

Turning the inside out: The microbiology of atopic dermatitis

by

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Abstract

Allergy is worldwide on the rise. The hygiene hypothesis of atopic diseases linked microbes with atopic dermatitis (AD) both as drivers or modulators of skin pathology. The earlier literature favored an inside-outside model of AD where an immunological abnormality compounded by a gut microbiota dysbiosis is the primary event. Probiotic intervention trials with lactobacilli and bifidobacteria as well as the application of bifidogenic oligosaccharide prebiotics showed indeed promising clinical results, but no consistent gut microbiota dysbiosis could be linked with AD. An alternative hypothesis known as outside-inside model of AD considers a genetic skin barrier effect compounded by a skin microbiota dysbiosis as primary pathogenic event. Cultivation microbiology has demonstrated strong skin colonization with superantigen-encoding *Staphylococcus aureus* in AD patients; microbiota and molecular microbiome analyses demonstrated that *S. aureus* abundance fluctuates and parallels clinical symptoms. In a mouse model delta-toxin of *S. aureus* induced mast cell degranulation leading to AD-like symptoms. Mutant mice developing AD symptoms showed increased skin colonization with *S. aureus*; antibiotic treatment alleviated symptoms. Clinical trials showed that various treatments reducing *S. aureus* skin load also reduced AD symptoms suggesting *S. aureus* as a potential critical driver of AD and a target for anti-microbial interventions other than antibiotics.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.13050

24 **Introduction**

25
26 The striking rise of atopic diseases in industrialized countries over the last decades and its relatively
27 low prevalence in populations living under more primitive conditions led to the formulation of the
28 « hygiene hypothesis » of atopic diseases by Strachan (1989) (Fig. 1). He investigated a large British
29 cohort of children for the occurrence of hay fever and childhood eczema. The prevalence of both
30 conditions was significantly related to the number of older children in the household. Strachan
31 defined allergy as a post-industrial revolution epidemic and suggested its relentless increase over
32 recent decades by a decrease in the protective effect of early childhood infections transmitted by
33 unhygienic contact with older siblings. Declining family size and improvements in household
34 cleanliness would curtail childhood infections and their postulated protective influence on allergy
35 development. A leading medical journal recommended to “Eat Dirt” (Weiss 2002) based on inverse
36 trends of infections on one side and allergic diseases on the other side (Bach 2002) and the decrease
37 of asthma development in children living on farms compared to rural controls (Braun-Fahrlander et
38 al. 2002). Higher environmental loads of bacterial endotoxins in the farm environment were linked
39 with the strong immunomodulating effects of bacterial LPS (Baker et al., 1992), which led recently to
40 clinical trials testing bacterial lysates from two enteric commensals, *Escherichia coli* and *Enterococcus*
41 *faecalis*, against atopic dermatitis (Lau et al., 2012). Also animal experiments demonstrated that the
42 intestinal microflora was an important inducer for the maturation of the immune system (Chung et
43 al., 2012). It was therefore straightforward to postulate an impact of aberrant intestinal colonization
44 during early postnatal development on deviant immune maturation leading to allergic diseases. The
45 association between deviant gut microbiota development and allergy has been intensively
46 investigated for atopic dermatitis (AD) (see Fig. 2 for clinical features and Fig. 3 for histology).

47 48 **Gut microbiota and atopic dermatitis**

49 *Decrease in bifidobacteria?*

50 A case-control study from Sweden and Estonia revealed a lower prevalence of fecal lactobacilli
51 ($P<0.01$) and bifidobacteria and higher counts of intestinal coliforms ($P<0.01$) and *Staphylococcus*
52 *aureus* ($P<0.05$) in allergic (skin prick test (SPT) positive for food antigens) compared to non-allergic
53 children (Björkstén et al., 1999). When serial stool samples from 18 allergic and 26 controls were
54 studied, only Bifidobacteria showed consistently higher prevalence ($P<0.05$) in the stool samples of
55 controls compared to children with allergy as defined by AD or positive skin prick test (Björkstén et

al., 2001). When studying a dozen of bacteria by cultivation methods, only 1 case compared to 7 control children showed higher counts of bifidobacteria in the stool. Fecal *Lactobacillus* and *Clostridium* titers were higher ($P=0.02$) in allergic children (AD, asthma, allergic rhinitis) with food-specific serum IgE antibody titers (Sepp et al. 2005). However, German children did not show fecal microbiota differences between AD patients with and without food-specific IgE antibody ($P>0.1$) (Kendler et al., 2006). In Japanese children with clinically defined AD, fecal bifidobacteria counts were significantly lower in patients than in controls ($P<0.05$) and the severity score was inversely related with fecal *Bifidobacterium* counts ($P<0.05$) but titer differences were small (Watanabe et al., 2003). A prospective study on atopy and gut microbiota development was also conducted in 76 Finnish infants with a family history of atopic disease (AD, asthma, allergic rhinitis) (Kalliomäki et al., 2001a). By cultivation-independent methods (FISH) subjects with positive prick test showed a 3-fold higher *Clostridium* ($P=0.04$) and a non-significant 3-fold lower *Bifidobacterium* count ($P=0.1$) than controls. Ouwehand and colleagues (2001) recommended that for a better diagnostic resolution bifidobacteria should be investigated at the species level since a stool comparison of 7 allergic (AD with positive SPT) with 6 control children (both were breast-fed) showed that the diseased children were dominated by *B. adolescentis* ($P=0.005$) while the control children were dominated by *B. bifidum* ($P=0.03$).

While these early data seem to implicate a protective role of fecal bifidobacteria against the development of AD, later studies did not always concur with this conclusion. For example Gore et al. (2008) did not observe a difference ($P>0.8$) between pediatric AD patients from UK and New Zealand and matched controls for fecal bacterial profiles when using a molecular technique (TTGE). When using a *Bifidobacterium* species-specific PCR, they did not detect differences in *B. adolescentis* and *B. bifidum* ($P>0.2$), but a significant higher prevalence of *B. pseudocatenulatum* ($P=0.04$) in AD patients.

Increase in E. coli and Clostridium?

A large prospective study from the Netherlands (KOALA in Dutch acronym) did not observe a difference ($P>0.15$) between children with atopic eczema and controls with respect to fecal bifidobacteria, even when pushing the analysis to the species level. However, *E. coli* was significantly ($P=0.01$) enriched in cases compared to controls (Penders et al., 2006). The risk of eczema by parental report was significantly higher in infants colonized with *E. coli* (OD (Odd Ratio)=1.87) and *C. difficile* (OR=1.4) and the risk of developing eczema increased with fecal *E. coli* titers ($P=0.02$) (Penders et al., 2007). Also an Asian at-risk birth cohort with parent history of eczema, asthma or allergic rhinitis with positive SPT to dust mites showed a higher abundance of Enterobacteriaceae

($P=0.02$) and *Clostridium perfringens* ($P=0.03$), but lower abundance of bifidobacteria ($P=0.02$) in children with eczema compared to controls (Yap et al. 2014).

Since the development of atopic diseases also depends on genetic factors, 700 children from the KOALA study were genotyped for genetic variations in the CD14 and Toll-like receptor 4 (TLR4) genes, known to be involved in the immune recognition and signal transduction of bacterial endotoxin. Most of the single nucleotide polymorphisms (SNPs) showed no interaction with *E. coli* exposure and AD ($P>0.2$) (Penders et al., 2010). The association of fecal *Clostridium difficile* detection at 1-month of age with eczema development remained significant after a follow-up of 7 years ($OR=1.45$, $P<0.05$). However, this association was only significant ($P<0.05$) in the subgroup of children with a history of parental atopy (asthma, wheeze, eczema) as a genetic marker for risk. Vaginal delivery at home was a significant protective factor against eczema development ($OR=0.84$, $P<0.05$) when compared with vaginal delivery at a hospital, pointing to the hospital as source for early *C. difficile* colonization (van Nimwegen et al., 2011). In a high risk population (single or double heredity for AD, allergic rhinitis or asthma) from Germany colonization with *Clostridium* cluster I ($OR=2.3$, $P<0.05$), but not *E. coli* ($OR=1.12$) and *C. difficile* ($OR=1.24$, $P>0.05$), were associated with a higher risk to develop AD (Penders et al., 2013). In contrast to the KOALA study a prospective study from Norway showed for allergen-specific immunoglobulin E sensitization a transient association with a decreased *E. coli* and increased *B. longum* levels in the stool ($P<0.05$), but no association between microbiota and AD development (Storro et al., 2011). In summary, no clear data identify specific gut microbes as risk factors for AD development.

Decreased gut microbiota diversity?

To address the problem of a distinct genetic background modifying a gut microbiota-AD link, gut microbiota composition and its impact on AD development was studied by using identical laboratory tests in three European birth cohorts from Sweden, UK and Italy. Using cultivation methods, AD development was not associated with significant changes in ten common gut bacteria ($P>0.1$). There were only trends ($P=0.06$) for early gut colonization with *S. aureus* and *Bacteroides* and later development of AD (Adlerberth et al., 2007). With culture-independent molecular methods AD development was associated with a reduced ($P<0.05$) early fecal microbiota (Wang et al., 2008). Australian researchers concurred with these observations when using a similar study design: gut microbiota were assessed at 1 week of age and allergy was assessed at 12 months of age. A significant ($P<0.03$) reduction of early bacterial diversity was seen in children with later eczema development when compared to controls (Ismail et al., 2012). However, not all studies associated low gut microbiota diversity with AD development. A Danish prospective study enrolled 400 children

122 at high risk of allergy development (their mothers had asthma) and they found a reduced diversity of
123 gut microbiota expressed in children with positive specific IgE and skin prick test ($P=0.03$), allergic
124 rhinitis ($P=0.007$), but notably not with AD ($P>0.1$) when compared to control children.

125 Enterobacteria, enterococci and staphylococci determined by fecal culture did not differ ($P>0.1$)
126 between AD and control children (Bisgaard et al., 2011).

127 Finally, 100 children from Norway who provided 4 stool samples over the 2 years observation period
128 and were analyzed by quantitative real-time PCR for 12 bacterial species did not show a significant
129 difference in colonization pattern for the investigated bacterial species between children with and
130 without eczema ($P>0.1$) (Storro et al., 2011).

131 ***Confounding genetic aspects***

132 From the available evidence no clear, consistent association between gut microbiota composition
133 and AD development could be deduced. This could mean that no such association exists. However, it
134 could also mean that AD is not a uniform, single clinical entity. Clinicians are aware of this problem
135 and distinguish AD according to history (age of onset, outgrowth vs. persistence) or serum IgE levels
136 (extrinsic vs. intrinsic AD). Progress in the elucidation of the genetic basis for AD has raised hopes
137 that in the near future endotypes can be defined in patients with AD and that these new subtypes of
138 AD can be used in clinical study design and drug development to target therapies to patients most
139 likely to benefit from a mechanism-based treatment (Leung and Guttman-Yassky, 2014).

140 Molecular geneticists have defined filaggrin mutations as a causal candidate gene for AD
141 susceptibility since this mutation affects directly the skin architecture and are thus at the basis of the
142 pathogenesis of AD (Irvine et al., 2011). However, not all AD patients show filaggrin mutations. A
143 meta-analysis of genome wide association studies have added three further genetic risk loci for AD
144 including two genes (*OVOL1*, *ACTL9*) involved in epidermal barrier function and in a cytokine gene
145 cluster (*KIF3A*) underlining the important role of immune dysregulation in AD (Paternoster et al.,
146 2012). A recent report extended the number of candidate genes to 11 implicating also functions in
147 transcriptional regulation and apoptosis suggesting possible links to autoimmune diseases (Ellinghaus
148 et al. 2013). By utilizing genome-wide association study and ImmunoChip data from >19,000
149 individuals with AD and psoriasis, the two most common immune-mediated inflammatory disorders
150 affecting the skin, distinct genetic mechanisms with opposing effects in shared pathways influencing
151 epidermal differentiation and immune response were associated with the two diseases (Baurecht et
152 al., 2015).

Only few authors have stratified their patients with respect to genetic traits defined by molecular tests, the KOALA study is a rare example (Penders et al., 2010). Some authors tried to account for the genetic background by differentiating AD children having parents who suffered or did not suffer from allergy. When stratified for this genetic background, they could link a decreased colonization by lactobacilli with the later development of AD in high risk children ($P=0.01$). However, this relationship was only statistically significant for the gut microbiota in the first week of life ($P=0.03$) and not of the subsequent time points (Johansson et al., 2011).

Gut microbiota and AD: insight from probiotic and prebiotic intervention trials

Is it possible to assess the validity and strength of an association between gut microbiota and AD by investigating the impact of probiotic or prebiotic interventions on both AD prevention and gut microbiota development?

Many probiotic intervention trials were conducted with probiotics, some with impressive results, particularly in Finland where high allergy rates are observed. In one trial *Lactobacillus rhamnosus* strain GG was given orally to pregnant Finnish mothers (10^{10} cfu (colony forming unit)/ day for 2-4 weeks before delivery) and their children (10^{10} cfu/ day for 6 months) and AD was assessed at 2 years of age in their children who all had a history of parental allergic disease (40% parents with AD). In this high risk population 23 % in the probiotic arm and 46 % in the placebo arm developed AD ($P=0.008$). Notably, no difference ($P=0.2$) was seen for immunological parameters of sensitization (IgE, RAST, prick test) between both groups (Kalliomäki et al., 2001b). This substantial difference in AD prevalence was maintained until 4 years of life (relative risk $RR=0.57$) (Kalliomäki et al., 2003). Combining *L. rhamnosus* LPR with *Bifidobacterium longum* BL999 or *Lactobacillus paracasei* ST11 with BL999 (10^9 cfu/day, 2 months before and after delivery, only to mothers) led to similar impressive reduction in allergy development in Finnish children ($OR=0.17$, $P<0.001$ for both preparations compared to placebo) (Rautava et al., 2012). Unfortunately, these groups did not study the gut microbiota, however, others did.

In Sweden children were enrolled into a placebo-controlled allergy prevention trial with the probiotic *Lactobacillus reuteri* strain ATCC 55730. The probiotic was given at a dose of 10^8 cfu per day during the last month of pregnancy and then 12 months with the same daily dose to the children. The *L. reuteri* group had less IgE-associated eczema during the second year (8 v. 20%, $P=0.02$) and less SPT reactivity (14 vs. 31%, $P=0.02$) (Abrahamsson et al., 2007). Stool samples were taken at 1 week, 1 and

185 12 months and evaluated by barcoded 16S rDNA 454-pyrosequencing. There was no significant
186 difference between children with AD and controls for any of the dominant bacterial genera; only in
187 the 1-month stool samples AD children showed a lower Shannon diversity index for total microbiota
188 when compared to controls (Abrahamsson et al., 2011). A Finnish placebo-controlled intervention
189 trial with *L. rhamnosus* GG (10^{10} cfu/ day for 1 month before delivery and 6 months after delivery to
190 the breastfeeding mother or the bottle-fed child) showed that 15 subjects who developed AD
191 differed from 19 healthy controls by showing a higher diversity index for the gut microbiota as
192 determined by microarray analysis ($P=0.03$). Bacteroidetes were more abundant in healthy children
193 ($P=0.01$), while *Clostridium* clusters IV and XIVa were found with higher prevalence in AD patients
194 ($P=0.03$). Yet these bacterial groups represented less than 5 per cent of the total bacteria (Nylund et
195 al., 2013). However, the low abundant bacterial species often imply important consequences.

196 Australian newborns from a high risk group received daily 3×10^9 cfu *Lactobacillus acidophilus* LAVRI-
197 A1 or placebo for 6 months and AD was assessed at 6 and 12 month of age. No difference in AD
198 development was seen (26 vs. 23 %) ($P=0.63$). Probiotic-treated infants showed an increased fecal
199 *Lactobacillus* colonization ($P=0.04$) and no change for fecal bifidobacteria (Taylor et al., 2007).
200 *Bifidobacterium* supplementation in pregnant Japanese women and their infants (5×10^9 cfu each of
201 *B. longum* BB536 and *B. breve* M-16V, twice daily, one month before delivery; and once daily to their
202 infants for 6 months) showed a significant reduction in AD of their children at 10 months of age
203 ($P=0.007$), but no effect on asthma and allergic rhinitis ($P>0.1$). At 4 months ($P=0.05$), but not 10
204 months of age ($P=0.48$) children with eczema showed a significantly lower number of fecal
205 Actinobacteria (Enomoto et al., 2014).

206 In a prebiotic trial, Italian children born to a parent with a history of AD were randomized to a
207 mixture of bifidogenic oligosaccharides (galacto-oligosaccharides GOS/fructo-oligosaccharides FOS,
208 0.8 g / 100mL reconstituted formula, given ad libitum over 6 months) or placebo (0.8 g maltodextrin
209 /100 mL). At 6 months of age, fewer children showed AD in the treatment than in the control group
210 (10 vs. 23 %) ($P=0.014$). Prebiotic-treated children showed a 10-fold increase in fecal bifidobacteria
211 ($P<0.0001$) over controls, but no effect on lactobacilli (Moro et al., 2006). A blind follow-up of the
212 children after intervention until 2 years of life demonstrated a protective effect of early prebiotic
213 feeding against allergic manifestations (AD, wheezing, urticarial, $P<0.05$) and respiratory infections
214 ($P<0.01$) (Arslanoglu et al. 2008). A Finnish study used a synbiotic approach by combining a probiotic
215 mixture (containing 10^{10} cfu *L. rhamnosus* GG and LC705, 2×10^8 cfu *B. breve* Bb99 and 2×10^9 cfu
216 *Propionibacterium freudenreichii* JS) with a prebiotic (0.8 g GOS) daily for 6 months after birth.
217 Treated children showed a significant reduction of both eczema ($P=0.015$) and atopic eczema
218 ($P=0.012$) compared to the placebo group, but the difference was small (26 vs. 32 % and 12 vs. 18 %, 7

respectively). The probiotic bacteria showed large increases at the end of the intervention period (P=0.001), which did not persist to the time when the allergy assessment was done (Kukkonen et al., 2007).

As also these data from intervention trials do not provide a clear gut microbiota-AD association, researchers were turning their coat, and this literally.

Outside-to-Inside mechanism of AD: The dominance of Staphylococcus aureus on the skin of AD patients

The postulated gut microbiota changes were assumed to reflect downstream consequences of a primary immunological abnormality which became known as the inside-to-outside view of AD pathogenesis. Other researchers have proposed that the permeability barrier abnormality in AD is not merely an epiphenomenon but rather a driver of disease activity (Elias et al., 2008). This notion became known as outside-inside view of AD (Elias and Steinhoff, 2008). In fact, since both immunological and skin disturbances are key to AD as suggested by genetic association studies, it makes sense to focus on skin microbiota. Clinicians knew for long time that AD patients are more susceptible to certain infections specifically severe bacterial infections with *Staphylococcus aureus* and disseminated viral infections with Herpes simplex and Poxviruses (Ring 2012). It was also known for a long time that *S. aureus* had a high prevalence on the skin of AD patients (Leyden et al., 1974). Early reports showed that 85 to 93 % of the lesion sites in AD patients are colonized by *S. aureus* with titers exceeding 10^4 bacteria per cm^2 of skin (Aly et al., 1977; Bibel et al., 1977). In these lesions 90 % of skin bacteria were *S. aureus*. Peak titers in lesion areas from AD patients yielded up to 10^7 *S. aureus* per cm^2 ; in these cases practically 100 % of the skin bacteria were *S. aureus*. In adjacent non-lesion areas of AD patients, *S. aureus* was found in 55 % of the sites with titers of about 10^4 *S. aureus* per cm^2 . Normal skin from controls was in contrast colonized by coagulase-negative staphylococci (mainly *Staphylococcus epidermidis*), followed by *S. aureus*, micrococci, and lipophilic diphtheroids. Overall, healthy skin carried fewer, but a more diversified skin flora. A case-control study confirmed that *S. aureus* was enriched on the skin of AD patients and the authors concluded that *S. aureus* found favorable growth conditions on the altered horny layer of AD patients (Gloor et al., 1982). Similar data were reported from Japan: 86 % of AD patients compared to 25 % of controls yielded *S. aureus*, while *S. epidermidis* showed an inverse ratio with 38 to 83 %, respectively. *S. aureus* counts correlated with disease severity, while a reverse tendency was seen for *S. epidermidis* (Higaki et al., 1999). Also AD patients from Singapore showed clear evidence for *S. aureus* skin colonization: 70 %

of AD patients harbored *S. aureus* on eczematous lesions (100 % in more severely affected patients). This percentage was 42 % on non-eczematous skin of AD patients, whereas only 5 % of healthy controls showed *S. aureus* on the skin ($P=0.003$) (Goh et al. 1997). A case-control study from Germany concurred with these observations thus confirming a general trend: in AD patients *S. aureus* was found on the affected skin of 80 % and 52 % of non-atopic eczema patients harbored *S. aureus* on their lesions. *S. aureus* was only found on the skin from 3 % of healthy controls. Non-affected skin from 63 % of AD and 24 % of eczema patients showed *S. aureus* (Masenga et al., 1990). A Chinese study came up with similar data except that *S. aureus* skin colonization was similarly high in non-atopic eczema and atopic eczema patients. For both eczema groups, skin *S. aureus* titers correlated with lesion severity ($P=0.01$) (Gong et al., 2006). Another case-control study also reported high rates of *S. aureus* colonization on both involved and normal skin of AD patients; here the *S. aureus* counts increased linearly with the severity of the skin disease. Skin-associated diphtheroid bacteria showed the opposite trend: they were high on healthy skin and low on AD skin (Williams et al., 1990). One should mention that all these results were obtained by culture-based techniques.

Molecular skin microbiome analysis confirms the association

The skin microbiome analysis of AD patients confirmed this very consistent picture obtained with cultivation techniques. During an untreated flare of AD, the skin microbiota in lesion areas is represented to 90 % by staphylococci (*S. aureus* was dominant followed by *S. epidermidis*), while *S. aureus* was rare and *S. epidermidis* a minor species on control skin ($P=10^{-4}$) (Kong et al., 2012). These researchers followed the skin microbiota in lesional areas of AD patients during a cycle of AD eruption covering the baseline state which still largely resembled the healthy control skin (although with a larger *S. aureus* contribution) over an untreated and a treated flare and a post-flare period. The latter two conditions again resembled the baseline state of AD skin microbiota. The increase in the proportion of *S. aureus* on the skin and the decrease in microbial diversity did not only correlate with the clinical score ($P=10^{-4}$), but actually preceded the worsening of AD disease severity. The association of *S. aureus* with AD was confirmed by the same authors in an interesting follow-up study where the skin microbiome was investigated in patients with genetically defined primary immunodeficiencies (PID) where AD patients served as positive controls (Oh et al. 2013). The PID patients showed eczema that superficially resembled AD eruptions both in appearance and predilection for the popliteal region. However, in contrast to AD patients, *Serratia marcescens* and *Staphylococcus epidermidis* and *S. haemolyticus* dominated the skin of PID patients suggesting a specific association of *S. aureus* with the skin of AD patients.

284

285 ***S. aureus* as driver of AD skin pathology: intervention trials**

286 However, an association does not yet establish whether *S. aureus* colonization is a consequence of
287 the altered skin physiology or histology in AD patients or actually a potential driver of AD
288 pathogenesis. Again, intervention studies might shed some light on this question. If *S. aureus*
289 colonization is a cause for the disease, its reduction or elimination should lead to an amelioration of
290 the clinical symptoms. If it is a consequence, reduction of *S. aureus* will not necessarily be followed
291 by an amelioration of the clinical symptoms.

292 A number of intervention modes were tested: the antiseptic dye gentian violet reduced significantly
293 the *S. aureus* density in lesional and unaffected skin from AD patients ($P < 0.001$) and reduced
294 subsequently also the disease severity score (Brockow et al., 1999). A 3-week course of hydrotherapy
295 ameliorated at the same time clinical scores ($P = 10^{-4}$) and *S. aureus* colonization of lesional sites as
296 determined by 16S rRNA profiling ($P < 0.05$) (Bourrain et al., 2013). A 3-month emollient treatment in
297 AD patients decreased the abundance of *Staphylococcus* on the skin as assessed by 16S rRNA gene
298 sequencing and in parallel also improved the clinical symptoms in 72 % of the patients. Interestingly,
299 responders also showed an increase in a skin commensal, *Stenotrophomonas maltophilia* (Seite et al.,
300 2014). However, researchers differed with respect to the identification of candidate “protective” skin
301 commensals. When comparing children from Finnish and Russian Karelia, who differ dramatically in
302 AD incidence, the skin commensal *Acinetobacter* was associated with protection (Haahtela et al.,
303 2015).

304 A placebo-controlled intervention with the antibiotic mupirocin in AD patients, who were heavily
305 colonized with *S. aureus*, resulted not only in a reduced colonization but also in a reduction of clinical
306 severity in the treated patients (Lever et al., 1988). Recolonization with *S. aureus* occurred rapidly,
307 but was not accompanied by a rapid clinical deterioration of the skin suggesting that *S. aureus* might
308 be a necessary, but not sufficient condition for AD recurrence.

309 Children were also treated with a bleach bath (0.005% sodium hypochlorite, twice weekly) which
310 resulted in a significant decrease in severity score for the submerged lesional areas ($P = 0.03$ after 1,
311 $P = 0.005$ after 3 months of treatment) (Huang et al., 2008). Two follow-up studies confirmed the
312 efficacy of bleach both with respect to AD skin scores and a reduction of *S. aureus* skin load
313 ($P < 0.01$) (Ryan et al., 2013; Wong et al. 2013). A number of intervention trials compared the addition
314 of an anti-microbial agent (topical antibiotic mupirocin and fusidic acid, $P < 0.0001$ Ravenscroft et al.,
315 2003; $P = 0.05$ Gong et al., 2006) or an anti-septic agent (chlorhexidine or potassium permanganate

316 P<0.05; Stalder et al., 1992) to an anti-inflammatory treatment with a corticosteroid. In all three
317 studies a clinical amelioration was observed in parallel with a reduction in *S. aureus* colonization.
318 Exclusive corticosteroid treatment also resulted in dramatic *S. aureus* reduction and AD symptom
319 amelioration (Gong et al., 2006) confirming observations in earlier trials (Stalder et al. 1994).
320 Corticosteroids are one of the most widely prescribed medicines against AD; they have a strong anti-
321 inflammatory effect which decreases the concentration of antimicrobial peptides in the skin induced
322 by inflammation providing a possible explanation for their efficacy against AD. Another intervention
323 trial combining topical corticosteroid with oral anti-histamine treatment during a disease flare in AD
324 patients differentiated two AD patient subgroups by clinical observation. All patients were initially
325 heavily colonized with *S. aureus* on the skin lesion; 70 per cent of patients showed a decrease from
326 10^7 *S. aureus* to <10 *S. aureus* / cm^2 ($P<0.01$). These patients showed a gradual and statistically
327 significant decrease in AD symptoms (SCORAD index) with treatment ($P<0.05$). Thirty per cent of
328 patients who were characterized by an initially much higher *S. aureus* lesion density of $>10^9$ / cm^2 did
329 not show a decrease in *S. aureus* colonization density by this treatment and also failed to
330 demonstrate a significant decrease in disease score with this treatment (Guzik et al., 2005).
331 Apparently, two different associations of *S. aureus* are found in AD patients: one that can be
332 interrupted by a combined topical corticosteroid/ anti-histamine treatment and one that resists this
333 intervention. In summary, whatever treatment considered, a close correlation between *S. aureus* skin
334 colonization and AD clinical scores was observed suggesting a possible causal relationship.

335

336 ***S. aureus* involvement in AD skin pathogenesis: superantigens and toxins**

337 The concept of *S. aureus* as a driver in AD skin pathology was further supported by the observation
338 that the clinical score in AD patients was not only significantly correlated with the density of skin
339 colonization by *S. aureus*, but also with colonization by a toxigenic strain suggesting a pathogenic
340 mechanism. The basic observation was already made 20 years ago: the skin of AD patients was
341 colonized to a much higher degree with toxigenic *S. aureus* strains than that of healthy controls (37
342 vs. 5 %) (Hoeger et al., 1992). Another study confirmed this conclusion reporting toxigenic *S. aureus*
343 in 57 and 33 percent of AD patients and controls, respectively. In addition, the detection of
344 superantigen (SAg)-producing *S. aureus* was associated with a significant higher SCORAD index and
345 with T-cell activation (Zollner et al., 2000). A third group concurred with that conclusion: the majority
346 of the AD patients were colonized with toxigenic *S. aureus*; many toxins were super-antigens (SEC,
347 SEA, TSST-1, SEB) (Bunikowski et al., 2000). In addition, the skin from AD patients showed a dermal
348 infiltration with superantigen-responsive T-cells. Other researchers showed a correlation between

the detection of *S. aureus* superantigens and severity score and serum IL-4 levels (Nada et al., 2012) or selection for superantigen-producing *S. aureus* isolates in corticosteroid-resistant AD patients (Schlievert et al., 2008). Many groups found a high prevalence of superantigen-encoding *S. aureus* in AD patients (46 to 71%), but not all researchers observed a correlation with disease severity (Arkwright et al., 2000; Lomholt et al., 2005; Mempel et al., 2003). In another study 63 % of *S. aureus* colonized AD patients produced α -toxin which was correlated with a higher total IgE titer (Wichmann et al., 2009). Researchers isolated 36 independent *S. aureus* strains from AD patients; they represented 14 different sequence types and 20 *spa* types demonstrating a great genetic heterogeneity in strains colonizing AD patients. However, despite this overall genetic heterogeneity the survey demonstrated a predominance of strains carrying *sea* and *tsst-1* super-antigens suggesting a selection for specific toxin genes in AD patients (Kim et al., 2009).

***S. aureus* as driver of AD pathogenesis: preclinical studies**

The concept of *S. aureus* as a driver in AD got very strong mechanistic support from mouse experiments. In a landmark study, researchers demonstrated how *S. aureus* isolated from AD patients manipulates the mast cell. This report integrates a number of scattered observations into a consistent and comprehensive picture how *S. aureus* might orchestrate important aspects of AD pathogenesis (Nakamura et al., 2013). The pathogenesis of AD is dominated by skin barrier dysfunction and an abnormal IgE immune response. Upon activation by IgE, bound to mast cells and cross-linked with an allergen, mast cells release their membrane-bound cytosolic granules, which liberate mediators important for the allergy development in AD. Interestingly, also culture supernatants from *S. aureus* induced a rapid, dose-dependent mast cell degranulation (Nakamura et al., 2013). With this bioassay at hand, the researchers isolated the mast cell inducer which turned out to be *S. aureus* δ -toxin, also known as δ -hemolysin or phenol-soluble modulins. Knock-out of the *hld* gene encoding this gene in *S. aureus* prevented mast cell degranulation and complementation of this gene on a plasmid restored the phenotype (Nakamura et al., 2013). *S. aureus* δ -toxin was also active in an *in vivo* test of passive cutaneous anaphylaxis in mice; δ -toxin induced mast cell degranulation through a signaling pathway different from that induced through antigen and IgE. Mice colonized with wild type and δ -toxin deficient mutant *S. aureus* were challenged with ovalbumin. The wild type, but not the mutant bacterium led to the production of higher amounts of IgE, IL-4 and a severe inflamed skin showing the histopathological hallmarks of AD: spongiosis, parakeratosis and neutrophil infiltration (Nakamura et al., 2013).

Mice lacking metalloproteinase 17 (ADAM17) develop a dry skin which progresses to pruritic eczema. Increased trans-epidermal water loss indicated a skin barrier dysfunction, and serum IgE is elevated

as in human AD (Kobayashi et al., 2015). Mutant mice show an emergence of *S. aureus* and *Corynebacterium bovis* within the skin microbiota immediately prior to the onset of eczematous dermatitis. Clinical skin scores, trans-epidermal water loss and IgE increase could be prevented by an antibiotic treatment that reduced *S. aureus* dramatically and *C. bovis* to a lesser extent. Interestingly, in mutant mice that had already developed eczema, a later antibiotic treatment reversed dysbiosis and had therapeutic effects. Conversely, withdrawal of antibiotics led to a marked skin inflammation and dysbiosis in mutant mice, but the development of pathology took some time. Inoculation of these mice with *S. aureus* after antibiotic withdrawal accelerated the return of skin symptoms. Further experiments in these mutant mice identified *S. aureus* as potent inducer of eczematous dermatitis via an immunological pathway involving Langerhans cells (Kobayashi et al., 2015).

Outlook

Based on epidemiological data of skin colonization with *S. aureus* in AD patients, results of clinical intervention studies and observations in a mouse model of AD, a pathogenic model of AD must now integrate genetic aspects of the host leading to skin barrier functions, immune-regulatory deviations, skin colonization by *S. aureus* and the impact of staphylococcal effector molecules. Based on available data one might envision a model where mutations in the corneal layer of the skin open a breach in the skin barrier, which allows *S. aureus* access to deeper layers of the dermis where immune cells are patrolling the skin (Nakatsuji et al., 2013). Seeding skin altered by the filaggrin mutation with *S. aureus* is not a problem since *S. aureus* is already present in many humans on the skin and particularly in the anterior nares (Kuehnert et al. 2006) from where it can be transmitted to the AD child by its caregivers (Chiu et al., 2010) and subsequently maintained in the AD patient by finger contact between nose and skin. Interestingly, self-reported eczema was nearly 3 times more prevalent among nasal carriers of *S. aureus* than in non-carriers (15% vs 6%; $P=0.005$, based on the total sample size of 405, Sakwinska et al., 2009). The bacterial inoculation of the skin is particularly favored by the scratching reaction on the itchy skin. The subsequent expansion of *S. aureus* on the AD skin could be aided by a defect in endogenous antimicrobial peptide synthesis observed in AD patients, but not in patients with other severe skin disease like psoriasis (Ong et al., 2002). This defect could allow the outgrowth of *S. aureus* on the AD skin. However, the defect in antimicrobial peptides in the skin of AD is still under discussion (Harder et al., 2010). *S. aureus* might play with its super-antigens and toxins an active role in manipulation this skin niche to its colonization benefit. As suggested by mouse experiments, *S. aureus* is endowed with genes that could set in motion an allergy reaction by stimulating the degranulation of mast cells and IgE antibody production. To what extent *S. aureus* superantigens have also a role in the development of other forms of allergy (Gould

et al., 2007) and become thus the pacemaker for the “atopic march” (Li 2014) must be addressed by future research.

Overall, it seems that AD is not any longer a disease of altered skin barrier and immune dysfunction (Boguniewicz and Leung, 2011), but also a disease of skin microbiota dysbiosis with *S. aureus*. Leading dermatologists and allergologists have announced a translational revolution for new treatment options with therapies targeting the T_H22, T_H17 and T_H2 immune pathways (Leung and Guttman-Yassky, 2014). To this list one should now add interventions that reduce *S. aureus* colonization on the skin of AD patients. In view of their broad action on the entire skin microbiota, the problem of antibiotic-resistance of *S. aureus* and the lack of efficacy of antibiotics against AD (Bath-Hextall et al., 2010), classical antibiotics should not be considered. Microbiologists are now facing a challenge to design strategies decreasing *S. aureus* skin load in order to take out one potential microbial driver of AD pathology.

Acknowledgements

I thank my colleagues from the Nestle Research Center Lausanne, Nutrition and Health Department, Carine Blanchard (Allergy group) for encouragement and helpful discussions and Olga Sakwinska (Host-Microbe Interaction group) for critical reading of the manuscript and Isabelle Pelisson (Galderma; Sophia Antipolis) for providing clinical pictures from AD patients.

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Figures

Fig. 1 The hygiene hypothesis of atopic diseases

Typical skin manifestation in the knee pit of an atopic dermatitis patient is displayed (A). Over the last century a steady increase of atopic dermatitis was seen in industrialized countries (B, incidence in %, mixed reports from UK) Epidemiologists have seen an inverse trend for incidence of infections and allergy manifestations in developed countries (Bach 2002) (C). Strachan (1989) attributed this trend to a reduced microbial exposure due to reduced family size (D). Exposure to a farm environment (E) protected children against allergy development (Braun-Fahrländer et al., 2002). Typical nasal swabs from a cow (top left) and a farmer (top right) and a city dweller (bottom) on chromogenic agar plates for *Staphylococcus* detection (picture credit: Dr. Valérie Bourdes, Galderma Research&Development, Sophia Antipolis, France and Dr. Olga Sakwinska, Nestlé Research Center, Lausanne, Switzerland).

Fig. 2 Clinical features of Atopic dermatitis

AD patients have frequently dry and scaly skin displaying itchy red areas with raised lesions that weep, crack and crust over. These areas are then at risk for infections with specific bacteria, fungi and viruses. The lesions are typically found in bend skin areas of arms, legs and the neck. The panel shows typical lesional skin areas of AD in the popliteal fossa (kneepit) (A), the antecubital fossa (elbow pit) (B), neck (C) and trunk next to elbow pit (D). Panel A and D show pencil marks made for clinical evaluation. Picture credit: Dr. Valérie Bourdes, Galderma Research&Development, Sophia Antipolis, France.

Fig. 3 Histopathological features of Atopic dermatitis

At the left side the skin of a control subject (A) is compared with the skin of an AD patient (B). In comparison with the healthy control the epidermis of AD patients shows typical changes like acanthosis, a skin thickening due to a benign overgrowth of the stratum spinosum (prickle cells); hyperkeratosis, a hypertrophy of the stratum corneum (horny layer) of the skin; parakeratosis, the persistence of nuclei in the keratinocytes as they rise into the horny layer of the skin; elongation of the rete ridges (epithelial extensions that project into the underlying connective tissue of the skin); spongiosis (abnormal accumulation of fluid in the epidermis, not visible in this skin section) and a prominent inflammatory infiltrate. Panel C and D show the histology from a non-lesional skin area (C)

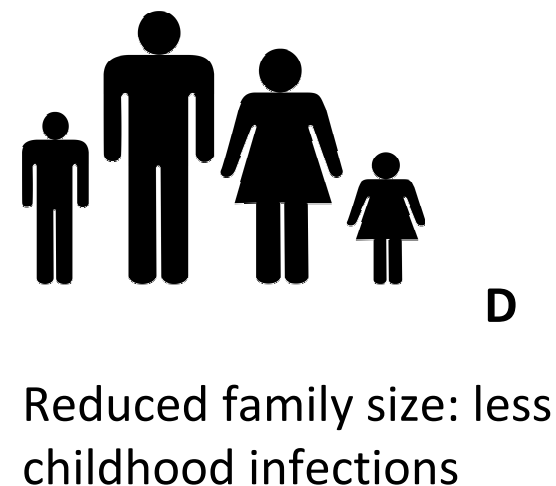
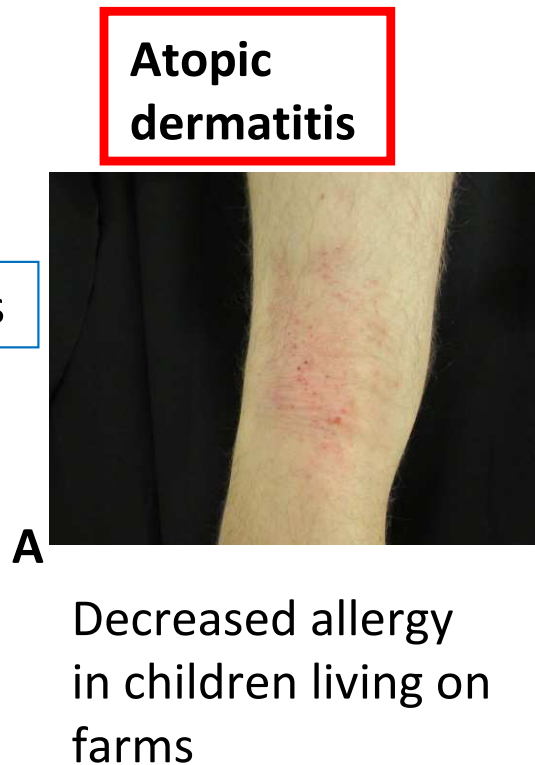
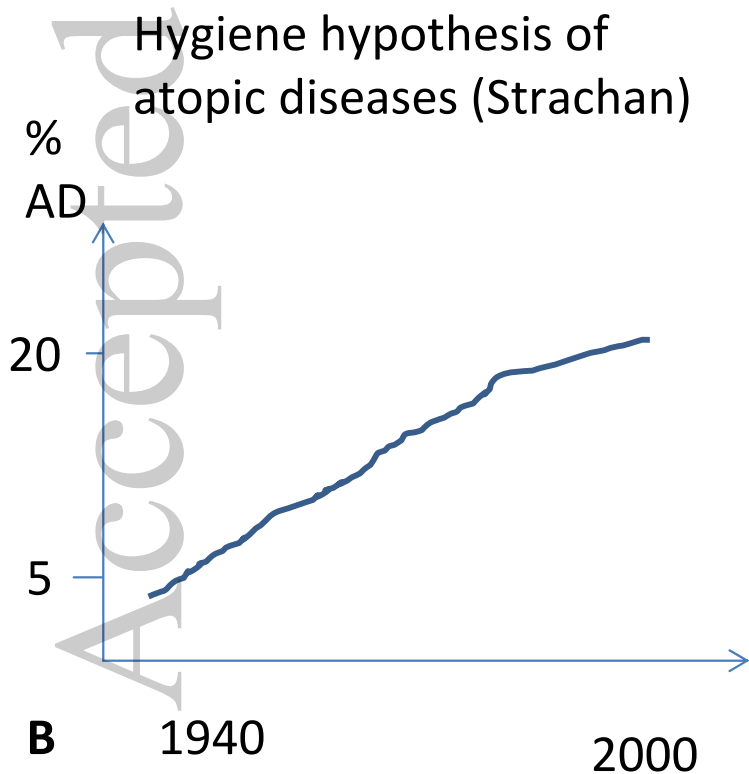
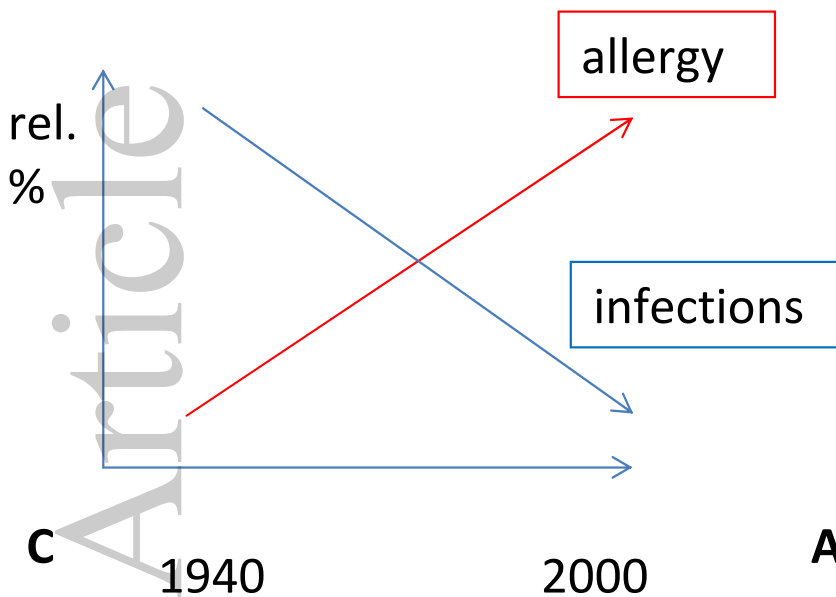
and a lesional area (D) from the same AD patient demonstrating the dynamic character of the disease. Panel A and B and C and D are given at the same magnification. Picture credit: Dr. Béatrice Bertino, Galderma Research & Development, Sophia Antipolis, France.

Fig. 4 Microbiota and atopic dermatitis

The impact of the gut microbiota (left panels) and of the skin microbiota (right panels) on AD has been studied leading to the inside-out and outside-in hypotheses of AD, respectively. Bifidobacteria (top left scanning microscopy) in feces have been linked to AD occurrence by association studies (Björkstén et al., 1999) and intervention trials with bifidogenic prebiotics represented by fructo-oligosides (FOS) and galacto-oligosaccharides (GOS) molecular schemes (fructose: green, galactose: grey, glucose: black) (Moro et al., 2006). The association of *Lactobacillus* (bottom left scanning microscopy, elongated cells mixed with streptococci) was shown by the effects of probiotic intervention trials (Kalliomäki et al., 2001b). The association of *Staphylococcus aureus* with AD was demonstrated by both culture techniques (top right, diversity of staphylococci on a chromogenic *Staphylococcus* agar) and 16S microbiota analyses (top middle). Picture credits: Dr. Valérie Bourdes, Galderma Research&Development, Sophia Antipolis, France, Dr. Norbert Sprenger, Dr. Olga Sakwinska, both Nestlé Research Lausanne; and Servier Medical Arts).

Fig. 5 *Staphylococcus aureus* and atopic dermatitis

A current model for the sequence of events leading to AD is depicted in the flow scheme on the top. Superantigen- and toxin-producing *S. aureus* has been associated with AD by microbiological analyses and by a recent study linking δ -toxin secretion by *S. aureus* with stimulatory effects on both mast cell degranulation and IgE production leading via neutrophil recruiting and skin pathology (scheme depicted at the bottom). The microbiological data are supported by clinical observation showing a parallel decrease in *S. aureus* colonization and clinical skin scores in patients treated with a combination of topical corticosteroid and oral anti-histamine (Guzik et al., 2005, top right) and the beneficial effect of bleach bath on AD symptoms (Huang et al., 2008, middle right panel). (picture credits: Drs. Valérie Bourdes & Dr. Béatrice Bertino, Galderma Research&Development, Sophia Antipolis, France; and Servier Medical Arts).



Higher microbial exposure from farm animals:
«Eat dirt» ?



A



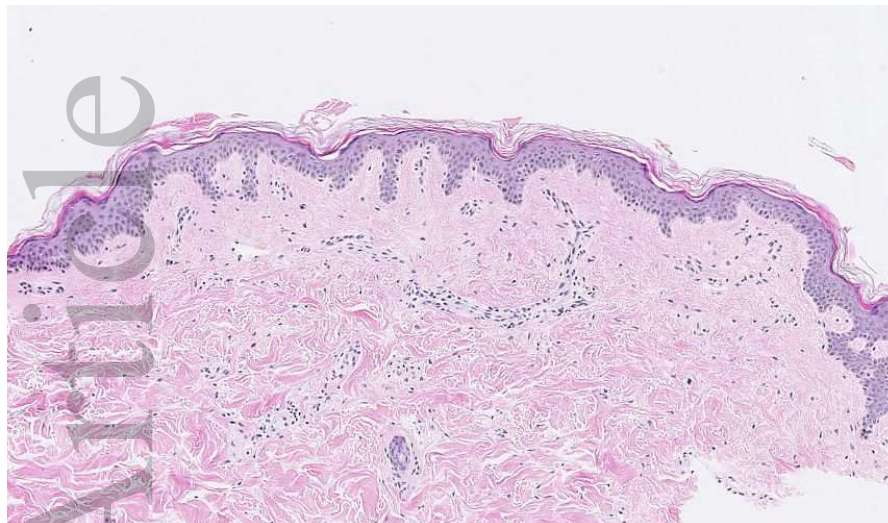
C



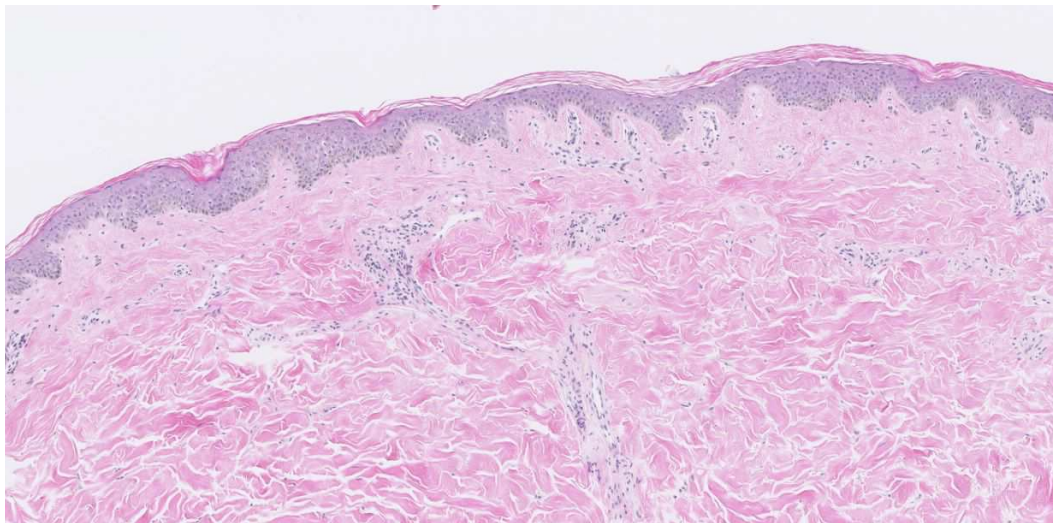
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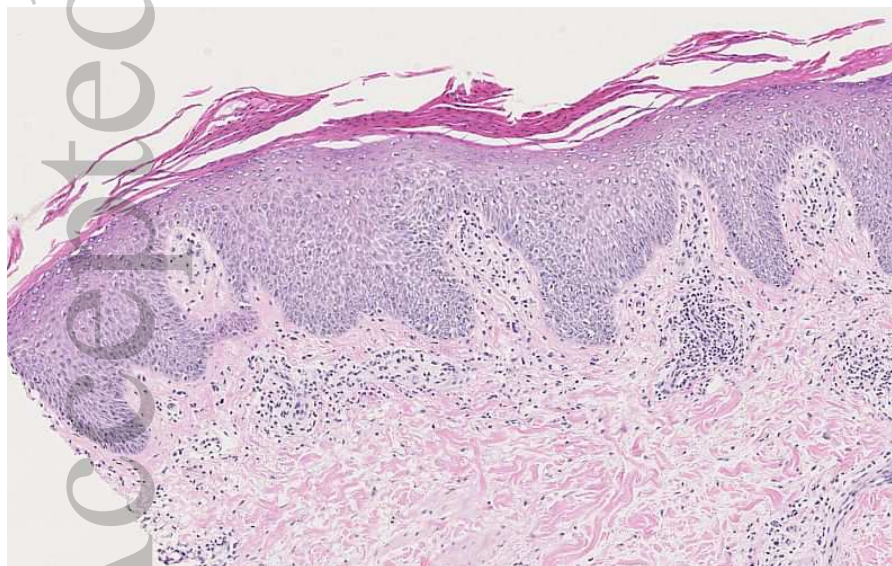
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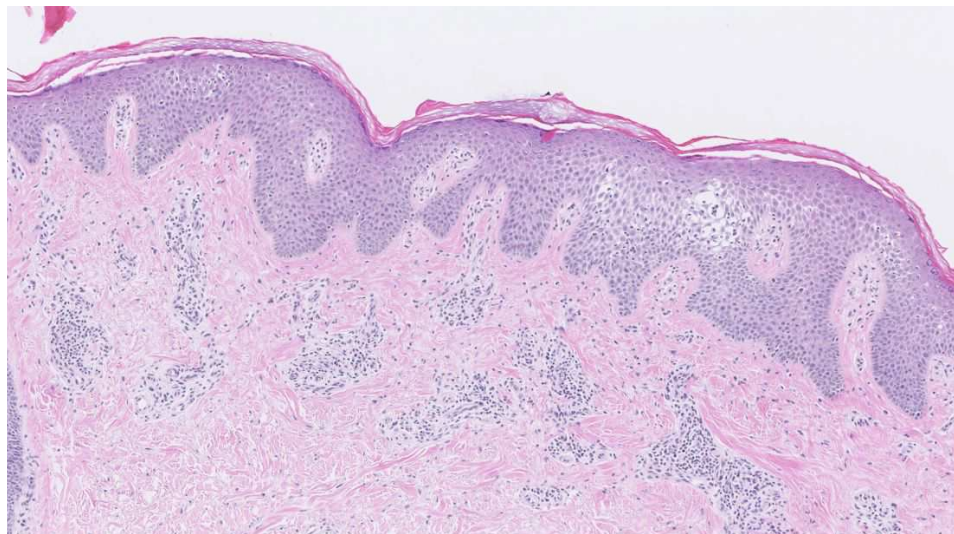
A



C



B



D

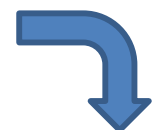
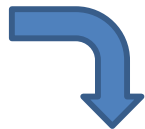
FOS



GOS



Bifido-
genic
prebiotics



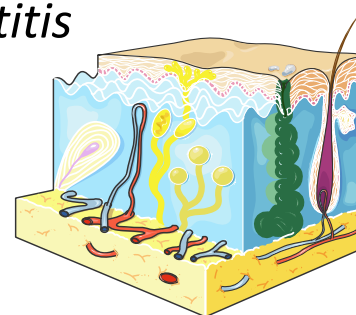
**Bifidobacterium
probiotics**

gut
microbiota ↔
AD
association
studies

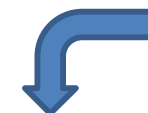
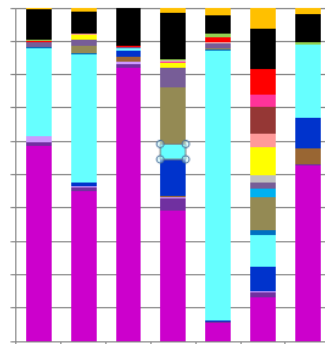


**Lactobacillus
probiotics**

Atopic dermatitis



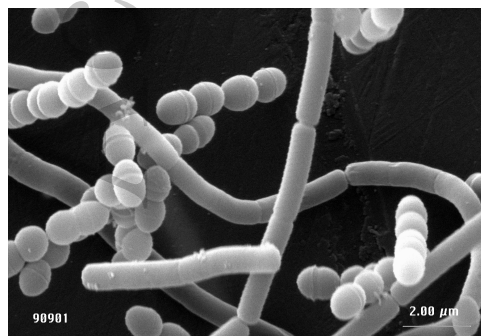
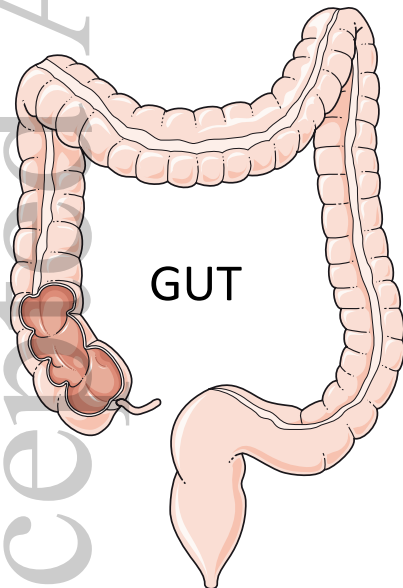
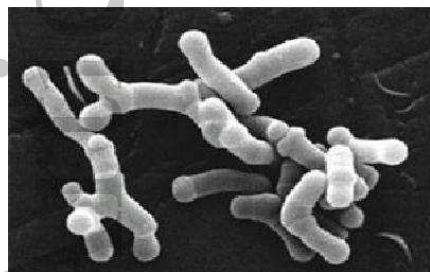
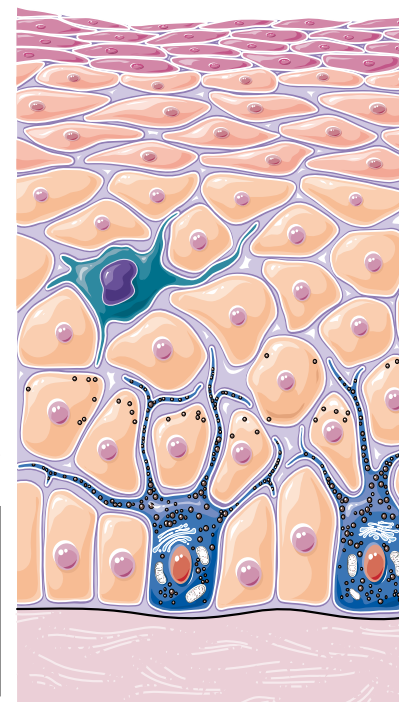
Microbiota
analyses



skin *S. aureus* ↔ AD
association studies



SKIN



Inside-out versus outside-in hypothesis of AD

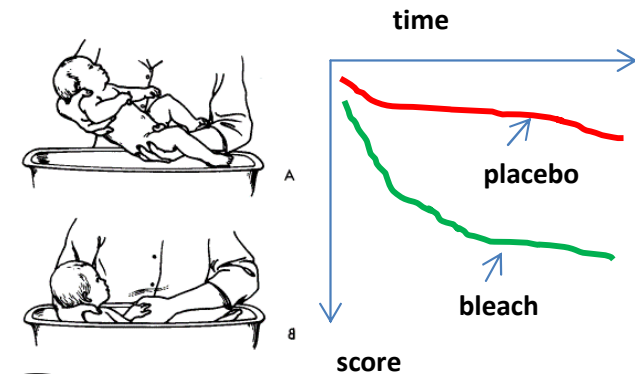
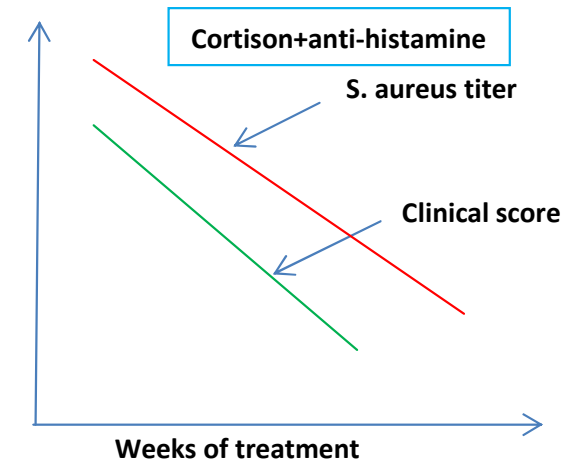
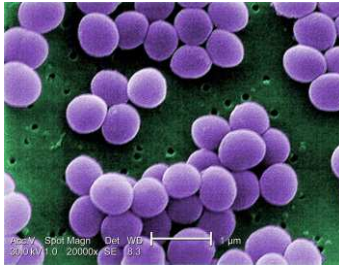
Genetic disposition (filaggrin mutation)

Skin barrier defects

Increased *S. aureus* colonization

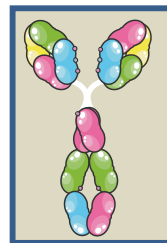
Inflammation & superinfection

With superantigen producing *S. aureus*



...secretes δ -toxin

...stimulates IgE production

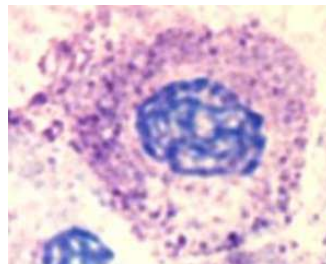


...recruits neutrophils



...leads to AD pathology

...induces mast cell degranulation



KO of δ -toxin gene:
No degranulation

