See related commentary on pg 2460

Skin Colonization by *Staphylococcus aureus* Precedes the Clinical Diagnosis of Atopic Dermatitis in Infancy

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Atopic dermatitis (AD) has a well-established association with skin colonization or infection by *Staphylococcus aureus*, which can exacerbate the disease. However, a causal relationship between specific changes in skin colonization during the first years of life and AD development still remains unclear. In this prospective birth cohort study, we aimed to characterize the association between skin colonization and AD development in 149 white infants with or without a family history of atopy. We assessed infants clinically and collected axillary and antecubital fossa skin swabs for culture-based analysis at birth and at seven time points over the first 2 years of life. We found that at age 3 months, *S. aureus* was more prevalent on the skin of infants who developed AD later on. *S. aureus* prevalence was increased on infants' skin at the time of AD onset and also 2 months before it, when compared with age-matched, unaffected infants. Furthermore, at AD onset, infants testing positive for *S. aureus* were younger than uncolonized subjects. In conclusion, our results suggest that specific changes in early-life skin colonization may actively contribute to clinical AD onset in infancy.

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INTRODUCTION

Atopic dermatitis (AD) is the most common skin disease in children. It occurs in the first year of life in 60% and before 5 years in almost 90% of patients (Akdis et al., 2006; Spergel, 2003). Despite recent advances in the understanding of the pathophysiological mechanisms leading to AD, the onset of this multifactorial disease remains poorly understood. Two main hypotheses have been proposed regarding AD development: (i) a primary immune dysfunction resulting in IgE sensitization and epithelial barrier disturbance and (ii) a primary defect in the epithelial barrier leading to immune dysregulation and inflammation. One important factor that may be related to epithelial barrier disturbance or immune dysfunction is the alteration of skin microbiota, particularly *S. aureus* overexpansion (Kong et al., 2012). *S. aureus* is highly prevalent in AD patients, both on lesional and nonlesional skin (Hoeger, 2004; Totté et al., 2016). It remains to be established, however, whether *S. aureus* colonization merely follows from AD-related skin alterations or plays an active role in AD development (Brüssow, 2016). Few studies have investigated the role of *S. aureus* (Lebon et al., 2009; Skov et al., 2009) or skin microbiota (Kennedy et al., 2017) in infants developing AD, and in all these reports microbial sampling was performed 3 times at most.

In this prospective birth cohort study, we aimed to determine whether differences in skin colonization during the first 2 years of life are associated with clinical AD onset. We also evaluated the impact of key epidemiological factors.

RESULTS

Out of 1,433 identified white pregnant women, 605 agreed to participate in the study. Newborns were enrolled between July 2010 and November 2012. Of them, 416 did not meet the inclusion criteria, and 40 were lost to follow-up. Of 149 included children, 36 developed AD at a mean age of 9.4 months (range = 1–24 months), with a mean SCORAD (i.e., SCORing Atopic Dermatitis) score of 20.1 (range = 8.0–35.5). In 20 (56%) of those 36 infants, AD started before age 6 months. Cumulative AD incidence during the first 2 years of life was 2.6 times as high (31% vs. 12%) in the high-risk as in the low-risk group, with the disease tending to begin more frequently in winter than summer (36% vs. 14%) (Table 1). AD risk was around 1.7 times as high in boys as in girls ($P = 0.089$), and we found no association between season of birth and AD risk (Table 2).

Infants' exposure to systemic antibiotics and emollients

Prevalence of systemic antibiotic intake during pregnancy and infants' first month of life was 27% and 7%, respectively,

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Abbreviations: AD, atopic dermatitis; CoNS, coagulase-negative staphylococci

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Table 1. Demographic and clinical characteristics of the study population

Characteristics	Patients, n (%) All (n = 149)	High risk[1] (n = 97)	Low risk (n = 52)	*P*-Value[2]
Sex, n (%)				
Female	71 (48)	47 (46)	24 (48)	0.79
Male	78 (52)	50 (54)	28 (52)	
Gestational age, weeks				
Mean	39.2	39.1	39.2	0.93[3]
SD	1.09	1.04	1.19	
Birth mode				
Vaginal	103 (69)	67 (69)	36 (69)	0.98
Cesarean	46 (31)	30 (31)	16 (31)	
Season of birth, n (%)				
Spring	30 (20)	21 (22)	9 (17)	0.14
Summer	29 (19)	22 (23)	7 (13)	
Fall	56 (38)	30 (31)	26 (50)	
Winter	34 (23)	24 (25)	10 (19)	
AD development, n (%)				
Yes	36 (24)	30 (31)	6 (12)	**0.008**
No	113 (76)	67 (69)	46 (88)	
Season of AD onset, n (%)				
Spring	10 (28)	7 (23)	3 (50)	0.29[4]
Summer	5 (14)	4 (13)	1 (17)	
Fall	8 (22)	7 (23)	1 (17)	
Winter	13 (36)	12 (40)	1 (17)	
Age at AD onset in months, n (%)				
0–6	20 (56)	17 (57)	3 (50)	0.44[3]
7–12	5 (14)	5 (17)	0	
13–18	4 (11)	2 (6.7)	2 (33)	
19–24	7 (19)	6 (20)	1 (17)	

Bold text indicates significance.
Abbreviations: AD, atopic dermatitis; SD, standard deviation.
[1]At least one first-degree relative with atopy.
[2]High- versus low-risk comparison, using Pearson chi-square test, except as noted.
[3]Wilcoxon-Mann-Whitney test.
[4]Pearson chi-square test on all AD patients, with the null hypothesis that all seasons of onset are equally likely.

without any significant association with clinical outcome (see Supplementary Table S1 online). Systemic antibiotic therapy in the first year of life was more common in the 11 infants developing AD after 1 year compared with healthy control subjects, but the difference was not significant.

Up to age 6 months, 75% of all children had emollient application more than once weekly and 50% at least once daily; application frequency decreased thereafter (see Supplementary Figure S1 online). At age 3 months, frequency of emollient application was significantly higher in subjects who subsequently developed AD.

Site-related differences in skin colonization can be observed within 24 hours after birth

Over the whole study, 1,138 axillary (mean per subject = 7.6) and 1,083 antecubital fossa (mean = 7.3) bacterial swabs were collected. On average, only 3.7% (range across visits = 0.68–7.4%) of axillary and 4.7% (range = 0.68–9.3%) of antecubital fossa swabs were missing. Among the 71 bacterial species identified, 16 were retained for analysis based on their overall higher prevalence over the whole study period. When comparing skin sites on the first day of life, *Escherichia coli*, *Staphylococcus aureus,* and *Staphylococcus capitis* were significantly more prevalent in axillary than in antecubital fossa, whereas coagulase-negative staphylococci unidentified at the species level were detected only in the latter (Figure 1a). These site-related differences in skin colonization at birth were no longer apparent by age 1 week for *S. aureus* and *S. capitis* (see Supplementary Figure S2 online). By contrast, the prevalence of *E. coli* became similar in axillary and antecubital skin only at age 6 months. Coagulase-negative staphylococci remained lowly prevalent in the antecubital fossa until age 2 years.

We found no evidence of seasonal variations in skin colonization in the first 24 hours of life, with two exceptions: *S. hominis* axillary prevalence was lower in winter-born neonates ($P = 0.064$, Pearson chi-square test) (Figure 1c), and *E. coli* antecubital prevalence was 5 times higher in summer-born neonates (see Supplementary Figure S3b).

Birth mode affects skin colonization but not risk of subsequent AD development

When comparing birth mode, *Staphylococcus epidermidis*, *Staphylococcus hominis,* and *E. coli* prevalence on axillary skin at birth was significantly lower in cesarean- than vaginally born infants (Figure 1b). *Staphylococcus lugdunensis* was detected in 7% of cesarean- but not in vaginally born children. Birth mode-related differences in skin colonization at birth were no longer apparent by age 1 week except for *E. coli*, whose prevalence became similar in all infants at age 3 months (see Supplementary Figure S4 online). The antecubital data showed a generally similar picture, with differences owing to birth mode unnoticeable by age 3 months (see Supplementary Figures S3a and S5 online).

Neither birth mode (Table 2) nor the bacteria strongly influenced by it (Figure 1d, and see Supplementary Figure S3c) were significantly associated with AD onset later in infancy. Nonetheless, lactobacilli were 1.8 times as prevalent in the vaginal swabs from mothers of AD as in those of non-AD infants ($P = 0.13$) (see Supplementary Table S2 online).

Skin colonization by *S. aureus* in infancy is positively associated with subsequent AD development

Among all 149 infants, most bacteria displayed zero or low prevalence on both axillary and antecubital skin at most follow-up visits, with modest deviations between the two sites at one or two time points (see Supplementary Figure S2). By contrast, the prevalences of *E. coli*, *S. epidermidis,* and *S. hominis* displayed substantially different patterns of evolution in early life, with significant differences between the two skin locations.

We evaluated the association between skin colonization by each of the 16 selected bacteria and AD development using logistic and Cox regressions as well as cross-sectional comparisons of prevalences. The logistic models showed that positive test results for *S. aureus* in axillary skin at least once during the follow-up was associated with increased AD risk (Table 3). This result was corroborated by the Cox models, which yielded

Table 2. Association of clinicodemographic variables with AD development

Characteristics	Patients, n (%) AD (n = 36)	Non-AD (n = 113)	Bivariate OR (95% CI)	*P*-Value[1]	Multivariate[2] OR (95% CI)	*P*-Value[1]
Family history of atopy						
Yes	30 (83)	67 (59)	3.4 (1.4–9.7)	**0.006**	3.6 (1.4–10)	**0.005**
No	6 (17)	46 (41)	Reference	—	Reference	—
Sex						
Female	13 (36)	58 (51)	0.54 (0.24–1.2)	0.11	0.51 (0.22–1.1)	0.089
Male	23 (64)	55 (49)	Reference	—	Reference	—
Mode of birth						
Vaginal	27 (75)	76 (67)	1.5 (0.64–3.6)	0.37	—	—
Cesarean	9 (25)	37 (33)	Reference	—	—	—
Season of birth						
Spring	6 (17)	24 (21)	0.74[3] (0.26–1.9)	0.54	—	—
Summer	9 (25)	20 (18)	1.6[3] (0.61–3.7)	0.35	—	—
Fall	15 (42)	41 (36)	1.3[3] (0.58–2.7)	0.56	—	—
Winter	6 (17)	28 (25)	0.61[3] (0.21–1.5)	0.30	—	—
Sterile axilla swab within 24 hours after birth						
Yes	12/35 (34)	32/107 (30)	1.2 (0.53–2.7)	0.63	—	—
No	23/35 (66)	75/107 (70)	Reference	—	—	—

Bold text indicates significance.
Abbreviations: CI, confidence interval; OR, odds ratio.
[1]Likelihood ratio test.
[2]The model includes family history of atopy and sex as independent variables.
[3]Each season compared with all others.

a hazard rate of AD almost 5 times higher when testing positive for *S. aureus* (Table 3), and by the prevalence peak of *S. aureus* observed at 3 months, which was significantly higher in infants who subsequently developed AD (20% vs. 5.7%; $P = 0.035$, Fisher exact test) (Figure 2a). The odds of developing AD when colonized by *S. aureus* at age 3 months were increased in the low- and high-risk groups to a similar extent ($P = 0.79$, Breslow-Day test).

Skin colonization by *S. hominis* in infancy tends to be negatively associated with subsequent AD development

Contrary to *S. aureus*, *S. hominis* prevalence at age 3 months tended to be lower in infants who developed AD (16% vs. 34%; $P = 0.079$, Pearson chi-square test), a difference strongly dependent on atopy predisposition (Figure 2b). Although the negative association of *S. hominis* with AD was confirmed by logistic regression, the time-to-event analysis showed only modest risk reduction (Table 3); this discrepancy may be due to odds ratio underestimation from the regression based on testing positive at least once because, by design, healthy control subjects were followed up longer than AD infants. *Enterococcus faecalis* prevalence tended to be greater ($P = 0.16$) at age 3 months in high-risk AD infants (Figure 2c), but a link between *E. faecalis* colonization and AD was not supported by our regression analyses. *S. epidermidis* colonization was overall very frequent (Figure 2d) and was not associated with AD development.

Our axillary and antecubital data were overall in good agreement for *E. faecalis*, *S. hominis*, and *S. epidermidis*, but less so for *S. aureus* (see Supplementary Figure S6 and Supplementary Table S3 online). Although *S. aureus* prevalence at age 3 months was greater on the antecubital skin of infants who subsequently developed AD ($P = 0.061$), the logistic and Cox regressions provided weaker evidence of an association between *S. aureus* and AD (see Supplementary Table S3).

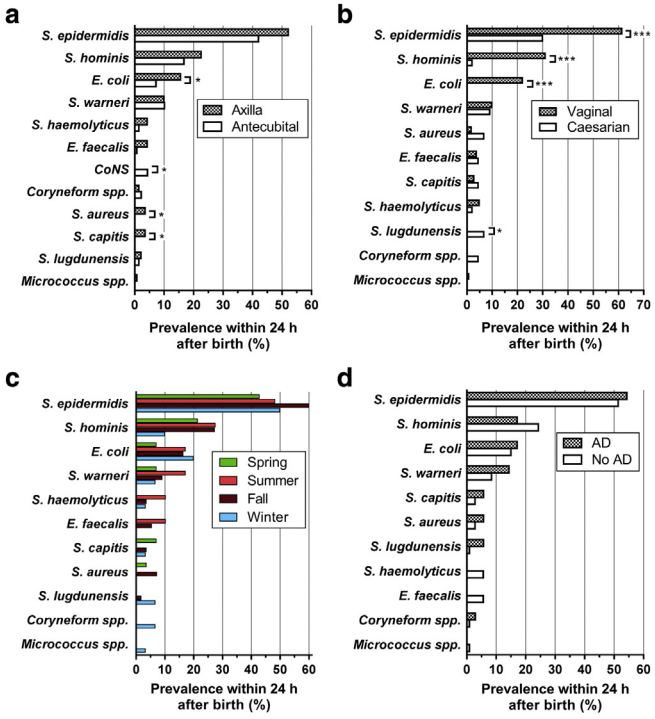

Figure 1. Skin colonization within 24 hours after birth by the most prevalent bacterial species. (**a**) Prevalence according to skin site. Axillary skin prevalence according to (**b**) birth mode, (**c**) season of birth, and (**d**) outcome. $^{*}P < 0.05$, $^{***}P < 0.001$. AD, atopic dermatitis; h, hour; spp, species.

Table 3. Association of AD with axillary fossa skin colonization by various bacteria during the two-year follow-up

Bacterial Species	OR n ≥ 1[1] (95% CI)	*P*-Value	Crude HR[2] (95%)	Adjusted[3] HR (95% CI)	*P*-Value[4]
Staphylococcus hominis	0.25 (0.095–0.63)	**0.003**	0.62 (0.29–1.3)	0.69 (0.32–1.5)	0.35
Staphylococcus aureus	3.0 (1.3–7.3)	**0.013**	4.6 (1.6–14)	**4.5 (1.5–13)**	**0.003**
Staphylococcus warneri	2.1 (0.87–4.8)	0.10	3.7 (0.49–28)	3.1 (0.41–24)	0.25
Escherichia coli	1.8 (0.78–4.0)	0.18	1.8 (0.41–7.6)	1.5 (0.34–6.4)	0.61
Micrococcus luteus	0.38 (0.084–1.8)	0.22	1.2 (0.16–8.6)	1.0 (0.13–7.5)	1.0
Enterococcus faecalis	0.62 (0.20–2.0)	0.41	1.3 (0.17–9.5)	1.3 (0.18–9.9)	0.79
Coryneform spp	0.83 (0.34–2.0)	0.68	0.58 (0.079–4.2)	0.58 (0.079–4.2)	0.59
Staphylococcus capitis	0.82 (0.25–2.6)	0.74	—[5]	—[5]	—
Actinomyces spp	0.77 (0.16–3.8)	0.75	—[5]	—[5]	—
Staphylococcus haemolyticus	0.91 (0.31–2.7)	0.87	1.8 (0.24–13)	1.6 (0.22–12)	0.64
Actinomyces neuii	0.93 (0.34–2.5)	0.89	2.5 (0.72–8.4)	2.0 (0.59–7.1)	0.25
Micrococcus spp	1.1 (0.27–4.1)	0.94	1.5 (0.20–11)	1.7 (0.23–13)	0.60
Staphylococcus lugdunensis	0.99 (0.21–2.7)	0.98	1.7 (0.40–7.1)	1.7 (0.40–7.3)	0.46
Staphylococcus epidermidis	—[6]	—[6]	0.69 (0.24–2.0)	0.68 (0.24–2.0)	0.47

Bold text indicates significance.
Abbreviations: AD, atopic dermatitis; CI, confidence interval; HR, hazard ratio; OR, odds ratio; spp, species.
[1]Odds ratio of developing AD if swab is positive at least once versus never, as calculated with a bivariate logistic model.
[2]Hazard ratio of AD from a bivariate extended Cox regression, with positive axillary swab as a time-varying covariate.
[3]Adjusted for risk group (familial history of atopy).
[4]Log rank test from the stratified Cox model.
[5]No accurate model could be fitted.
[6]All 149 patients tested positive at least once for *S. epidermidis*.

Skin colonization by *S. aureus* is more frequent at AD onset and 2 months before, compared with age-matched, unaffected subjects

To gain further insight into the relationship between skin colonization and AD development, we calculated the prevalences of *S. aureus*, *S. hominis*, *E. faecalis*, and *S. epidermidis* in infants at AD onset and at the two visits before it and then compared these prevalences to those in age-matched healthy children. The median intervals between AD diagnosis and the last and second to last visits before it were 2 and 4.5 months, respectively. Prevalence of *S. aureus* in axillary skin was 5 times as high in AD as in healthy subjects both at disease onset and at the last visit before it and was also noticeably higher at the second to last visit (Figure 3a). Furthermore, at AD onset, infants testing positive for *S. aureus* were younger than uncolonized subjects (Figure 3e). *S. hominis* prevalence was about 1.5 times lower in infants at AD onset and at the prior visits, a difference that was more pronounced when only high-risk AD subjects were considered (Figure 3b). *E. faecalis* tended to be more prevalent in infants developing AD (Figure 3c), whereas *S. epidermidis* test results were positive in almost all subjects regardless of outcome (Figure 3d).

The same analyses performed on antecubital data showed similar patterns, with some variations in the magnitude and chronology of the differences (see Supplementary Figure S7 online).

DISCUSSION

In this study, we observed a distinct increase of *S. aureus* prevalence at age 3 months in infants who later developed AD. In these infants, *S. aureus* prevalence was increased not only at clinical AD onset but also within an average of 2 months before. Furthermore, infants testing positive for *S. aureus* were younger at AD onset than uncolonized ones. Altogether, our findings suggest that *S. aureus* may be an important driver of AD onset in infancy.

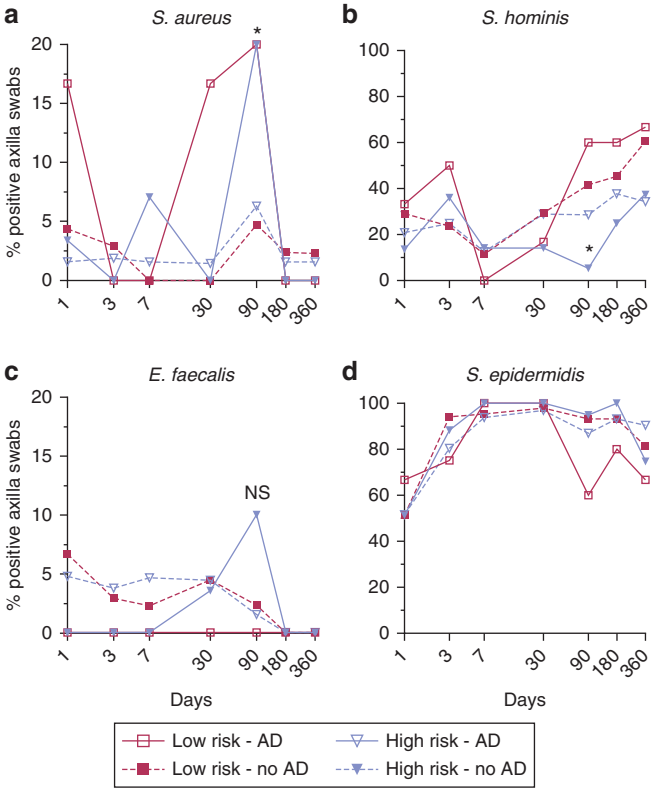

Figure 2. Evolution of axillary skin colonization over the first 2 years of life according to risk and outcome. *$P < 0.05$ (AD vs. no AD for *S. aureus*, high-risk AD vs. high-risk no AD for *S. hominis*). AD, atopic dermatitis; NS, not significant.

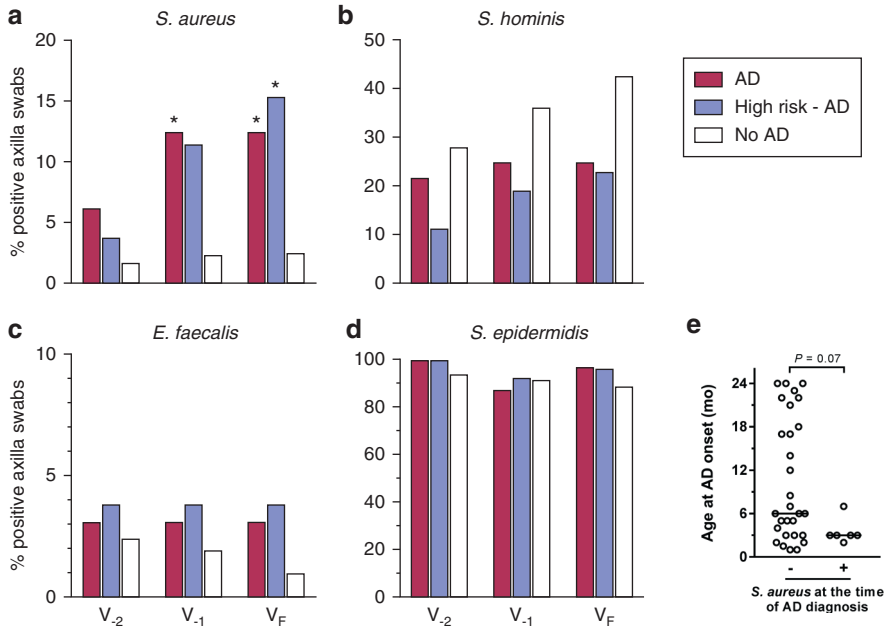

Figure 3. AD development is associated with a higher prevalence of *S. aureus* in axillary skin. (**a**–**d**) Prevalence at time of AD onset and at the two previous follow-up visits. V_F indicates final visit (immediately after AD diagnosis); V_{-1} and V_{-2} indicate the last and second to last scheduled visits before the final one, respectively. $^*P < 0.05$ (AD or high-risk AD vs. no AD). (**e**) Age at AD onset in children testing positive or not for *S. aureus*; lines represent medians. The two groups were compared with a Mann-Whitney test. AD, atopic dermatitis.

Although the association of *S. aureus* with AD flares and severity is firmly established, only a handful of studies have investigated whether changes in early-life skin colonization are linked to subsequent AD development (Gong et al., 2006; Higaki et al., 1999; Hill et al., 2011; Totté et al., 2016). In the large cohort study by Lebon and colleagues (2009), a positive culture result for *S. aureus* from nasal swabs taken at age 6 months was associated with a higher incidence of AD later (Lebon et al., 2009). By contrast, another cohort study of children born of asthmatic mothers found no association between colonization with *S. aureus* and AD incidence (Skov et al., 2009). Kennedy et al. (2017) recently arrived at the same conclusion, using 16S rRNA gene sequencing on skin swabs taken at several sites between ages 2 days and 6 months; they noted a virtual absence of *S. aureus* sequences in their cohort's samples.

Despite these conflicting data, an active role of *S. aureus* in AD initiation is supported by several lines of evidence. At the molecular level, *S. aureus* strains isolated from AD patients have been shown to secrete various exotoxins with key immunomodulatory functions, and severe dermatitis appears to be more frequent in children carrying toxigenic strains of *S. aureus* than in those carrying non-toxigenic strains (Bunikowski et al., 2000). Staphylococcal toxins can act as superantigens with potent immunostimulatory properties, induce specific IgE synthesis (Breuer et al., 2000; Bunikowski et al., 1999; Leung et al., 1993), and even directly impair the skin barrier (Gutowska-Owsiak et al., 2011; Niebuhr et al., 2010). Experiments on murine models have also provided strong support to a causal relationship between *S. aureus* and AD. For example, Kobayashi et al. (2015) have shown that antibiotics given to mice with pre-established eczematous dermatitis strongly reduce inflammation and that *S. aureus* inoculation can accelerate the development of dermatitis (Kobayashi et al., 2015). In filaggrin mutant mice, dermal penetration of *S. aureus* has been shown to correlate with increased expression of various cytokines classically associated with AD (Nakatsuji et al., 2016). Additionally, several intervention trials aimed at reducing *S. aureus* colonization have shown a beneficial effect on AD symptoms (Huang et al., 2009; Ryan et al., 2013; Wong et al., 2013), even though the clinical usefulness of antistaphylococcal interventions in noninfected AD remains controversial (Bath-Hextall et al., 2010).

Previous research has suggested a relative undifferentiation of bacterial communities across skin sites in the first 3 months of life (Capone et al., 2011; Dominguez-Bello et al., 2010), a notion recently challenged by a study showing site-specific differences on the second day of life (Kennedy et al., 2017). In good agreement with that study, we found site-specific differences in skin colonization as early as within 24 hours after birth. Despite both axillary and antecubital fossae generally considered as "moist", we noted striking differences between the two locations for *S. epidermidis* and *S. hominis*, starting from around age 1 week and persisting or increasing until age 2 years. This suggests that even subtle variations in the skin microenvironment could rapidly lead to measurable differences in the microbial ecosystem. Even though the two sampling sites we chose are not typically affected by AD in infancy, they had the advantage of not being heavily exposed to feces, wipes, or emollients, which could have substantially increased interindividual variability in skin colonization. Furthermore, our findings are in agreement with previous studies, which have shown significant anomalies in the nonlesional skin of AD patients (Pellerin et al., 2013; Seidenari and Giusti, 1995; Suárez-Fariñas et al., 2011). Our data do not rule out the possibility that the higher *S. aureus* prevalence we observed before AD onset was a consequence of subclinical skin alterations occurring months before the diagnostic criteria of AD would be fulfilled, an issue beyond the scope of this work. Further "multi-omics" studies will be required to better understand the complex interplay between the skin and its

microbial ecosystem and to evaluate precisely the impact of subclinical skin alterations on the microbiota.

S. hominis prevalence in infants developing AD was smaller at and before disease onset, pointing to a putative protective role of *S. hominis* against AD. Prevalence curves suggest that the role of this bacterium might vary throughout infancy, which could explain the nonsignificant association of *S. hominis* with AD development in the time-to-event analysis. Further research is warranted to establish whether *S. hominis* plays a protective role in AD pathogenesis, but a recent study has reported a beneficial role of early-life skin colonization by commensal staphylococci on AD incidence (Kennedy et al., 2017). It is reasonable to hypothesize that *S. hominis,* like the ubiquitous skin commensal *S. epidermidis,* may actively contribute to skin homeostasis and protection against infections (Cogen et al., 2010; Gallo and Nakatsuji, 2011; Lai et al., 2009; Wanke et al., 2011).

S. epidermidis colonized most infants by age 1 week and remained highly prevalent in all individuals. At AD onset, all infants testing positive for *S. aureus* also carried *S. epidermidis*. An inhibitory effect of *S. epidermidis* on *S. aureus*, not directly addressed in our study, has often been reported (Iwase et al., 2010; Otto et al., 1999). We cannot exclude that children more heavily colonized with *S. aureus* actually had a lesser burden of *S. epidermidis*. Nonetheless, the exact role that *S. epidermidis* plays in AD pathogenesis remains unclear (Hon et al., 2016; Kong et al., 2012).

The influence of birth mode on skin colonization was evident at birth but became unnoticeable between ages 3 days and 3 months, depending on the particular location and bacterial species. Despite these early-life changes in skin microbiota, we found no evidence of an association between mode of birth and AD development. The influence of cesarean birth on early-life gut (Adlerberth et al., 2007; Biasucci et al., 2010; Grönlund et al., 1999) and skin (Dominguez-Bello et al., 2010) microbiota has been investigated but remains poorly understood, and it may have long-lasting immunological effects (Jakobsson et al., 2014). Many studies have looked at a possible association between cesarean birth and AD, because the incidence of both has been increasing in the past decades. These heterogeneous studies have unsurprisingly yielded conflicting results: whereas an influence of cesarean birth on allergy development has been shown, most studies found no significant association between this birth mode and AD (Bager et al., 2008; McKeever et al., 2002; Negele et al., 2004; Papathoma et al., 2016; Park et al., 2010; Renz-Polster et al., 2005). Some studies, however, observed a higher risk of AD in cesarean-born children, which could be influenced by host genetics (Lee et al., 2014; Yu et al., 2015).

In summary, this prospective study adds robust evidence to the association between cutaneous colonization by *S. aureus* in infancy and subsequent AD development, even though it remains to be determined whether subclinical skin anomalies preceding AD can affect the cutaneous microbiota. Disturbances in the skin microbiota before complete maturation of the immune system could have long-term effects on cutaneous and general health, a question that should be addressed in future studies. Establishing a clear causal relationship between early-life alterations of skin microbiota and AD development could lead to primary prevention strategies in patients at risk.

MATERIALS AND METHODS

This prospective cohort study was approved by the Swiss Ethics Committee and was conducted according to the Declaration of Helsinki principles.

Subjects and study design

Healthy, term (≥37 weeks) newborns were enrolled in the Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland). Nonwhite newborns, those suffering from disorders affecting the epidermal barrier or immune system, and those whose cord blood could not be obtained were excluded. Study subjects were divided into two groups: infants having at least one first-degree relative with atopy (physician-diagnosed AD, allergic rhinoconjunctivitis, and/or allergic asthma) were assigned to the high-risk group and those with no family history of atopy to the low-risk group. Written informed consent was obtained from all parents.

Clinical follow-up and endpoints

From birth and throughout the first 2 years of life, infants were monitored for clinical signs of AD based on Hanifin and Rajka diagnostic criteria, and AD severity was assessed using the SCORAD clinical tool (Kunz et al., 1997). Follow-up examinations were scheduled at 1, 3, and 7 days and at 1, 3, 6, 12, and 24 months, with study endpoint being either the eighth follow-up visit or time point of AD development.

Analysis of skin and vaginal colonization

Bacterial flocked swabs (Copan, Brescia, Italy) were collected at each visit from moist sites (right axillary and antecubital fossae) not heavily exposed to feces, wipes, or emollients (e.g., face); skin sampled before AD diagnosis was not inflamed. Emollients were not applied for 24 hours and systemic antibiotics were not given for 2 weeks before swabbing. Mothers' vaginal swabs were collected from the high-risk group. Skin swabs were gently rolled on a 2-cm^2 surface for 5 seconds, immersed in Amies medium, inoculated onto blood agar, and incubated at 37 °C in a 5% CO_2 atmosphere. Agar plates were read at 24 and 48 hours for the presence of bacterial colonies, and each colony presenting a specific morphotype (more likely to represent different bacterial species) was identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (Croxatto et al., 2012). Morphotype selection depended on size, color, pigmentation, hemolytic activity, consistency, and shape (flat or bulging). Bacteria were considered either absent or present for all statistical analyses.

Statistical analysis

Seasons of birth and onset were defined as spring (March—May), summer (June—August), fall (September—November), and winter (December—February). Potential risk factors for AD were analyzed with logistic models and likelihood ratio tests. A best-fitting multivariate model was built using Akaike information criterion—based stepwise variable selection. Two different approaches were used to evaluate, longitudinally, the link between skin colonization and AD development: (i) logistic regression models of AD development as a function of testing positive at least at one visit versus none and (ii) time-to-event analysis using extended Cox regression, with swab positivity as a time-varying factor. We calculated both crude and risk group-adjusted hazard ratios and tested their significance using log-rank tests. Curves showing the prevalence of selected bacterial

species across time according to outcome and risk group were scrutinized, and post hoc tests were performed where interesting differences were noticed. Differences between AD children and age-matched healthy control subjects in terms of skin colonization at and before AD onset were assessed using Fisher exact test; because the times of AD onset were variable, the control group was synthetically built from a weighted average of the prevalences in healthy control subjects over the whole follow-up. All analyses were conducted with R version 3.3.0 (R Development Core Team, 2008). $P < 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at 10.1016/j.jid.2017.07.834.

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Table S1. Exposure of AD infants and healthy controls to systemic antibiotics

Systemic antibiotic use		Patients, n (%)		
		AD (n=36)	**Non-AD (n=113)**	***P* value**[1]
During pregnancy	Yes	8 (23)	32 (29)	0.66[2]
	No	27 (77)	80 (71)	
	Missing data	1	1	
Before age 1 month	Yes	2 (6)	8 (7)	1.0
	No	33 (94)	103 (93)	
	Missing data	1	2	
Before age 1 year	Yes	7 (64)	41 (38)	0.12
	No	4 (36)	67 (62)	
	Missing data	0	5	
	Censored data[3]	25	0	
Before age 2 years	Yes	2	62 (59)	-
	No	0	43 (41)	
	Missing data	0	8	
	Censored data[3]	34	0	

[1]Fisher's exact test except [2]Pearson's χ^2 test; [3]children who developed AD before the specified time point

Table S2. Association between vaginal microbial species in mothers from the high-risk group who delivered naturally and subsequent AD development by their child

	Patients, %		
Microbial species	**AD (n=16)**	**Non-AD (n=35)**	***P* value**[1]
Lactobacillus spp.	56	31	0.13
S. milleri group	13	2.9	0.23
S. haemolyticus	6.3	0	0.31
E. faecalis	6.3	20	0.41
S. agalactiae	6.3	2.9	0.53
Coryneform spp.	6.3	2.9	0.53
E. coli	0	8.6	0.54
S. epidermidis	13	5.7	0.58
CoNS	0	2.3	1
C. albicans	19	20	1
α-hem.[2] streptococci	0	5.7	1

[1]Fisher's exact test; [2]α-hemolytic

Table S3. Association of AD with antecubital fossa skin colonization by various bacteria during the two-year follow-up

Bacterial species	OR 'n≥1'[1] (95% CI)	*P* value	Crude HR[2] (95%)	Adjusted[3] HR (95% CI)	*P* value[4]
S. hominis	**0.40 (0.17-0.98)**	**0.042**	0.70 (0.35-1.4)	0.72 (0.36-1.4)	0.35
S. aureus	2.8 (0.93-8.1)	0.06	1.3 (0.17-9.2)	1.5 (0.21-11.5)	0.67
Coryneform spp.	0.36 (0.054-1.3)	0.18	-[5]	-[5]	-
M. luteus	1.6 (0.63-3.9)	0.3	1.6 (0.49-5.3)	1.6 (0.48-5.1)	0.46
S. haemolyticus	1.88 (0.47-6.6)	0.34	2.7 (0.37-20)	2.5 (0.33-19)	0.37
S. epidermidis	0.47 (0.075-3.7)	0.42	1.6 (0.73-3.7)	1.5 (0.68-3.5)	0.3
S. warneri	1.4 (0.60-3.1)	0.43	1.3 (0.31-5.3)	1.1 (0.26-4.5)	0.93
E. faecalis	1.6 (0.42-5.6)	0.44	2.1 (0.28-15)	1.5 (0.21-11)	0.68
S. capitis	1.6 (0.33-6.5)	0.51	-[5]	-[5]	-
CoNS	0.71 (0.19-2.1)	0.56	-[5]	-[5]	-
S. lugdunensis	0.77 (0.11-3.3)	0.75	-[5]	-[5]	-
E. coli	1.2 (0.25-4.4)	0.8	-[5]	-[5]	-
Micrococcus spp.	0.98 (0.30-2.7)	0.97	1.5 (0.36-6.3)	1.6 (0.38-6.7)	0.53

[1]odds ratio of developing AD if swab is positive at least once versus never, as calculated with a bivariate logistic model; [2]hazard ratio of AD from a bivariate extended Cox regression, with positive antecubital swab as a time-varying covariate; [3]adjusted for risk group (familial history of atopy); [4]log-rank test from the stratified Cox model; [5]no accurate model could be fitted

Figure S1. Evolution of emollient use in AD children and healthy controls over the first two years of life. **, $P < 0.01$ (Pearson's χ^2 test). Note that only two AD patients were still followed up at age two years, so the bar is not shown.

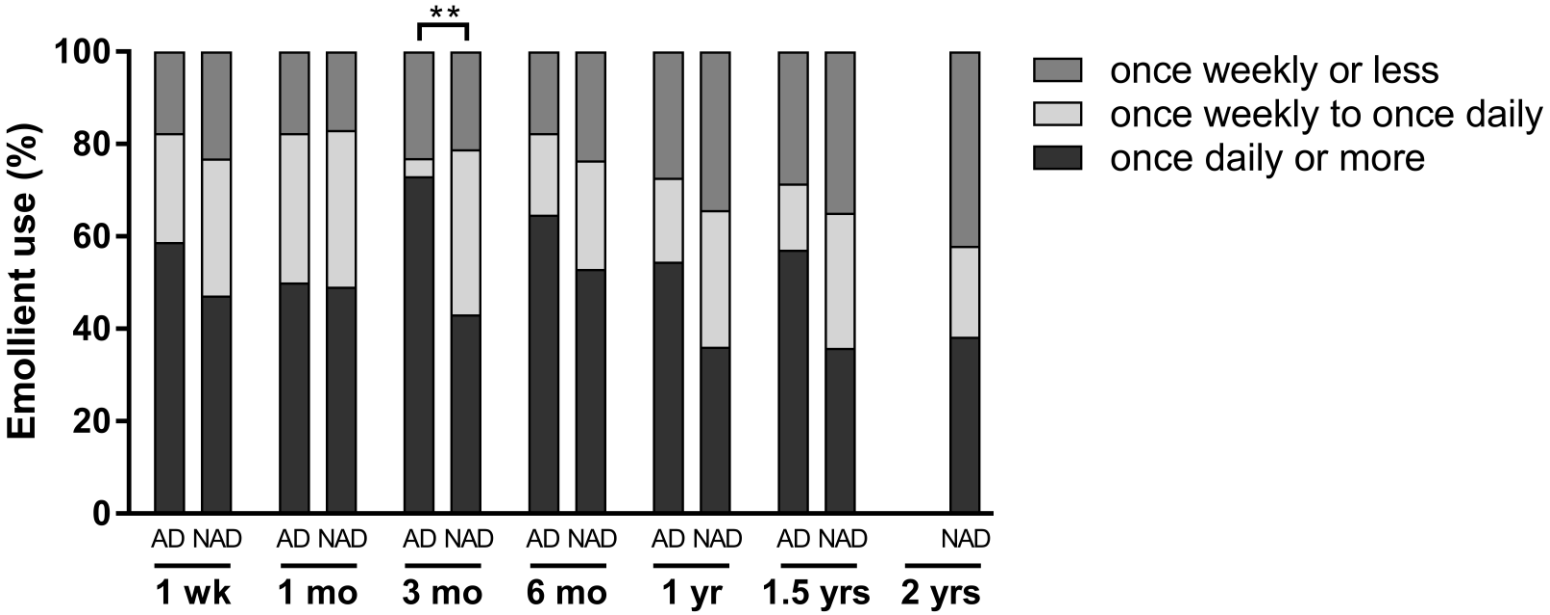

Figure S2. Evolution of axillary (solid lines) and antecubital (dashed lines) fossa skin colonization over the first two years of life in all 149 children. P-values were calculated using McNemar's test.

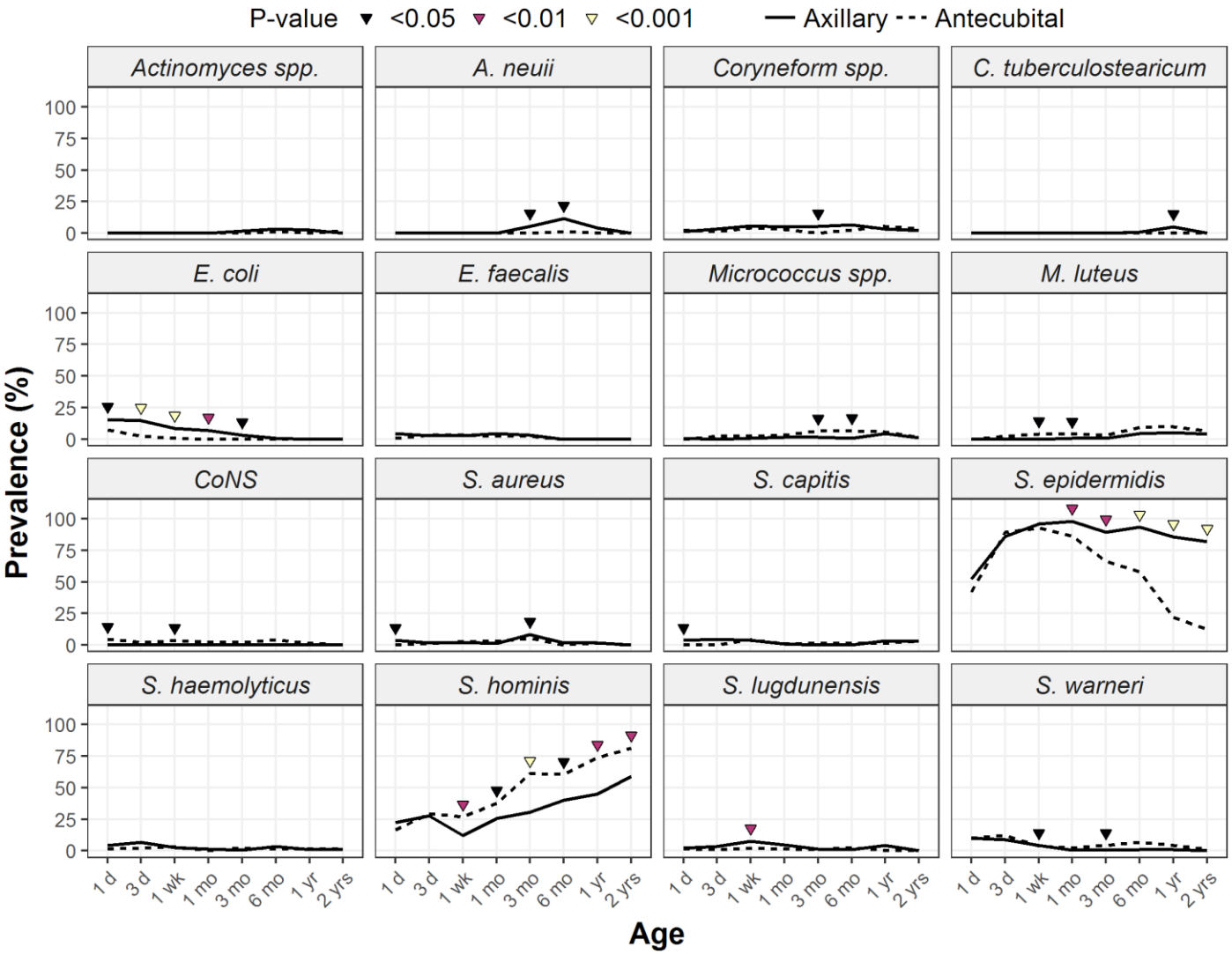

Figure S3. Antecubital fossa skin colonization on the first day of life, according to (a) delivery mode, (b) season of birth and (c) outcome. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

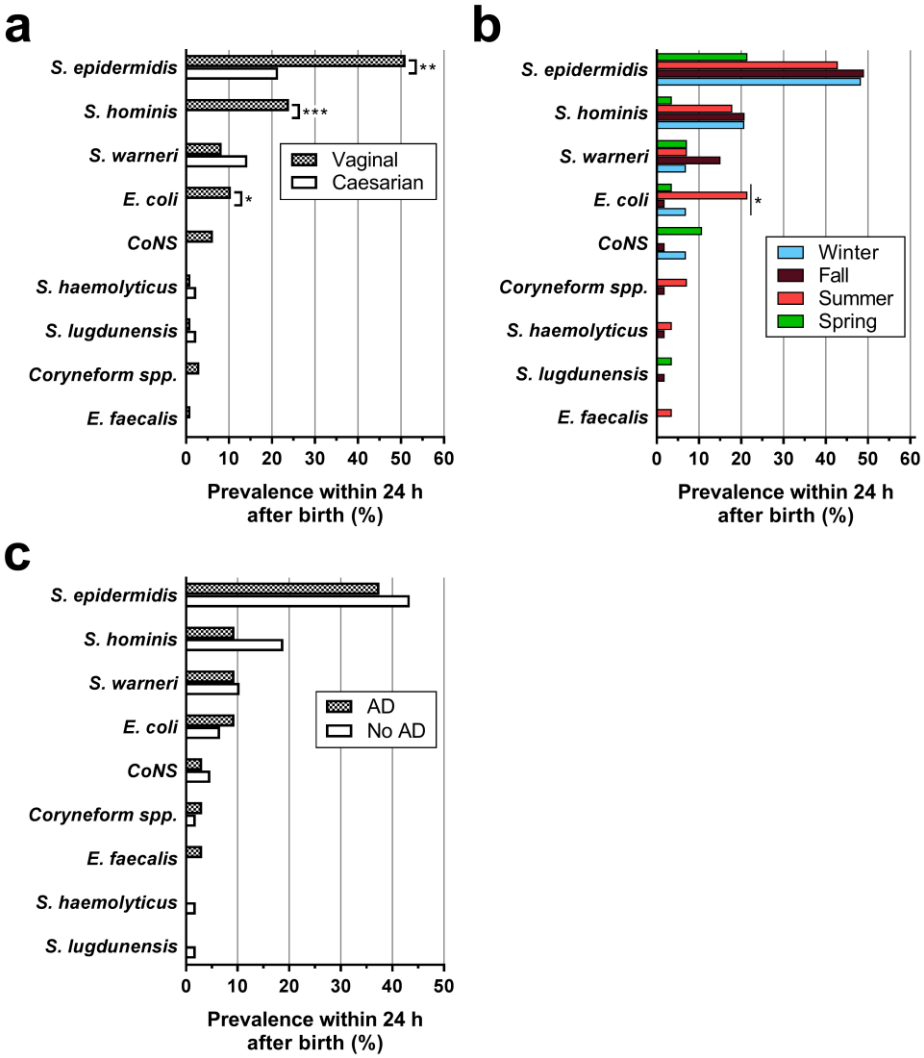

Figure S4. Evolution of axillary fossa skin colonization from birth to age three months in vaginally born (solid lines) and cesarean-born (dashed lines) infants

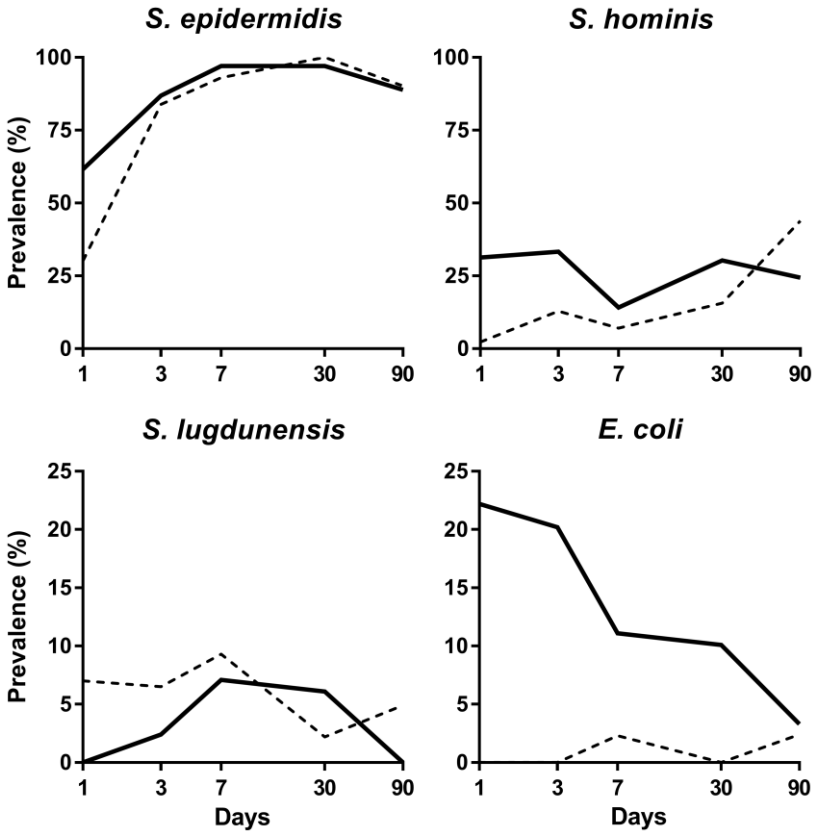

Figure S5. Evolution of antecubital fossa skin colonization in vaginally born (solid lines) and cesarean-born (dashed lines) infants

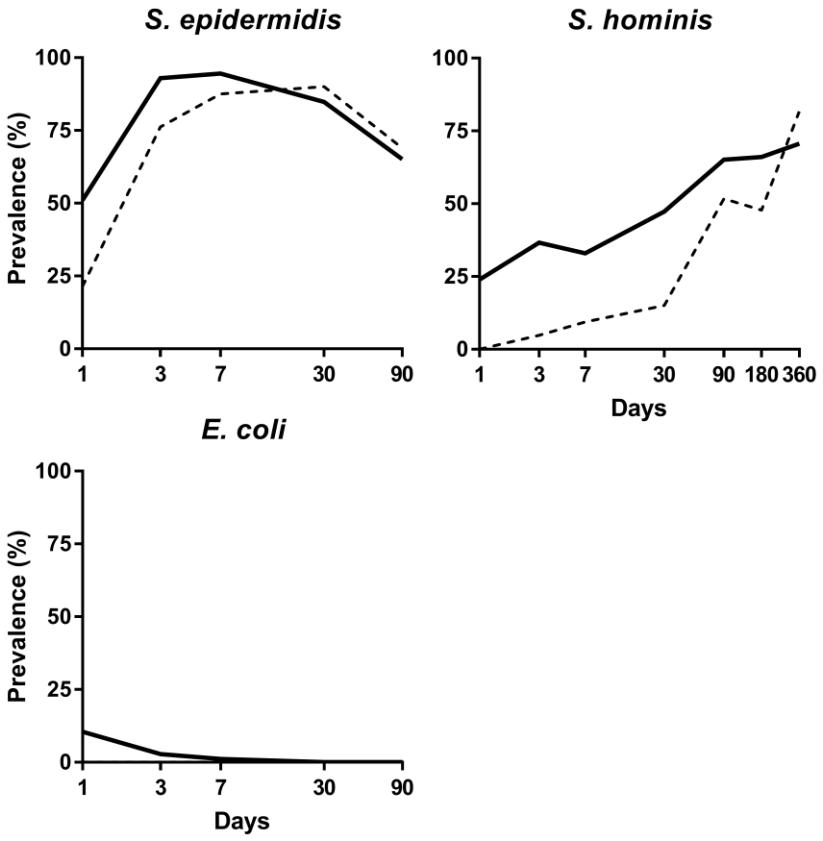

Figure S6. Evolution of antecubital fossa skin colonization over the first two years of life according to risk and outcome. ** $P < 0.01$ (high-risk AD versus high-risk no AD).

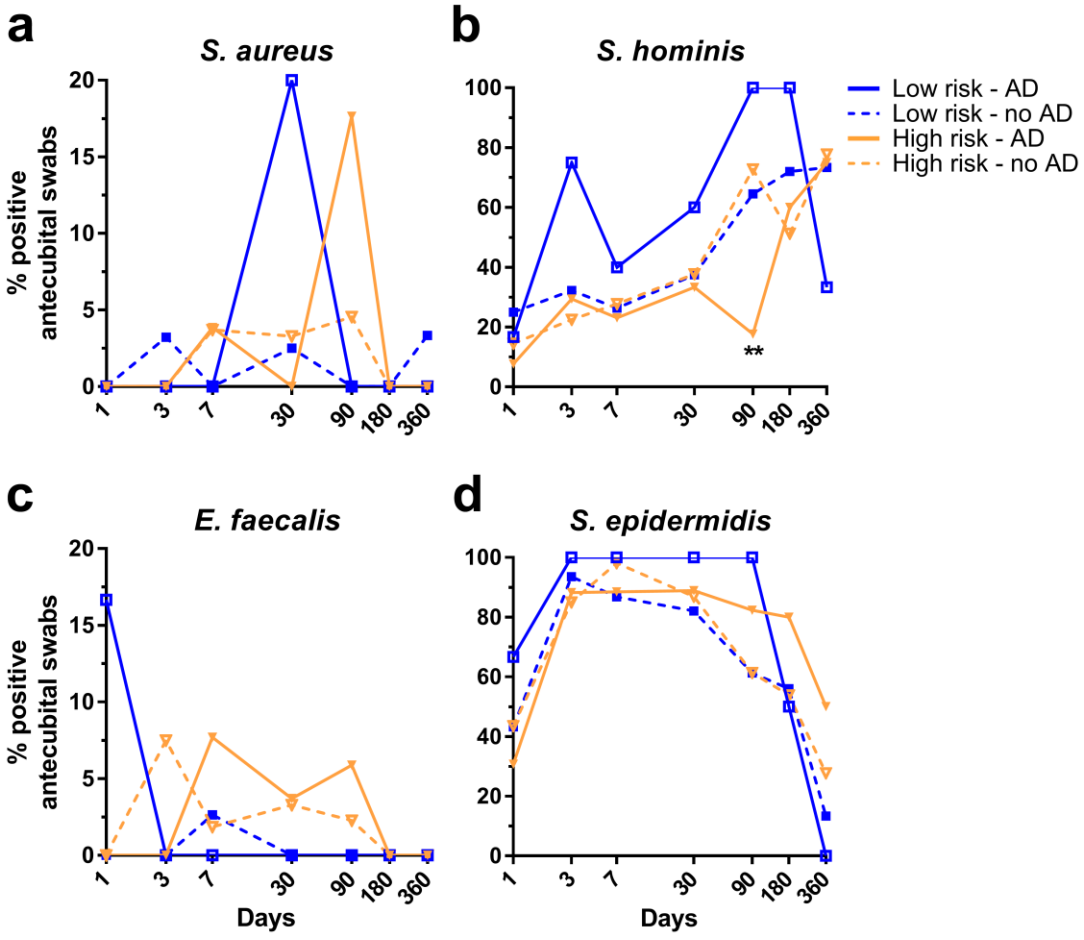
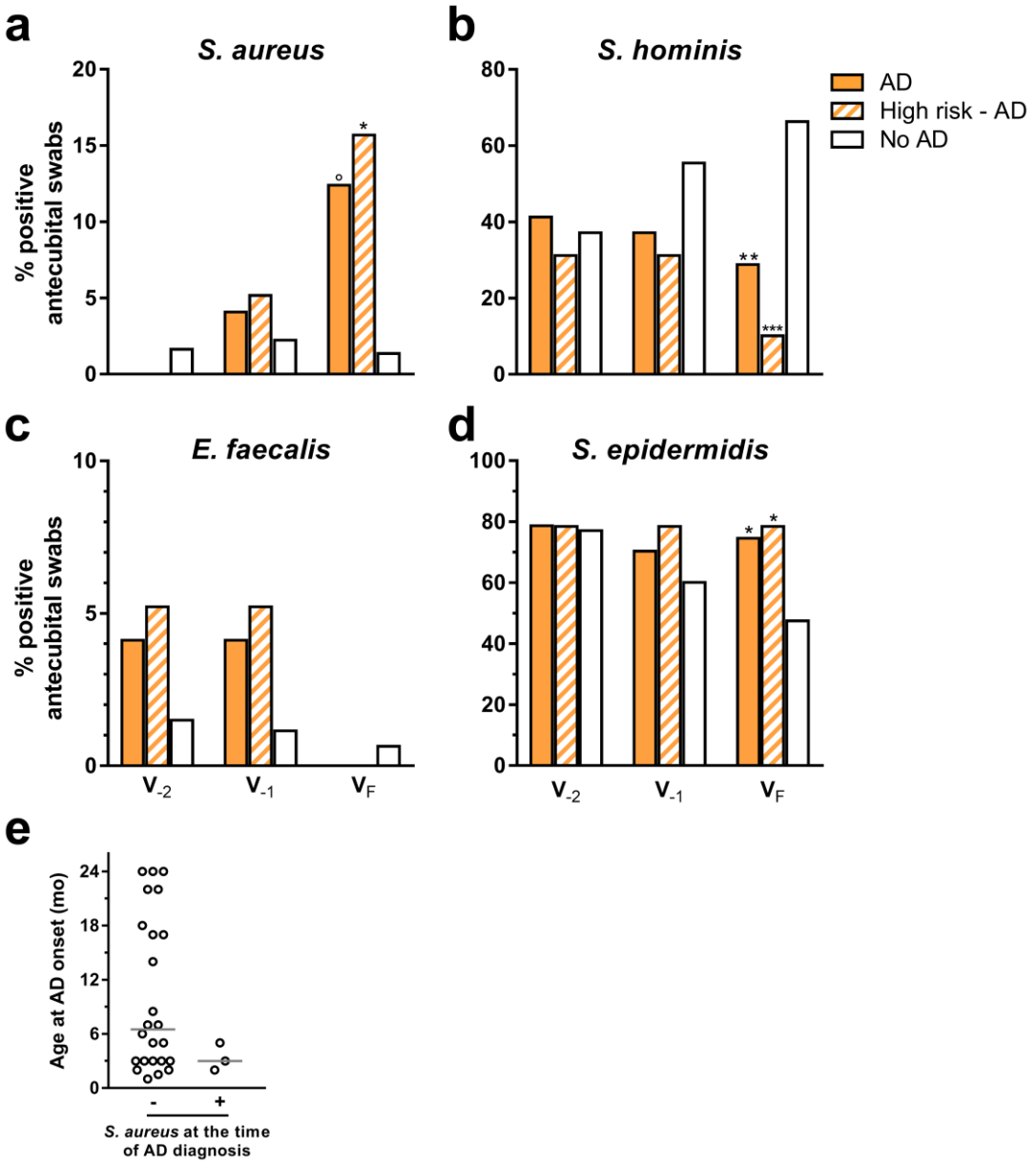
Figure S7. (a–d) Prevalence at time of AD onset and at the two prior follow-up visits. V_F, final visit (immediately following AD diagnosis); V_{-1} and V_{-2}, last and second to last scheduled visits prior to the final one, respectively. ° $P = 0.06$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (AD or high-risk AD versus no AD). (e) Age at AD onset in children testing positive or not for *S. aureus*; lines represent medians.