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Skin *Staphylococcus aureus* detection and relationship to atopic dermatitis outcomes using culture and metagenomic sequencing

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***Staphylococcus aureus* (*SA*) skin colonization in pediatric atopic dermatitis (AD) increases risk for severe AD and development of other allergic diseases. Despite this, there is no consensus regarding the optimal method to detect *SA*. Studies comparing metagenomic shotgun sequencing (MSS) and culture-based methods in *SA* detection and relationships to AD outcomes are lacking. In the Mechanisms of Progression of Atopic Dermatitis to Asthma in Children (MPAACH) cohort, we defined *SA* colonization categories by contact agar plate sampling/culture and skin tape sampling/MSS: double negative, sequencing only positive, contact plate only positive, and double positive ($n = 759$). We assessed AD severity, sensitization, total IgE, and atopic outcomes across categories. Culture missed 69% of samples detected by MSS and MSS missed 54% of samples detected by culture. The double positive group exhibited higher AD severity, sensitization load, and total serum IgE, and was more likely to develop allergic rhinitis (AR) compared to other groups. Detection of *SA* by MSS or culture missed over half of the *SA* detected by the other method. Importantly, detection via both methods correlated with increased AD severity, sensitization, total IgE, and AR. Thus, these methods are complementary and both may be necessary for comprehensive evaluation of *SA* and its clinical and biologic impact.**

Atopic dermatitis (AD) is a chronic, inflammatory skin disorder characterized by skin barrier defects and immune dysregulation, as well as environmental exposure to allergens and microbes. Prevalence of *Staphylococcus aureus* (*SA*), a gram-positive opportunistic pathogen, has been shown to be positively associated with AD disease severity and flares. Patients with AD are also more likely to be colonized with *SA* than healthy controls[1,2]. These effects are mediated in part by *SA* cell-surface and secreted virulence factors, including superantigens such as enterotoxins[3,4]. While literature suggests that skin microbial dysbiosis and increased *SA* colonization are key modulators of AD development, there is a lack of concordance in methods for skin sampling and detecting *SA*. Various non-invasive sampling methodologies are utilized including skin swabs, contact agar plates, and skin tapes with subsequent downstream assays including culture and non-culture-based methods[5,6]. Metagenomic shotgun sequencing (MSS) is commonly utilized and thought to better enhance ability to capture the complete skin microbial profile compared to culture-based methods, which may incur a bias towards specific organisms depending on the culture methods[7–9]. However, the utility of MSS in detecting *SA* and its relationship to AD disease outcomes has not been directly compared to other methods. We aimed to directly address this gap in knowledge by comparing detection of *SA* on the skin of children with AD using skin tape sampling followed by MSS versus direct skin sampling followed by culture on blood agar and subsequent in vitro assay verification. We hypothesized that the ability to culture the *SA* through contact agar plate/culture detection may be equally or more clinically relevant to AD and atopic march outcomes than detecting *SA* through MSS.

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To address these aims, we studied children participating in the **M**echanisms of **P**rogression of **A**topic Dermatitis to **A**sthma in **CH**ildren (MPAACH) cohort across five annual visits ($n = 759$). We first compared *SA* detection using the two methods, and then compared AD clinical features and atopic march outcomes between individuals with *SA* detected via the culture-dependent vs. sequencing-based approaches.

Methods

Participants

MPAACH is a prospective longitudinal early-life cohort of children with AD and has been previously described[10,11]. Eligible children were aged ≤ 3 years upon enrollment, had a gestation of ≥ 36 weeks, and either a diagnosis of AD or the parent(s)/legal authorized representative indicated a positive response to each of the 3 questions from the Children's Eczema Questionnaire (CEQ)[12,13]. All children are seen annually and during each visit, clinical data and biospecimens are collected. This study was approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center, and all parents (or legal guardians) signed informed consent prior to participation. All methods were performed in accordance with the relevant guidelines and regulations.

Contact blood agar plate sampling and detection of *SA*

Lesional skin was defined by the Hanifin and Rajka diagnostic criteria for atopic dermatitis[12]. Never-lesional (NL) skin was defined as at least 10 cm from lesional (L) sites and had no history of lesions at that site according to parental report as previously described[14].

Sheep blood agar contact plates (Hardy Diagnostics, Santa Maria, CA) were used to collect host skin biome samples from NL skin at each annual visit as previously described[14]. Each morphologically distinct colony was further isolated on individual sheep blood agar plates (Remel, San Diego, CA) and incubated for an additional 24 h. Colonies were further cultured on mannitol salt agar (Becton Dickinson, Franklin Lakes, NJ). Putative *SA* colonies were further subjected to a coagulase/agglutination assay using Staphaurex™ Latex Agglutination Test (Thermo Fisher Scientific, Waltham, MA). Contact plate phenotypes were determined by presence or absence of *SA* by the coagulase assay.

Skin tape sampling and metagenomic shotgun sequencing

SmartSolve skin tape strips measuring 1 inch x 1 inch were taken from NL-skin at each annual visit as previously described[14]. Briefly, skin cells were sampled by placing the tape strip on the skin, gently massaging the tape for 15–20 s, removing the tape, and storing it in ice-cold in BL buffer supplemented with 2% thio-glycerol (Promega, Madison, WI). This process was repeated to provide a total of 12 tape strips for each sampled skin site.

Genomic DNA was isolated from NL tape strips 1–3 using the Promega Wizard DNA isolation kit and pooled for sequencing. Sequence library generation was performed using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). Libraries were sequenced targeting an average of 7.5 million paired end reads per sample on the NovaSeq 6000 S4 Flow Cell (Illumina). Raw sequence reads were demultiplexed and filtered for quality and for removal of human contaminant sequences using Kneaddata v0.12.0 (bioBakery) at the default setting[15]. Approximately 19% of the reads across samples passed quality control and human contaminant removal. Taxonomic profiling was performed using MetaPhlan v4.0.6 (bioBakery) under the default settings for samples with > 500k microbial reads[15]. Taxa prevalent in less than 5% of samples were filtered out. Sequencing phenotypes were determined by presence or absence of reads in each sample mapping to *SA*-specific marker genes[16,17]. In a sensitivity analysis, data was rarefied/subsampled to the lowest count across samples using MicrobiomeStat[18,19].

Outcome definitions

AD severity was assessed by SCORAD: mild AD severity was defined as a SCORAD < 25, moderate as SCORAD ≥ 25 and < 50, and severe AD as SCORAD ≥ 50. Skin prick testing (SPT) was performed on each child at each MPAACH annual visit to 11 aeroallergens (mold mix 1 (Alternaria tenuis, Hormodendrum Cladosporioides, Helminthosporium interseminatum, Aspergillus fumigatus, Aspergillus niger, Penicillium notatum), mold mix 2 (Rhizopus nigricans, Pullularia pullulans, Fusarium vasinfectum, Mucor racemosus), ragweed, grass, tree mix 1 (Pecan, Maple BHR, Oak RVW, American Sycamore, Black Willow), tree mix 2 (White Ash, Birch Mix [Red & White], Black Walnut, Common Cottonwood, and American Elm), weeds, mite mix (Dermatophagoides pteronyssinus and Dermatophagoides farinae), cockroach, cat, dog) and 6 foods (peanut, soy, wheat, milk, egg white, egg yolk). Sensitization was defined a positive SPT with a wheal measurement ≥ 3 mm than the saline control to a food or aeroallergen. Sensitization load was defined as the total number of positive SPTs. Allergic rhinitis was defined as a combination of allergy symptoms and a positive SPT to an aeroallergen. Food allergy is defined as parental report of doctor diagnosed food allergy. The Pediatric Asthma Risk Score (PARS) was calculated as published[20]. Additional questionnaires and clinical assessments have been previously described[14].

Quantification of total and staphylococcus enterotoxin A (SEA) specific IgE

Total serum IgE and serum *Staphylococcus* enterotoxin A (SEA) specific IgE were quantified via Thermo Fisher ImmunoCap system (Labcorp, Cincinnati, OH). SEA-specific IgE was detectable at levels > 0.10 kU/L.

Statistical analysis

For individuals with multiple time points available, we included only the visit during which the individual had their worst AD severity as assessed by SCORAD. We used the following step-by-step algorithm to define the individual-level subset: (1) if participant was double positive at one or more visits, we utilized data from the double positive visit with the highest matched SCORAD value, (2) if participant was contact plate only positive or sequencing only positive at one or more visits, we utilized the data from the contact plate only positive or

sequencing only positive visit with the highest matched SCORAD value, and (3) if participant was only double negative at one or more visits, we utilized data from the double negative visit with highest matched SCORAD value (Fig. 1).

The MSS and clinical data was organized into a single object using the *phyloseq* package and used for subsequent analyses[21]. Generalized linear mixed-effect regression model (glmer) was used to assess associations between *SA* detection categories and outcome variables. Self-reported race, age at visit, sex, season at visit and contact plate and skin tape strips sampling areas were included as fixed effect terms and sampling area was treated as a random effect to account for the nesting of samples within sampling areas. Sampling sites were grouped into areas as follows: dry (hand, wrist, forearm, arm, upper arm, leg, thigh, lower leg, shin, knee, foot), moist (abdomen, antecubital fossa, popliteal fossa) and sebaceous (face, neck, chest, back, shoulder). To ensure independent samples that would not affect the results of other methodology, sampling could not be performed on the exact same anatomical site. In order to further mitigate any effect of potential *SA* site selectivity, we also performed additional sensitivity analysis by utilizing only contact agar plate and skin tape strips matched dry and matched sebaceous samples. The *lmer* and *glmer* function from the *lme4* package were used for continuous outcomes that followed a normal distribution (identity link function) and categorical outcomes (logit link function), respectively. The *lsmeans* package was used to determine least squares mean and standard error values (lsmean [SE]) for all continuous data in the analysis[22,23]. Total serum IgE was log-transformed to meet the normality criteria of the model. Statistical significance was defined as p-value < 0.05. Statistical analyses were performed in R version 4.3.2 (R core team, 2023).

Results

Demographics of study participants

There were 682 MPAACH participants enrolled, and among these, there were 328 unique MPAACH participants who had *Staphylococcus aureus* (*SA*) detection data available from NL skin using both methods (contact agar plate/culture and skin tape strips/MSS) from at least one annual visit at the time of analysis. Compared to the full cohort of 682 participants, the subset of 328 was slightly older and included a higher proportion of children who used oral or topical antibiotics in the past year, but they did not differ significantly in other demographics, AD severity, skin barrier function, or comorbid allergic conditions (Table S1).

Comparison of *SA* detection by contact agar plate/culture assay versus skin tape strips/MSS assay

Among the 328 unique participants, there were 759 cumulative study visit samples across V1 through V5 (270 samples at V1, 178 samples at V2, 173 samples at V3, 107 samples at V4, and 31 samples at V5). We classified these samples based on the presence or absence of *SA* detected by the contact agar plate/culture and skin tape strips/MSS at each study visit into four categories (Fig. 2a). Their characteristics are summarized in Table 1: **double negative**: *SA*- by both methods ($n = 584$); **sequencing only positive**: *SA*- by contact agar plate/culture, *SA*+ by skin tape strips/MSS ($n = 90$); **contact plate only positive**: *SA*+ by contact agar plate/culture, *SA*- by skin tape strips/MSS ($n = 52$); **double positive**: *SA*+ by both methods ($n = 33$). The children across 759 study visits had a median age of 3.5 [2.3–5.0], 52.8% were male and 63.6% were Black (Table 1).

Out of the 759 samples, 175 were positive for *SA* by at least one method: 123 samples were *SA*+ by skin tape strips/MSS, 85 samples were *SA*+ by contact agar plate/culture, and 33 samples were *SA*+ by both methodologies. Out of the 123 samples detected by skin tape strips/MSS assay, 90 (73%) samples were missed by the contact agar plate/culture assay. Likewise, out of the 85 samples detected by contact agar plate/culture assay, 52 (61%) were

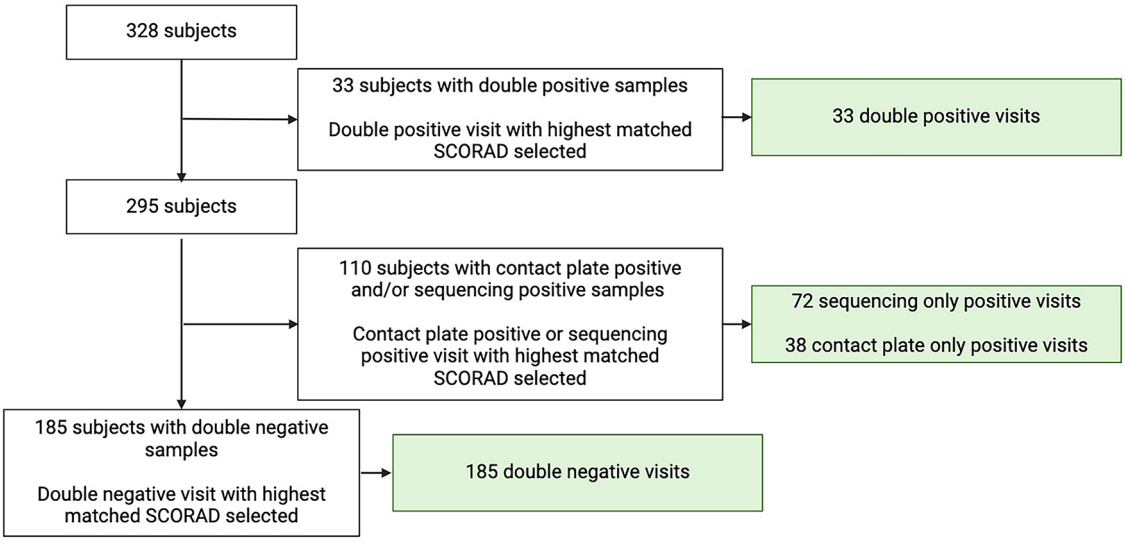

Fig. 1. Algorithm for selecting one visit per individual to create the individual-level subset.

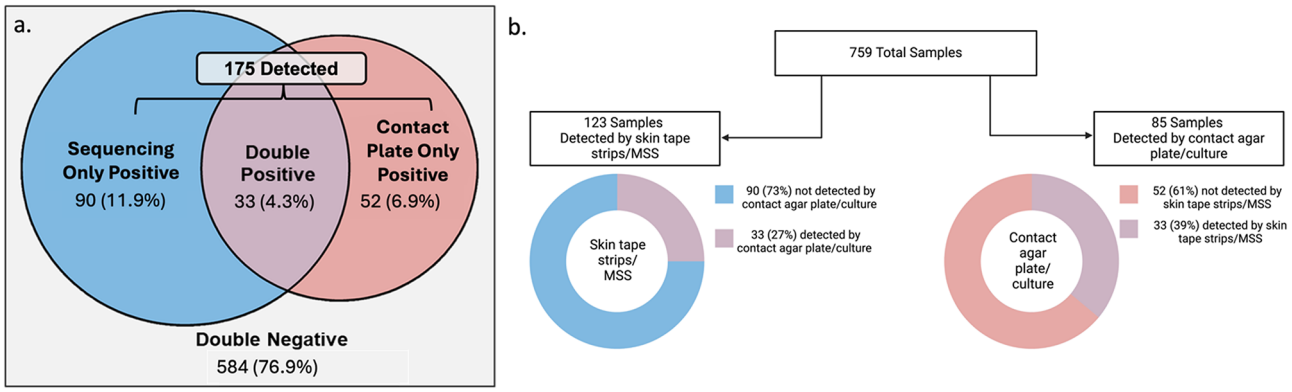

Fig. 2. Total samples categories and detection rates by different methodologies. **(a)** Venn diagram of contact agar plate/culture assay and skin tape strips/metagenomic shotgun sequencing assay detection groups in the total samples. **(b)** Overview of the detection rates of *SA* by different methodologies in the total samples.

	All samples	Double negative	Sequencing positive	Contact plate positive	Double positive
	n = 759	*n* = 584	*n* = 90	*n* = 52	*n* = 33
Demographic					
Age (y)	3.5 (2.3–5.0)	3.6 (2.3–5.0)	3.1 (2.2–4.8)	3.6 (2.4–5.3)	2.5 (1.6–3.7)
Male sex	401 (52.8)	312 (55.0)	46 (51.1)	25 (48.1)	18 (54.5)
Black race	483 (63.6)	362 (63.8)	67 (74.4)	35 (67.3)	19 (57.6)
Public insurance	500 (65.9)	377 (66.5)	68 (75.8)	32 (61.5)	23 (69.7)
AD Severity					
SCORAD (score)	17.1 (10.7–26.7)	16.8 (10.5–25.6)	18.0 (10.5–28.7)	19.3 (11.4–26.5)	29.8 (13.1–39.0)
Moderate/Severe AD (SCORAD ≥ 25)	221 (29.1)	155 (27.3)	31 (34.4)	15 (28.8)	20 (60.6)
PARS (score)	7 (4–9)	7 (4–9)	6 (5–9)	7 (5.5–9)	7 (6–9)
Sensitization					
Total serum IgE	63.5 (17.8–163.5)	68 (18–163)	46 (16–91)	137 (19–413)	83 (35–321)
Sensitization group					
Nonsensitized	376 (49.5)	292 (51.5)	51 (56.7)	27 (54.0)	12 (36.4)
Comorbid conditions					
Food allergy	593 (78.1)	130 (22.9)	17 (18.9)	10 (19.2)	6 (18.2)
Allergies or allergic rhinitis	214 (28.2)	167 (29.5)	25 (27.8)	8 (15.4)	14 (42.4)
Other					
AD onset < = 3 months of age	323 (42.6)	255 (45.0)	29 (32.2)	23 (44.2)	16 (48.5)
AD onset < = 6 months of age	510 (67.2)	395 (69.7)	55 (61.1)	35 (67.3)	25 (75.8)
Antibiotic usage in the past year	361 (47.6)	281 (49.6)	38 (42.2)	24 (46.2)	18 (54.5)
Current steroid cream usage	614 (80.9)	471 (83.1)	73 (81.1)	44 (84.6)	26 (78.8)
Visit season					
Spring	172 (23.2)	128 (22.6)	20 (22.2)	14 (26.9)	10 (30.3)
Summer	203 (26.7)	150 (26.5)	23 (25.6)	18 (34.6)	12 (36.4)
Fall	208 (27.4)	164 (28.9)	27 (30.0)	11 (21.2)	6 (18.2)
Winter	176 (23.2)	142 (25.0)	20 (22.2)	9 (17.3)	5 (15.2)

Table 1. Total sample characteristics (all samples)[a]. [a]Data are presented in median (25^{th} –75^{th}) or *n* (%). Data contains raw values (not least-square mean values) without covariate adjustment.

missed by the skin tape strips/MSS assay (Fig. 2b). Thus, skin tape strips/MSS does not capture all *SA* detected by contact agar plate/culture and vice versa, revealing that these methodologies are not equal and yield different detection results, potentially resulting in different clinical associations. The samples in each category remained consistent even after rarefaction/subsampling to ensure even sequencing depth between categories.

Due to the longitudinal nature of MPAACH where individuals are followed over five annual visits, the groups described in Table 1 could include samples from a single individual at multiple study visits. To ensure that inclusion of multiple samples from the same individual at different time points was not contributing to bias in our analysis, we conducted all further analyses with each participant represented at only one visit using the

algorithm outlined in Fig. 1. Using this algorithm, out of the 328 individuals, 185 were *SA*- by both methodologies (double negative), 72 were *SA*+ by skin tape strips/MSS only (sequencing only positive), 38 were *SA*+ by contact agar plate/culture only (contact plate only positive), and 33 were *SA*+ by both methodologies (double positive) (Fig. 3a). Out of the 105 samples detected by skin tape strips/MSS, 72 (69%) samples were missed by the contact agar plate/culture. Likewise, out of the 71 samples detected by contact agar plate/culture assay, 38 (54%) were missed by the skin tape strips/MSS assay (Fig. 3b). Thus, the two methodologies often missed detection of *SA* that was detected by the other method. These data mirrored what we observed when samples collected at different time points from the same individual were used (Fig. 2b). There were no differences in demographics, parental reported oral or antibiotic usage in the past 12 months, current topical steroid usage, or history of early onset AD between the individual-level subset categories (Table 2).

Children with *SA* detection with multiple methodologies had worse AD features

Given the lack of agreement in *SA* detection between the methods, we next examined associations between *SA* detected by each or both method(s) with AD severity and allergen sensitization using the individual-level subset data. Children with *SA* detection by both contact agar plate/culture and skin tape strips/MSS (double positive) had a higher SCORAD compared to children in whom *SA* was not detected by either method (double negative) (lsmeans[SE]: 34.9 [6.92] vs. 28.6 [6.55], $p = 0.016$), or to children in whom *SA* was detected only by skin tape strips/MSS (sequencing only positive, 34.9 [6.92] vs. 27.5 [6.68], $p = 0.010$), or to children in whom *SA* was detected only by contact agar plate/culture (contact plate only positive, 34.9 [6.92] vs. 25.8 [6.88], $p = 0.006$, Fig. 4a). No differences in SCORAD were observed between the sequencing only positive or contact plate only positive groups when compared to each other or to the double negative group. We also evaluated the proportion of children with mild vs. moderate/severe AD in each detection category. Children in the double positive group were more likely to have moderate/severe AD compared to double negative (60.6% vs. 38.4%, $p = 0.031$), sequencing only positive (60.6% vs. 35.2%, $p = 0.011$), and contact plate only positive groups (60.6% vs. 31.6%, $p = 0.011$) (Fig. 4b).

We next evaluated allergen sensitization to 11 aeroallergens and 6 food allergens based on skin prick testing results. There was a higher proportion of allergen sensitized children in the double positive group compared to the double negative (63.6% vs. 44.9%), sequencing only positive (63.6% vs. 45.1%), and contact plate only positive groups (63.6% vs. 47.4%). This difference was significant between the double positive and double negative groups ($p = 0.042$, Fig. 4c). We also measured the sensitization load, which was defined as the number of food/aero allergens to which a participant was sensitized. We observed a higher sensitization load in the double positive group (4.33 [0.63]) compared to the other groups (double negative: 2.51 [0.44], $p = 0.001$; sequencing only positive: 2.98 [0.54], $p = 0.040$; contact plate only positive: 2.70 [0.68], $p = 0.032$; Fig. 4d).

We next measured total serum IgE levels between the groups. Participants in the double positive group had higher total serum IgE (4.96 [0.55]) compared to participants in the double negative (4.17 [0.40]), sequencing only positive (4.27 [0.45]) and contact plate only positive groups (4.77 [0.49]) but this was not significant. Of note, total IgE levels in the contact plate only positive group were higher than double negative and sequencing only positive groups but did not achieve significance (Fig. 4e).

Previous research has shown that skin microbes inhabit specific skin niches based on factors such as temperature, moisture, sebum content and pH, with *SA* commonly present in moist areas including the antecubital and popliteal fossa[24–26]. Thus, while the data in Fig. 4a-e were adjusted for the skin tape strip and contact agar plate sampling areas (dry, moist, sebaceous), we also performed sensitivity analysis to ensure that sampling site differences were not contributing to the observed findings. Specifically, we restricted the analysis to matched dry and matched sebaceous samples in the individual-level subset for both the contact agar plate and skin tape strip samples. In this sensitivity analysis, 89 individuals were double negative, 34 individuals were sequencing only positive, 28 individuals were contact plate only positive, and 14 individuals were double positive. The distribution of matched dry and matched sebaceous individuals and samples in each category are

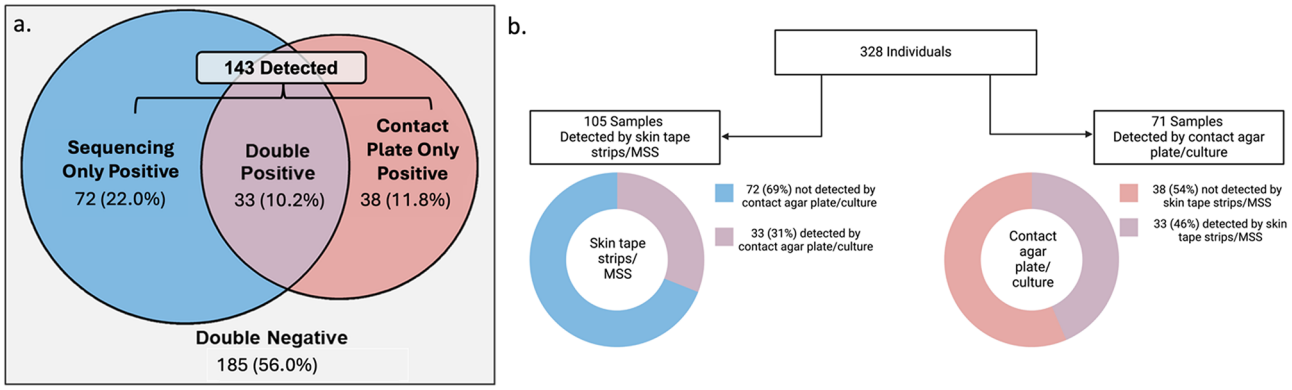

Fig. 3. Individual-level subset categories and detection rates by different methodologies. **(a)** Venn diagram of contact agar plate/culture assay and skin tape strips/metagenomic shotgun sequencing assay detection groups in the individual-level subset. **(b)** Overview of the detection rates of *SA* by different methodologies in the individual-level subset.

	All individuals	Double negative	Sequencing only positive	Contact plate only positive	Double positive	
	n = 328	*n* = 185	*n* = 72	*n* = 38	*n* = 33	*P*-value
Demographic						
Age (y)	2.7 (1.8–4.6)	2.6 (1.6–4.4)	3.0 (2.1–4.7)	3.1 (2.2–5.2)	2.5 (1.6–3.7)	0.36
Male sex	172 (52.4)	99 (53.5)	36 (50.7)	19 (50)	18 (54.5)	0.94
Black race	207 (63.1)	109 (58.9)	51 (71.8)	27 (71.1)	19 (57.6)	0.14
Public insurance	213 (64.9)	114 (61.6)	52 (73.2)	23 (60.5)	23 (69.7)	0.21
AD Severity						
SCORAD (score)	21.0 (13–31.1)	21.6 (13.6–30.6)	18.0 (10.8–28.8)	19.6 (11.4–27.6)	29.8 (13.1–39.0)	0.16
Moderate/Severe AD (SCORAD ≥ 25)	128 (39.0)	71 (38.4)	25 (35.2)	12 (31.6)	20 (60.6)	0.04
PARS (score)	6.5 (4–9)	6 (4–9)	6.5 (4–9)	7 (6–9)	7 (6–9)	0.31
Sensitization						
Total serum IgE	43 (14–140)	35 (11–133)	34 (16–80)	133 (18–339)	83 (35–321)	0.03
Sensitization group						0.23
Nonsensitized	174 (53.0)	102 (55.1)	39 (54.9)	20 (52.6)	12 (36.4)	
Comorbid conditions						
Food allergy	66 (20.1)	37 (20)	15 (21.1)	8 (21.1)	6 (18.2)	0.99
Allergies or allergic rhinitis	75 (22.9)	36 (19.5)	20 (28.2)	5 (13.2)	14 (42.4)	0.06
Other						
AD onset < = 3 months of age	142 (43.3)	83 (44.9)	25 (35.2)	17 (44.7)	16 (48.5)	0.55
AD onset < = 6 months of age	225 (68.6)	128 (69.2)	46 (64.8)	25 (65.8)	25 (75.8)	0.72
Antibiotic usage in the past year	161 (49.1)	97 (52.4)	22 (31.0)	17 (44.7)	18 (54.5)	0.28
Current steroid cream usage	264 (80.5)	147 (79.5)	58 (81.7)	32 (84.2)	26 (78.8)	0.89
Visit season						0.11
Spring	76 (23.2)	42 (22.7)	15 (21.1)	9 (23.7)	10 (30.3)	
Summer	83 (25.3)	38 (20.5)	17 (23.9)	15 (29.5)	12 (36.4)	
Fall	80 (24.4)	45 (24.3)	22 (31.0)	7 (18.4)	6 (18.2)	
Winter	89 (27.1)	60 (32.4)	17 (23.9)	7 (18.4)	5 (15.2)	

Table 2. Individual-level subset characteristics[a]. [a]Data are presented in median (25^{th} –75^{th}) or *n* (%). Data contains raw values (not least-square mean values) without covariate adjustment.

shown in Table 3. We observed a higher SCORAD in the double positive group (38.2 [7.40]) compared to the double negative (28.0 [6.71], $p = 0.0078$), sequencing only positive (27.4 [6.97], $p = 0.0104$) and contact plate only positive groups (22.7 [7.02], $p = 0.00038$, Fig. 4f). The results of AD severity, sensitization and total IgE in this sensitivity analysis paralleled our findings in the larger subset (Fig. 4g-j).

Children with *SA* detection with multiple methodologies had worse atopic march outcomes

Previous research has shown that increased AD severity and sensitization are linked to an increased risk of developing other atopic diseases, including allergic rhinitis (AR), asthma and food allergy (FA)[27]. Additionally, evidence shows that changes in the skin microbiome are also linked to the atopic march[11,28,29]. In our analysis, we observed the largest difference in AD severity and allergic sensitization between double positive group (*SA* detection through both skin tape strips/MSS assay and contact agar plate/culture assay) and the other groups, with children in the double positive group having the worst AD severity, sensitization, and highest total IgE levels. Thus, we hypothesized that being double positive for *SA* using two methodologies would also be associated with atopic march progression. We compared the development of AR, FA, or asthma in participants with *SA* detected by both skin tape strips/MSS and contact agar plate/culture (double positive) to those who were double negative or single positive (*SA* detected by skin tape strips/MSS or contact agar plate/culture, but not both). The double positive group had a significantly higher proportion of children with AR compared to the double negative/single positive group ($p = 0.022$, Fig. 5a).

The children were too young to diagnose asthma, so we assessed asthma risk using the Pediatric Asthma Risk Score (PARS)[30]. While we observed a higher PARS in the double positive group compared to the double negative/single positive group, this difference was not statistically significant. Since PARS includes self-reported Black race as a risk factor, we wanted to probe further to understand whether differences in self-reported race between the two detection groups could be affecting these results. While there was no difference in PARS for Black participants between the double negative/single positive groups (7.67 [0.35]) and double positive (7.50 [0.70]), the Non-Black participants in the double negative/single positive group had a significantly lower PARS (5.28 [0.37]) compared to Black participants in the double negative/single positive ($p = 4.33e\text{-}12$) and double positive groups ($p = 0.001$). There was no difference between the Non-Black double positive group (6.62 [0.76]) and the Black double negative/single positive and Black double positive groups (Fig. 5b). Lastly, we assessed doctor diagnosed FA and did not observe a significant difference in having FA between the double positive and double negative/single positive group (Fig. 5c). We also assessed AR, PARS and doctor diagnosed FA using

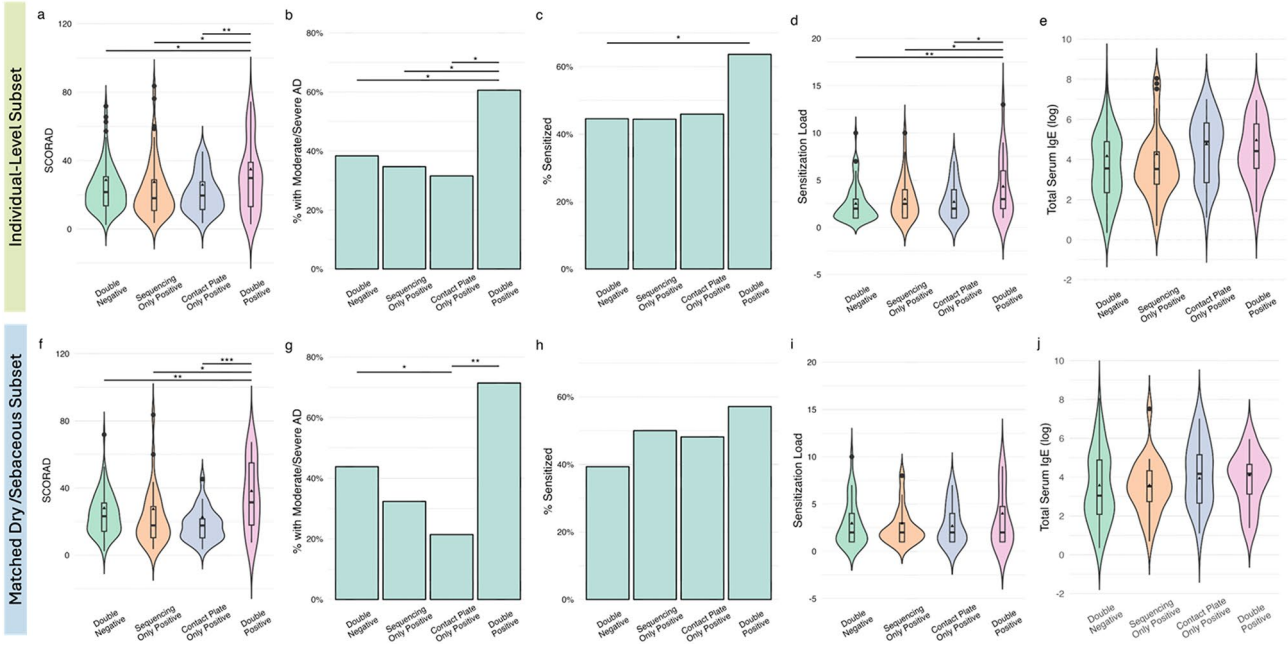

Fig. 4. Atopic dermatitis severity, allergen sensitization and total serum IgE across individual-level subset *SA* detection categories and matched dry-sebaceous *SA* detection categories. **(a**, **f)** SCORAD, (**B**, **G)** AD Severity, **(c**, **h)** sensitization, **(d**, **i)** sensitization load and **(e**, **j)** total serum IgE (log) values in the individual-level subset and the matched dry-sebaceous subset, respectively. Differences across categories were evaluated using logistic mixed-effects and linear mixed-effects models, with ▲ denoting least squares mean values. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

	Total	Double negative	Sequencing only positive	Contact plate only positive	Double positive
Total	165	89	34	28	14
Dry	131 (79.4)	68 (76.4)	30 (88.2)	23 (81.1)	10 (71.4)
Sebaceous	34 (20.6)	21 (23.6)	4 (11.8)	5 (17.9)	4 (28.6)

Table 3. Individuals with matched dry and sebaceous samples on contact agar plate and skin tape strips[a].
[a]Data are presented as n (%).

the matched dry and matched sebaceous samples in a sensitivity analysis and observed similar results to the individual level subset (Fig. 5d-f). Interestingly, the Non-Black double positive group had significantly higher PARS compared to the Non-Black double negative/single positive group in this subset (8.53 [1.41] vs. 5.30 [0.45], $p = 0.027$). These results suggest that being positive for *SA* by both methodologies is associated with an increased asthma risk compared to those who are double negative or single positive for *SA* in Non-Black participants.

SA detection with contact agar plate/culture showed higher detection of Staphylococcus enterotoxin A-specific IgE

To assess the biologic impact of *SA* detected by skin tape strips/MSS versus contact agar plate/culture, we quantified *Staphylococcus* enterotoxin A (SEA)-specific IgE levels in a subset of selected participants that were matched for both age and race between categories ($n = 90$). We observed that both double positive and contact plate only positive groups (groups with *SA* detected by contact plate/culture) had a higher proportion of children with SEA-specific IgE compared to the sequencing only positive and double negative groups (groups without any *SA* detected by contact plate/culture) but this did not reach significance (Fig. 6a-b). Notably, sequencing only positive had intermediate SEA-specific IgE proportion between the double negative and the double positive or contact plate only positive groups, but it did not reach significance. The SEA-IgE results of the matched dry and matched sebaceous samples ($n = 43$) were similar to the primary results (Fig. 6c-d).

Discussion

To our knowledge, this is the first study comparing multiple sampling methodologies for detecting *Staphylococcus aureus* (*SA*) and their relationship to AD severity, sensitization, and atopic march outcomes. Our data reveal that contact agar plate/culture and skin tape strips/metagenomic shotgun sequencing (MSS) methodologies yield substantially different *SA* detection rates, with each method missing cases that the other picks up, suggesting that

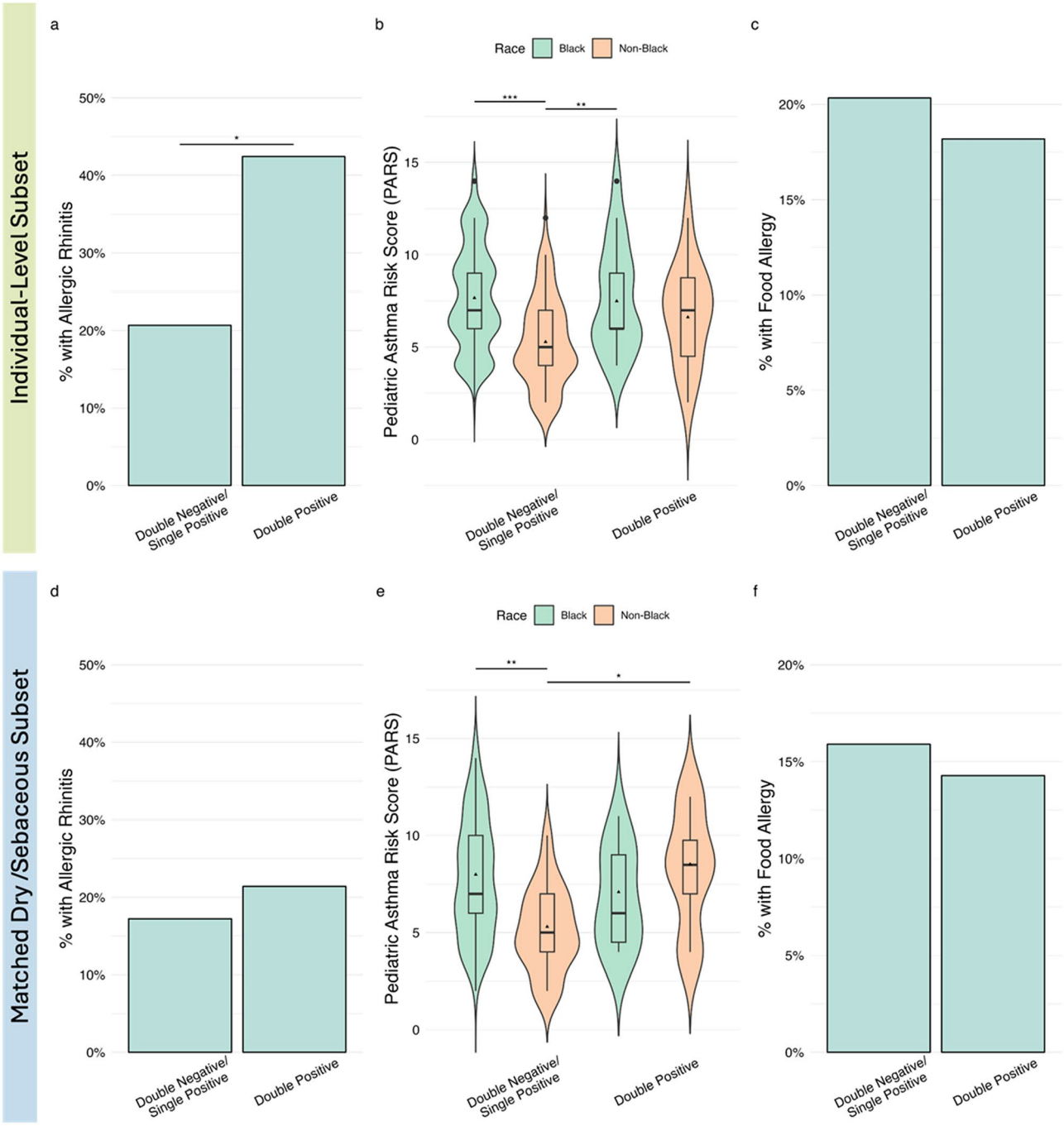

Fig. 5. Allergic rhinitis, Pediatric Asthma Risk Score (PARS) and food allergy across individual-level subset *SA* detection categories and matched dry-sebaceous *SA* double positive vs. double negative/single positive categories. **(a**, **d)** Allergic rhinitis, **(b**, **e)** PARS within the Black and Non-Black participants and **(c**, **f)** doctor diagnosed food allergy in the individual-level subset and the matched dry-sebaceous subset, respectively. Differences across categories were evaluated using logistic mixed-effects and linear mixed-effects models, with ▲ denoting least squares mean values. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

they may provide synergistic information. This is a novel finding and represents a paradigm shift, challenging the widely accepted idea that MSS more fully reflects the skin biome compared to culture-based methods. While contact agar plate/culture assay missed 69% of *SA* detected by skin tape strips/MSS assay, 54% of *SA* detected by contact agar/culture were missed by skin tape strips/MSS assay. Further, we observed that children with *SA* detected via both assays had worse AD severity, increased allergen sensitization and total serum IgE, worse allergic rhinitis (AR) and increased asthma risk in Non-Black children. Interestingly, the double positive and contact plate only positive groups both had higher prevalence of detectable serum *Staphylococcus* enterotoxin-A (SEA) specific IgE compared to the sequencing only positive and double negative groups (groups without any *SA*

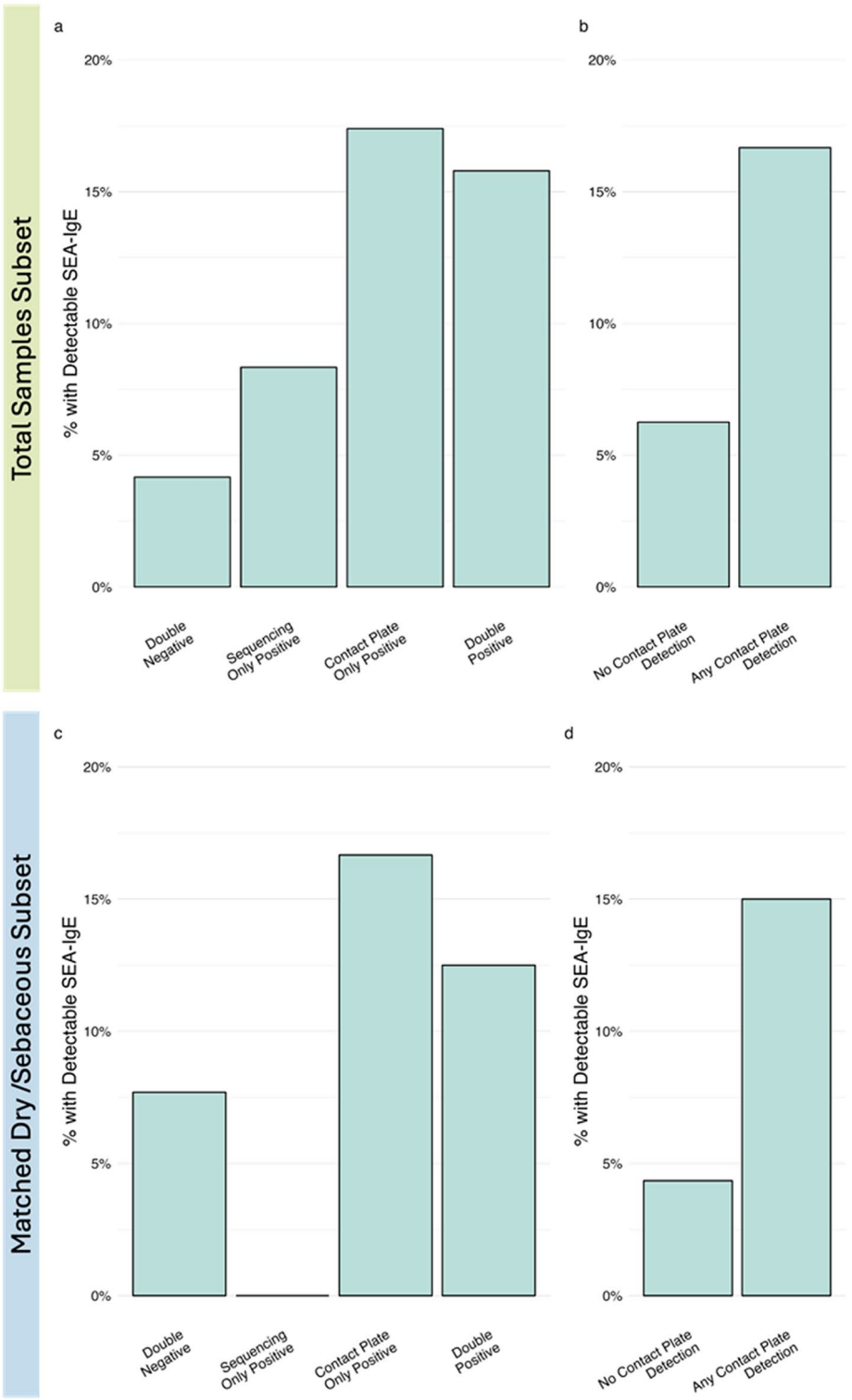

Fig. 6. *Staphylococcus* enterotoxin A (SEA) specific IgE in a subset of total samples across *SA* detection categories and in a subset of matched dry-sebaceous samples across *SA* detection categories. **(a)** Proportion of participants with SEA-specific IgE in a subset of samples in the total samples ($n = 90$) categories and **(b)** contact plate detection categories. **(c)** SEA-specific IgE in a subset of matched dry-sebaceous samples in the total samples ($n = 43$) categories and **(d)** contact plate detection categories. Differences between categories in SEA-specific IgE was evaluated using Chi-square tests.

presence on contact plate), suggesting that the contact agar plate/culture method may detect more biologically relevant *SA*. Collectively, our data support that both sampling methods provide useful information that are non-overlapping and necessary to fully capture the biological relevance of the skin biome.

The major finding of our study is that *SA* detection is not equivalent between contact agar plate/culture assay and skin tape strips/MSS assay. In fact, the majority of children with *SA* detected through contact agar plate/culture assay did not have *SA* detected through skin tape strips/MSS assay and vice versa. These results are highly relevant, given the intense focus on *SA* in clinical practice and research studies. In the clinic, anti-*Staphylococcal* treatments including antibiotics, steroids, bleach baths and even topical commensal organisms are utilized as a result of skin *SA* detection[31,32]. Further, many research studies on AD and the atopic march focus on the detection of *SA* on the skin. Our data shows that MSS, the current gold standard analysis methodology for comprehensive microbiome analysis, may not fully reflect *SA* presence on AD skin. This inequivalence in detection raises the question of which assay is most reflective of disease state.

Thus, we first investigated which detection group was most associated with AD features. The data revealed that utilizing both assays may be more useful in identifying patients with worse AD disease than utilizing just one assay. Indeed, children who were double positive by culture and sequencing showed worse clinical outcomes including increased AD severity and allergen sensitization prevalence and higher sensitization load. Children in the double positive group also exhibited higher total serum IgE compared to the other groups. These results not only reinforce previous data highlighting the link between *SA* colonization and AD disease severity, but also suggest that detecting *SA* by two methodologies may be crucial in this link[11,32] Interestingly, the contact plate only positive group had a higher IgE compared to the sequencing only positive and double negative groups, indicating that the *SA* captured by contact agar plate/culture may be more biologically significant.

In order to further explore the biological impact of *SA* detection, we examined the serum SEA-specific IgE in a subset of patients. A previous study showed that children with AD had higher levels and rates of serum SEA and SEB-specific IgE than atopic children without AD, and in another cross-sectional study of 74 children with AD, SEA-specific IgE levels correlated with AD severity[33,34]. These findings suggest that SEA-specific IgE is a reflection of the immunological response to pathogenic disease-inducing *SA*. In our cohort, we observed that among those with skin *SA* detected by contact agar plate/culture assay (contact plate only positive and double positive groups), there was a higher percentage of individuals with detectable serum SEA-specific IgE compared to those without *SA* detected by contact agar plate/culture assay (double negative and sequencing only positive groups). Children with detection by contact agar plate/culture assay had a higher immunological response (both total serum IgE and proportion with detectable SEA-specific IgE). These results indicate that contact plate sampling, which is more cost-effective and faster than sequencing, may be better at capturing biologically and clinically relevant viable bacteria. MSS on DNA from tape strips (and similar DNA sampling methods) provides comprehensive information on all microbes present on the skin surface (bacteria, viruses, fungi) but is not selective for living microbes and may be picking up some artifacts that are not biologically relevant. Our data support that there may be a key benefit in utilizing contact agar plate or similar culture-dependent methods of sampling that selectively focus on one or more microbes to enhance sequencing data.

The observed differences in AD features and total serum IgE and SEA-specific IgE are unlikely to stem from differences in *SA* load on the skin. The majority of samples that showed any relative abundance of *SA* via MSS did not have any *SA* detected by contact agar plate/culture assay (sequencing only positive group). Conversely, some samples with no *SA* relative abundance via skin tape strips/MSS assay had detectable *SA* on contact agar plate/culture assay (contact plate only positive group). These findings suggest that the relative abundance of *SA* assessed via MSS is not a complete or accurate representation of actual *SA* load on the skin.

In order to evaluate atopic march outcomes between categories, we next examined AR and observed an increased proportion of children with AR in the double positive group compared to the double negative/single positive group. Previous studies have shown that the nostrils have similar architecture to the skin, and both contain similar bacterial genera including *Corynebacterium*, *Cutibacterium*, *and Staphylococcus* (including *SA*). Driven primarily by *Staphylococci* in both environments, there is also an association between microbial composition in both the nose and skin with AD severity. Additionally, there is also an association between *SA* and AR; patients with AR have been shown to be more frequently colonized with nasal *SA* compared to healthy controls, leading to local IgE production and more severe AR symptoms[35–37]. Studies have shown that increased local IgE production may correlate with elevated antigen-specific serum IgE levels, potentially contributing to the total serum IgE pool[38,39]. Thus, detection of *SA* on skin may serve as an indicator of nasal *SA*, therefore resulting in a higher prevalence of AR and increased serum IgE.

Although the children were too young to diagnose asthma objectively, we next analyzed the asthma risk using PARS which has shown to be an effective asthma development estimate tool in a wide range of ethnicities, backgrounds, and susceptibilities[20,30,40]. Previous data in MPAACH demonstrated that Black children with AD were six times more likely to have asthma risk without AR or food allergy (FA) but with an increased genetic heritability for asthma and higher rates of exposures to secondhand smoke and traffic-related air pollution[10]. When we initially compared the double positive vs. double negative/single positive group, we did not observe a difference in PARS, likely due to the lower number of Black individuals in the double positive group. However, when stratified by race, Non-Black participants in the double positive group had comparable PARS to that of Black participants while the PARS of Non-Black participants in the double negative/single positive group was significantly lower than that of Black participants. This difference was even more apparent in the dry/sebaceous matched subset. Being double positive for *SA* likely drives the Non-Black individuals towards an increased asthma risk, an effect not seen in Black participants due to the already high risk of asthma. It is likely that the effect of the skin biome in Black participants is overshadowed by factors such as socioeconomic deprivation and environmental exposures that have previously been associated with asthma and confer a higher risk to Black children[41–43]. As such, environmental and socioeconomic influences may mask the impact of *SA* colonization in

Black participants. Our findings support a novel idea that the biome may also play a role in promoting asthma risk in Non-Black populations in the absence of these factors.

We also assessed doctor diagnosed FA and found no difference in proportion of children with FA between the double positive vs. double negative/single positive groups. While other studies have shown a relationship between *SA* skin colonization and FA[11,28,44], we hypothesize that the gut biome may be more relevant than the skin biome for development of FA in this subset. Indeed, previous literature has shown that individuals with FA have a distinct gut microbiota compared to healthy controls, and dysbiosis may precede the development of FA[45]. We likely don't see a relationship with FA because most studies showing a relationship between *SA* skin colonization and FA are performed predominantly in White populations. The subset of MPAACH participants in this study is made up of 63% Black children and Black individuals are less likely to have FA compared to Non-Black individuals in the MPAACH cohort. Thus, it is likely that the impact of the skin microbiome on FA may be diluted in our analysis due to self-reported race. However, we may also currently lack sufficient power to detect significant differences in FA in this subset. Previous studies have shown that the correlated variables of race, measures of socioeconomic status (SES), and environmental exposures also affect skin microbial composition and AD disease severity[46–49]. In the individual-level subset, approximately 63% of participants self-identified as Black and 64% were on public insurance, two variables that are highly correlated in our cohort. We adjusted for self-reported race in our primary analysis to account for microbial differences in the detection groups and outcomes of interest. However, future studies will need to be undertaken to identify distinct colonization patterns and AD outcomes specific to different race, exposures, and measures of SES.

The skin microbiome composition and diversity are influenced by site physiology, with differences in communities reported as early as a few days to weeks after birth[50,51]. *SA* colonizes moist areas including the antecubital fossa and the popliteal fossa, which are also common sites of AD lesions[52]. Sampling the same anatomical site using both contact plates and skin tapes strips would lead to inaccurate results (as any *SA* present might be sampled and removed from surface by one method). Thus, in the primary analysis, we included and adjusted for all three sampling areas (dry, moist and sebaceous) as fixed and random effects to account for potential effect of sampling areas on the outcomes. To further validate the robustness of our results and control for potential biases from common *SA* colonization areas, we conducted a sensitivity analysis excluding moist areas and only utilized samples with contact plate and tape strips sampled from either matched dry or matched sebaceous areas. While some of these results did not reach significance likely due to the lower sample size, the results of the sensitivity analysis were consistent with the primary results, indicating that the observed associations were robust regardless of sampling location.

There are numerous strengths and weaknesses of this study. The strengths include the large cohort size and the matched longitudinal clinical, sensitization, microbial, immunologic, and outcome data available in MPAACH. The study limitations include a focus on children restricting our ability to generalize our findings to older ages. Indeed, the skin microbiome varies over an individual's lifetime and over physiologic sites[24,53]. In this study, we found that children double positive for *SA* tended to be younger, but the observed associations in our study were robust regardless of age at visit, self-reported race, sex, season at visit and sampling areas. Other sampling/analysis pipelines (including qPCR, 16 S rRNA sequencing etc.) were not assessed in this study. However, our stringent quality controls measures following MSS (excluding samples with low sequencing depths and establishing a prevalence filter) ensured the use of only high-quality data for *SA* detection. Additionally, the samples in each category remained consistent after rarefaction/subsampling, ensuring that results were robust even after accounting for differences in sequencing depth between categories. Due to the nature of MPAACH, in which all participants have AD, we cannot make conclusions regarding *SA* detection patterns and AD onset. This study focused on *SA* and did not include presence and differential abundance of other microbes such as *S. hominis* and *S. epidermidis* which may affect *SA* colonization[54]. Additionally, other factors including variability in depth of sampling and biofilm production by *SA* and other microorganisms were not assessed in this study.

Collectively, our findings underscore the critical importance of selecting the appropriate microbial sampling methodology and downstream analysis techniques in AD for both clinical and research applications. Our novel findings suggest that detecting *SA* via multiple methodologies may be essential in determining AD and atopic march outcomes, irrespective of *SA* load. Furthermore, while MSS may be suitable for comprehensive microbial analysis, detecting *SA* on contact agar plates and other culture-dependent assays may be more practical and yields important insight on biologically and clinically relevant bacteria that influence the atopic disease course and outcomes.

Data availability

Raw count data is available at https://figshare.com/s/32b4d6eac5fd1ab063f6.

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Author contributions

AD processed samples and performed sequencing/analysis, performed the statistical analyses, prepared figures and tables and wrote the manuscript. WCC assisted in the statistical analyses and preparation of figures and tables and contributed to writing. EJ, BG, DM and DS processed samples and performed assays. LW and MH performed clinical assessments and collected biospecimen. SS processed samples and helped perform sequencing. LS contributed to the sample processing and editing. JMB, LJM, DBH and NJO contributed to conceptualization, methodology, statistical analyses and writing. GKKH contributed to the conceptualization, methodology, writing and supervision. All authors reviewed and revised the manuscript and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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