls have and NMF of 0.095 +/-0.029,[30] we hypothesized a 12% change in NMF in children developing AD compared with children without AD. With a 5% two-sided significance level and a power of 80%. AD, as we calculated at sample size of 366 children. In order to account for possible drop-outs we decided on a study population of 450 participants in total, whereas 150 were preterm and 300 mature children.

Data management

Study data are collected and entered directly into an online REDCap (Research Electronic Data Capture) database hosted at the Capital Region of Denmark.

Patient and public involvement

Patients and the public were not involved in the design of the study. All participants will be acknowledged and thanked for their contribution in future publications.

STRENGTHS AND LIMITATIONS

The major strength of this birth cohort study is the extensive and repeated skin barrier measurements. We will examine the skin barrier with multiple methodologies including Raman spectroscopy, TEWL and SC biomarkers. We will collect DNA and bacteria for genetic and skin microbiome analyses at several time points increasing the chance of finding a pathogenic role. We will include both preterm and term newborns allowing us to study the immature skin barrier and thymus in a large subset of children. We will use internationally accepted definitions to diagnose AD and assess severity.[25, 26] Collectively, the BABY cohort will cover a wide range of parameters with potential importance for

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the development of AD. Since approximately 80% of AD patients develop their disease within the first two years of life, we expect to identify children with both transient and more established AD, as well as being able to differentiate between early features and predictors. Furthermore, we already now plan for future follow-up studies on skin barrier functions, AD and allergic diseases in this birth cohort.

A potential limitation of the BABY Cohort is that all term children are recruited from Copenhagen only, possibly limiting the generalizability of the study to more rural areas. While we will register ambient room conditions including air humidity and indoor and outside temperature, seasonal and climatic variations will affect TEWL measurements. Since bathing habits prior to study visits are not standardized, but only registered, this might impact our skin barrier assessments. Children receiving incubator therapy have all measurements made directly in the incubator and the ambient conditions are recorded. As the study is strictly non-invasive, we will not make any blood measurements, and can therefore not assess the possible role of systemic inflammation. Due to our study design, we cannot discriminate clearly between early features and predictors. A concern in cohort studies is that participants may be lost to follow up. This is especially a concern for the premature children with many potential comorbidities who are recruited from Rigshospitalet; a highly specialized department responsible for treatment of all extremely premature children in eastern Denmark. To keep track of the included families, we gather contact information of both parents and contact them prior to follow-up visits. However, in case a family withdraws from the study, the date and reason for withdrawal will be recorded.

ETHICS AND DISSEMINATION

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Enseignement Supérieur (ABES).

For peer review only

The study is approved by the scientific Ethical Committee of the Capital Region (H-16042289 and H-16042294) and the local data protection agency (ID-no.: HGH-3017-040, I-suite no.:05578). Both parents or guardians will give written informed consent prior to entry to the study.

The BABY Cohort is conducted in accordance with the Declaration of Helsinki. All relevant study results will be presented in peer-reviewed publications and presented at national and international conferences.

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409 Figure legend 1:

410 Scheduled investigations for preterm children in the BABY Cohort

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412 Figure legend 2:

413 Scheduled investigations for term children in the BABY Cohort

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Figure 1 - Scheduled investigations for preterm children in the BABY Cohort



Figure 2 - Scheduled investigations for term children in the BABY Cohort



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BMJ Open

'Barrier dysfunction in Atopic newBorns studY' (BABY): protocol of a Danish prospective birth cohort study

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Primary Subject Heading:	Dermatology
Secondary Subject Heading:	Paediatrics, Dermatology, Immunology (including allergy)
Keywords:	atopic dermatitis, birth cohort, Raman, skin barrier, preterm, TEWL





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**‘Barrier dysfunction in Atopic newBorns studY’ (BABY): protocol of
a Danish prospective birth cohort study**

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30 Atopic dermatitis, birth cohort, preterm, Raman, skin barrier, TEWL.

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41 Foundation, during the conduct of the study.

43 **Author contributions**

44 TG, LS and JPT designed the study, created the study protocol, and obtained approval of the study
45 design. ST, CMB, CE, IJ and SK contributed to revision and refinement of the study design. TG,
46 AH, MRR, NHR, MHK and JPT were responsible for data collection. TG, AH, MRR, LS and JPT
47 drafted the manuscript. All authors critically revised the manuscript. All authors supervised the
48 study.

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11 52 Rigshospitalet and Nordsjællands Hospital who have contributed.
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63 **ABSTRACT**

64 Introduction:

65 Skin barrier development and dysfunction in premature and mature newborns is important for the
66 risk of atopic dermatitis (AD).

67 Methods and analysis:

68 BABY Cohort is a prospective birth cohort study of 150 preterm children (gestational age (GA)
69 below 37+0) and 300 term children (GA 37+0 to 41+6). Skin barrier is assessed through
70 transepidermal water loss, tape stripping, Raman-spectroscopy and microbiome sampling. Clinical
71 examinations are done and DNA from buccal swabs is collected for genetic analyses. Thymus size
72 is assessed by ultrasound examination. Information on pregnancy, delivery and parental exposures
73 and diseases are collected and structured telephone interviews are conducted at 18 and 24 months to
74 assess exogenous exposures in the child and onset of AD. Hanifin and Rajka criteria as well as The
75 U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis are used to diagnose AD. Severity
76 of AD is assessed using the Eczema Area and Severity Index (EASI) and Patient Oriented Eczema
77 Measure (POEM).

78 Ethics and dissemination:

79 The study is approved by the scientific Ethical Committee of the Capital Region (H-16042289 and
80 H-16042294).

81 Outcomes will be presented at national and international conferences and in peer-reviewed
82 publications.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is a Danish prospective birth cohort study assessing skin barrier functions and risk factors for atopic dermatitis.
- The study includes both preterm and term newborns from the general population.
- Repeated and comprehensive measurements of skin barrier will be performed at several time points.
- A limitation is the lack of blood measurements as this study is strictly non-invasive.

INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing, inflammatory skin disease, characterized by dry and itchy skin that affects up to 20% of children in Northern Europe.[1] About 60-80% develop the disease in their first two years of life, and children with early onset are at increased risk of having severe and persistent disease.[2, 3] The risk of AD is increased in children of parents with atopic disorders such as AD, asthma and allergic rhinitis.[4-6]

Genetic and environmental risk factors contribute to the development of AD through skin barrier dysfunction and immune dysregulation.[2] While loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as the strongest genetic risk factor for AD,[7] genome wide association studies have only identified a relatively small proportion of the genetic risk effect.[8] The inflammation in AD is characterized by overexpression of Th2 cytokines, including IL-4 and IL-13.[9] that together with IL-1 may lead to increased secretion of thymic stromal lymphopoietin (TSLP), decreased epidermal antimicrobial peptides and filaggrin levels, in turn worsening skin inflammation and epidermal barrier functions.[10] Changes in the skin microbiome is also associated

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4 109 with worsening of AD, showing reduced bacterial diversity[11] and increased colonization with
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6 110 *Staphylococcus aureus* (*S. aureus*).[12]
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11 112 While several environmental risk factors have been identified, e.g. winter birth and exposure to hard
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13 113 domestic water, this has not yet led to prophylactic solutions.[13] Interestingly, the risk of AD is
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15 114 decreased in premature newborns and infants undergoing heart surgery, which often includes partial
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18 115 or total thymectomy, perhaps due their reduced number of total lymphocytes and circulation T-cells
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20 116 resulting in an inappropriate immune response to antigens encountered in the skin.[14-16]
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25 118 There is a need for birth cohort studies that closely examine the skin of newborns at several time
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27 119 points to identify infants at risk of developing AD early in life. The BABY Cohort is a prospective
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29 120 birth cohort study that investigates early skin barrier development in preterm and term newborns to
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31 121 identify early prognostic skin barrier changes for development of AD.
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36 123 **OBJECTIVES**
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39 124 Primary objective:
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41 125 To identify early predictors of AD during the first two years of life, including skin barrier dysfunction
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43 126 and exogenous exposures during pregnancy and in infancy. The study will assess patient and parental
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45 127 characteristics, family history of atopic comorbidities, exposures during pregnancy and in infancy
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48 128 and skin barrier function and development.
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52 130 Secondary objectives:
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55 131 To closely describe the normal skin barrier development including immune activity and skin
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57 132 microbiome in preterm and term newborns during the first two years of life.
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METHODS AND ANALYSIS

Study population and setting

The BABY Cohort is an ongoing prospective and observational birth cohort study recruiting 150 preterm and 300 term newborn infants. Recruitment began in August 2017. Parents of eligible children are recruited at the maternity and neonatal wards at Rigshospitalet, Copenhagen, and Nordsjællands Hospital, Hillerød, in Denmark. Children eligible for enrolment are preterm newborns (GA below 37+0) excluding preterm newborns with severe congenital abnormality or conditions affecting their life expectancy and fullterm healthy term singleton newborns (GA 37+0 to 41+6) excluding mature newborns receiving antenatal steroids for fetal lung maturation. Children with parents unable to communicate in Danish are excluded, since it is not possible to use (for practical and financial reasons) interpreters right after birth given that we have to be very flexible and recruit at odd hours. Children are included independently of their hereditary risk for AD

Cohort design

All study procedures are summarised in Figure 1 and 2, and each component of the visit is detailed below. Preterm children are scheduled for two study visits: during the first 31 days of life and approximately two months after their scheduled due date (Figure 1). Term children are scheduled for four study visits: during the first 3 days of life and approximately at 2, 6 and 12 months of age (Figure 2). Many premature born children continue to have many hospital visits after their discharge, and many of the families lives far away from the hospital, i.e. other parts of Denmark. Therefore, preterm

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11 159 If a child develops AD during the first 2 years of life, an additional follow-up visit is performed.
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13 160 Overall, all children are recruited and examined as soon as possible after their delivery. Very
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15 161 immature born children often receive intensive medical care, and we wait until the child is stable until
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17 162 we perform the examinations.
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23 164 For all study visits the time of the study visit is registered, to be able to adjust for any effects that
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27 166 is 18 and 24 months old. All study visits are conducted by trained medical doctors.
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32 168 **Baseline interview**
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34 169 During the first study visit, parents are interviewed to obtain information about the pregnancy and
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36 170 birth, including the type of delivery and maternal intrapartum antibiotic treatment. Furthermore,
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40 172 APGAR scores and medical treatment at the neonatal ward is obtained.
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45 174 **Study interview**
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48 175 At every study visit, we obtain detailed information about the child's health, vaccination status,
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55 178 **Parental questionnaires**
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Parents complete an online questionnaire on family structure, residential situation, pet exposure, occupation, maternal exposures during pregnancy, smoking and drinking habits, history about current and previous skin diseases and atopic diseases in the family.

Telephone interviews

At 18 and 24 months, parents participate in a structured telephone interview about the child's health, vaccination status, method of feeding, admittance to hospital, medical treatments, bathing habits, skin care, ultraviolet exposures and AD assessment using a modification to the U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis, with parental assessment of visible flexural dermatitis in the elbows or knees.[17] If AD is diagnosed during the telephone interview an extra study visit in the clinic is scheduled.

Anthropometric measures

At the first visit, birth information on height, weight and head circumference is retrieved from the birth record. At all follow-up visits, anthropometric measurements are made. A digital weight scale is used to record weight in kg without clothing and diaper. Height and head circumference are measured in cm using a flexible non-elastic measuring tape.

Skin barrier measurements

Transepidermal water loss

During all study visits, transepidermal water loss (TEWL) is assessed using a portable closed condenser-chamber device (Aquaflux model AF200, Biox Systems Ltd, UK).[18] TEWL is measured three times on the same skin area located on the central part of the volar forearm. No preference is given to the left or right arm but depends on how the baby is positioned.

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Natural moisturizing factors

Using a custom build device, the level of natural moisturizing factors (NMF) is measured on the thenar region using confocal Raman spectroscopy (RiverD International B.V., Rotterdam, The Netherlands).[19-21] Three values are recorded at all study visits, except the first study visit for the premature children. The thenar region of the child’s hand is placed on the device for approximately 60 seconds. Scattered light is sent towards the skin surface, exciting the molecules in the skin. Each molecule represents a specific spectrum of light, and the specific composition of molecules is thereby represented in the returned spectrum of light.[20] Again, the most accessible hand is measured, in turn depending on the child’s posture at the time of examination.

Superficial stratum corneum (SC) sampling

During all study visits, SC is collected by tape stripping as previously described.[22, 23] Eight consecutive tape stripping discs (22 mm) D-squame, CuDerm, Dallas, Texas) are applied on the skin followed by standardized pressure applied by a D-squame pressure application pen for 5 seconds and gently removed with tweezers. Tapes are stored at -80° C immediately after sampling. Preterm infants have SC collected from the skin between the shoulder blades, and at two months of age from the cheek as well. Term infants have SC collected from cheek skin and the dorsal surface of the hand. No preference is given to the left or right sides but depends on the positioning of the child. If a child develops AD, SC is collected from the dorsal surface of the hand and from a lesional skin site, preferably from the cheek, otherwise from a skin site with the most severe AD. SC samples will be analyzed for biomarkers of the immune response by multiplex immuno-assays, NMF using a liquid chromatography previously described by Kezic et al. [22] and corneocyte surface morphology by atomic force microscopy. [24]

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Clinical skin assessment

A complete examination of the skin is performed at each study visit to describe the normal skin barrier development.

Size, number and location of both congenital and acquired naevi are registered. Studies and meta-analysis have shown that the number of nevi is inverse with AD. However, we are not aware of prospective data collection. The palm of the hand is photographed to assess skin hyperlinearity at 2 months of age and in case the child develops AD.

Atopic dermatitis assessment

The skin is evaluated for signs of AD at each study visit. A diagnosis of AD is initially given by a physician and is subsequently diagnosed clinically using to the diagnostic criteria of Hanifin and Rajka except for IgE-levels and subcapsular cataract.[25] AD severity is assessed using the Eczema Area and Severity Index (EASI).[26] During all following visits, AD severity is assessed using EASI and Patient Oriented Eczema Measure (POEM) tool[27] and treatment for AD is recorded. As mentioned, during the structured telephone interviews, AD is diagnosed using The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis.[17]

Genetics

Buccal swabs (Isohelix, Harrietsham, U.K.) are used to collect DNA to screen for the most common *FLG* mutations in Northern European populations (R501X, 2282del4 and R2447X)[28] by TaqMan genotyping assay, a routine analysis in our Biochemical department, and for single nucleotide polymorphisms. For both analyses, cheek mucosa is rubbed for 60 seconds with a swab and stored at -80° C until analysis.

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Skin swabs

During all study visits, a bacterial swab is collected from the cheek skin (ESwab Collection and Transport System Copan Italia, Brescia, Italy) and cultured for bacterial growth by routine methodology at the Department of Microbiology, Herlev and Gentofte Hospital, Denmark. Only samples positive for β -Hemolytic Streptococci isolates (groups A, B, C, G) or *S. aureus* have antimicrobial susceptibility testing performed and are subsequently stored at -80° C for future analyses. In preterm children, skin microbiome is collected from the lumbar area of the back at first visit and from cheek and lumbar area at two months of age. Skin microbiome samples (Isohelix, Harrietsham, U.K.) are collected from cheek and dorsal surface of the hand in term children. If a child develops AD, skin microbiome is also collected from a lesional skin site, preferably from the cheek otherwise from the most severe AD lesion. Skin swabs are rubbed on the skin for 60 seconds and are immediately stored at -80° C until analysis.

Ultrasound

During all study visits, ultrasound examination is performed to visualize the thymus gland and measure its size. The thymus index is defined as the multiplication of the two measurements and represents an estimate of the thymic volume.[29] The largest transverse diameter of the thymus is measured in a horizontal scan plane and the area of the largest lobe is measured in a sagittal scan plane. Both measurements are performed twice. The best images with a full visualization of the gland are selected by a trained radiologist. Measurements are performed with a transportable LOGIQ V2 ultrasound system with a 2-5.5 MHz C4-RS transducer (GE Healthcare, Milwaukee, WI).

Study settings

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At each visit, air humidity, outdoor and indoor temperature is registered.

Sample size estimation

The sample size calculation was based on including preterm and mature children in a 1:2 ratio. The power calculation was based on an expected prevalence of AD in 20% of the cohort population, assessing changes in NMF, which is one of multiple important endpoints in our study. Based on a previous study, where adult controls had an NMF of 0.095 ± 0.029 , [30] we hypothesized a 12% change in NMF in newborns developing AD compared with children without developing AD. Using a two-sided parametric test with an alpha of 5% and a power of 80%, we calculated a sample size of 366 children. In order to account for possible drop-outs, and the intention to study many other predictors for AD and skin barrier function in general, we decided on a study population of 450 participants in total, i.e. 150 preterm and 300 mature children.

Data management

Study data are collected and entered directly into an online REDCap (Research Electronic Data Capture) database hosted at the Capital Region of Denmark.

Patient and public involvement

Patients and the public were not involved in the design of the study. All participants will be acknowledged and thanked for their contribution in future publications.

STRENGTHS AND LIMITATIONS

The major strength of this birth cohort study is the extensive and repeated skin barrier measurements.

We will examine the skin barrier with multiple methodologies including Raman spectroscopy, TEWL

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4 299 and SC biomarkers. We will collect DNA and bacteria for genetic and skin microbiome analyses at
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7 300 several time points increasing the chance of finding a pathogenic role. We will include both preterm
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9 301 and term newborns allowing us to study the immature skin barrier and thymus in a large subset of
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11 302 children. We will use internationally accepted definitions to diagnose AD and assess severity.[25, 26]
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13 303 Collectively, the BABY cohort will cover a wide range of parameters with potential importance for
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15 304 the development of AD. Since approximately 80% of AD patients develop their disease within the
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18 305 first two years of life, we expect to identify children with both transient and more established AD. as
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20 306 well as being able to differentiate between early features and predictors. Furthermore, we already
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23 307 now plan for future follow-up studies on skin barrier functions, AD and allergic diseases in this birth
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25 308 cohort.
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29 310 A potential limitation of the BABY Cohort is that all term children are recruited from Copenhagen
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31 311 only, possibly limiting the generalizability of the study to more rural areas. While we will register
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33 312 ambient room conditions including air humidity and indoor and outside temperature, seasonal and
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35 313 climatic variations will affect TEWL measurements. Since bathing habits prior to study visits are not
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37 314 standardized, but only registered, this might impact our skin barrier assessments. Children receiving
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39 315 incubator therapy have all measurements made directly in the incubator and the ambient conditions
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41 316 are recorded. As the study is strictly non-invasive, we will not make any blood measurements, and
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43 317 can therefore not assess the possible role of systemic inflammation. Due to our study design, we
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45 318 cannot discriminate clearly between early features and predictors. A concern in cohort studies is that
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47 319 participants may be lost to follow up. This is especially a concern for the premature children with
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49 320 many potential comorbidities who are recruited from Rigshospitalet; a highly specialized department
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51 321 responsible for treatment of all extremely premature children in eastern Denmark. To keep track of
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53 322 the included families, we gather contact information of both parents and contact them prior to follow-
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up visits. However, in case a family withdraws from the study, the date and reason for withdrawal will be recorded.

ETHICS AND DISSEMINATION

The study is approved by the scientific Ethical Committee of the Capital Region (H-16042289 and H-16042294) and the local data protection agency (ID-no.: HGH-3017-040, I-suite no.:05578). Both parents or guardians will give written informed consent prior to entry to the study.

The BABY Cohort is conducted in accordance with the Declaration of Helsinki. All relevant study results will be presented in peer-reviewed publications and presented at national and international conferences.

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414 Figure legend 1:

415 Scheduled investigations for preterm children in the BABY Cohort

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417 Figure legend 2:

418 Scheduled investigations for term children in the BABY Cohort

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Figure 1 - Scheduled investigations for preterm children in the BABY Cohort



Figure 2 - Scheduled investigations for term children in the BABY Cohort



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