Safety and immunogenicity of an investigational 4-component *Staphylococcus aureus* vaccine with or without AS03_B adjuvant: Results of a randomized phase I trial

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Abbreviations: AEs, adverse events; AT, α-toxin; ATP, According–to-Protocol; CIs, Confidence Intervals; ClfA, clumping factor A; CPS5 and 8, capsular polysaccharides types 5 and 8; GMC, geometric mean concentration; GMT, geometric mean titer; ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; LNR, laboratory normal range; MRSA, methicillin-resistant *S. aureus*; OPA, opsonophagocytic assay; pIMDs, potential immune-mediated diseases: PVL, Panton-Valentine leucocidin; rEPA, recombinant exoprotein A from *Pseudomonas aeruginosa*; SAEs, serious adverse events; TT, tetanus toxoid.

We assessed the safety, reactogenicity and immunogenicity of a staphylococcal vaccine combining capsular polysaccharides types 5 and 8 (CPS5/8), conjugated to tetanus toxoid (TT), with mutated detoxified α-toxin (AT) and clumping factor A (ClfA). In this phase I, randomized, placebo-controlled, observer-blind trial (NCT01160172), 88 healthy 18- to 40-year-olds received CPS5-TT/CPS8-TT/AT/ClfA vaccine (5/5/10/10 μg or 10/10/30/30 μg dose, each with or without AS03_B adjuvant) or saline, at months 0, 1, 6. Solicited and unsolicited adverse events (AEs) were recorded for 7 and 30 d post-vaccination, respectively; potential immune-mediated diseases (pIMDs) and serious AEs (SAEs) were recorded throughout the study. Humoral and antigen-specific CD4$^+$/CD8$^+$ T-cell immunity were assessed from Day (D) 0 to D540 post-vaccination. The most frequently reported solicited local and general AEs were pain (78.6%–100% of subjects), fatigue (36.4%–93.3% of subjects post-dose 1–2) and headache (20%–44.4% of subjects post-dose 3). Overall, 4 SAEs and 2 potential immune-mediated diseases (pIMDs) (none fatal or vaccine-related) were reported. For each antigen, pre-vaccination seropositivity rates were high (85.7%–100%) and geometric mean concentrations (GMCs) in vaccine recipients sharply increased from D0 to D14, then plateaued to study end. Exploratory group comparisons suggested higher GMCs with higher dosage, without AS03_B effect. Vaccine-induced antibodies were functional (CPS5 opsonophagocytic assays, and AT/ClfA inhibition assays). AT- and ClfA-specific CD4$^+$ T-cells with Th0/Th1 cytokine profile were induced at low levels (median <0.05%) by each formulation (intracellular cytokine staining). In conclusion, no safety concerns were identified and each vaccine formulation induced robust humoral immune responses after the first vaccine dose.

Introduction

Staphylococcus aureus, both a commensal and a pathogen to its human host, can cause a broad spectrum of diseases, from skin and soft tissues infections, joint and bone infections, to bacteremia, endocarditis, pneumonia or toxic shock syndrome.[1] Following increased use of broad-spectrum antibiotics,

methicillin-resistant *S. aureus* (MRSA) clones emerged, representing up to 50% of strains identified in hospital settings.[2,3] Up to 60% of the general population is either persistently or intermittently colonized with *S. aureus*. However, although subjects who permanently carry *S. aureus* are at increased risk of staphylococcal bacteremia, they have a lower infection-related mortality rate[4] suggesting that some protective immunity to *S. aureus*

develops during carriage. Therefore, vaccination against *S aureus* may contribute to the prevention of such infections but the challenges for developing a staphylococcal vaccine are enormous.[5]

S. aureus virulence factors include the expression of capsular polysaccharides (CPS) and several cell-surface and secreted proteins implicated in different aspects of pathogenesis.[6-8] The capsule contributes to virulence by promoting the ability of *S. aureus* to resist opsonophagocytic killing.[9] *S. aureus* has also developed other immune evasion mechanisms.[10-17] In addition, it can survive under the form of small colony variants in mammalian cells, triggering subacute latent infections,[18] and is able to form persistent biofilm infections that prevent macrophage phagocytosishence triggering persistent infection.[19]

Previous staphylococcal immunotherapy or vaccine candidates failed to show efficacy in humans.[20-26] The lack of efficacy against infection occurred despite of high vaccine-induced antibody levels.[23,24,26] Due to the complexity of *S. aureus* virulence mechanisms and previous failures in developing effective *S. aureus* vaccines relying on one single type of antigen, the development of vaccines targeting multiple virulence factors (e.g. toxins and adhesion proteins in addition to CPS) by induction of humoral but also T-cell-mediated immunity has been considered.[4,27-29]

GSK Vaccines has developed a 4-component staphylococcal vaccine combining CPS types 5 and 8 (CPS5 and CPS8), conjugated to tetanus toxoid (TT) (CPS5-TT; CPS8-TT), with mutant forms of hemolysin-1 (α-toxin; AT) and ClfA. CPS5 and CPS8 are the most common CPS types identified among clinical isolates (up to 75% of isolates).[30] Previous clinical trials with other polysaccharide conjugate vaccines have shown that TT conjugates induced high antibody levels and a robust immune memory response.[31] AT, a heptameric pore-forming exotoxin produced by the majority of *S. aureus* pathogenic strains, is involved in cell lysis, opsonophagocytosis impairment[32,33] and survival of bacteria inside phagocytic cells.[34,35] *S. aureus* ClfA is a major surface adhesion factor that binds to fibrinogen and has anti-phagocytic activity.[11,22,36] Although well-conserved across strains, genetic variants with strain-specific epitopes have been recently described.[37] In humans, antibodies to AT were shown to be lower in patients with *S. aureus* sepsis,[38] and to protect against recurrent skin infections.[39] The protective effect of AT and ClfA was demonstrated in different animal models of *S. aureus* pneumonia, bacteremia and skin infection.[33,40-42] The combination of AT and CPS conjugates enhanced protection in a rat model of osteomyelitis,[43] while passive immunization with both CPS5 and ClfA antibodies inhibited the emergence of unencapsulated strains and significantly reduced the appearance of small colony variants.[44-46] Of note, IL-17-mediated cellular immunity has been found to play a key role in the protective effect induced by immunization with ClfA against *S. aureus* infection.[47]

The majority of invasive MRSA infections are reported in persons aged 50 y and above, and patients with underlying diseases such as chronic renal insufficiency or diabetes are at increased risk.[44,48] An Adjuvant System was included in the investigational vaccine, to test if the magnitude and persistence of immune responses can be enhanced and to eventually overcome immunosenescence or impaired immunity in certain target populations. AS03 Adjuvant System, previously used in influenza vaccines, was selected as it had been shown to induce robust antibody and T-cell immune response.[49-53]

Here we report the first-time-in-human Phase I trial evaluating the safety, reactogenicity and immunogenicity of the 4 formulations of the staphylococcal 4-component vaccine differing in terms of antigen dose and presence of $AS03_B$ adjuvant.

Results

Study population

88 subjects were enrolled, received dose 1 and were included in the total vaccinated cohort and according-to-protocol cohort for immunogenicity. Due to strict criteria related to abnormal safety lab parameters for administration of the subsequent doses of the vaccines, 72 subjects received dose 2 and 56 dose 3, but all 88 subjects were kept in the study for safety follow-up (**Fig. 1**). Subjects vaccinated with the second or the third vaccine dose were evenly distributed across groups except for the 10/30 group for which only 5/15 subjects received the third vaccine dose. Nine subjects withdrew, none because of an adverse event (AE). The mean age of the study subjects ranged from 30.1 to 31.9 y and majority were of Caucasian heritage. The gender ratio was unevenly distributed (**Table S1**).

Safety and reactogenicity

As shown in **Figure 2**, the most frequently reported solicited local AEs in the vaccine groups was pain (the majority was grade 1 after doses 1 and 2). Grade 3 pain was reported after dose 1 in 1 subject (5/10AS group) and after dose 3 in 2 subjects (1 in each of the high dose groups). Grade 3 redness was reported following 19 doses: 3 of 35, 4 of 36, 4 of 31 and 8 of 39 doses in the 5/10, 5/10AS, 10/30 and 10/30AS groups, respectively; 5 cases occurred after dose 1, 7 after dose 2 and 7 after dose 3. Grade 3 swelling was reported following 9 doses: 2 of 35, 3 of 31 and 4 of 39 doses in the 5/10, 10/30 and 10/30AS groups, respectively; 3 cases occurred after each vaccine dose. All Grade 3 solicited local AEs were reported in the vaccine groups and lasted for 1 to 3 d

The most frequently reported solicited general AEs in the study groups were fatigue (up to 93.3% of subjects following dose 1; up to 50% of subjects following dose 2 and 3;) and headache (up to 71.4% of subjects following dose 1; up to 44.4% of subjects following dose 2 and 3). Reported fever was usually mild (<38.5°C), except for one event in group 5/10AS and one in group 10/30AS where grade 2 fever (>38.5°C–39.5°C) was recorded (**Fig. 2**). Grade 3 general symptoms were reported 2 times: malaise in the placebo group after dose 1 and fatigue in the 10/30AS group after dose 3.

The percentage of subjects reporting at least 1 unsolicited AE was comparable in the vaccine groups vs placebo (46/58 [79.3%] subjects vs 25/30 [83.3%] subjects after dose 1–2; 20/35 [57.1%] subjects vs 9/21 [42.9%] subjects after dose 3) (**Table S2**). None of the grade 3 unsolicited AEs was considered by the investigators as vaccine-related.
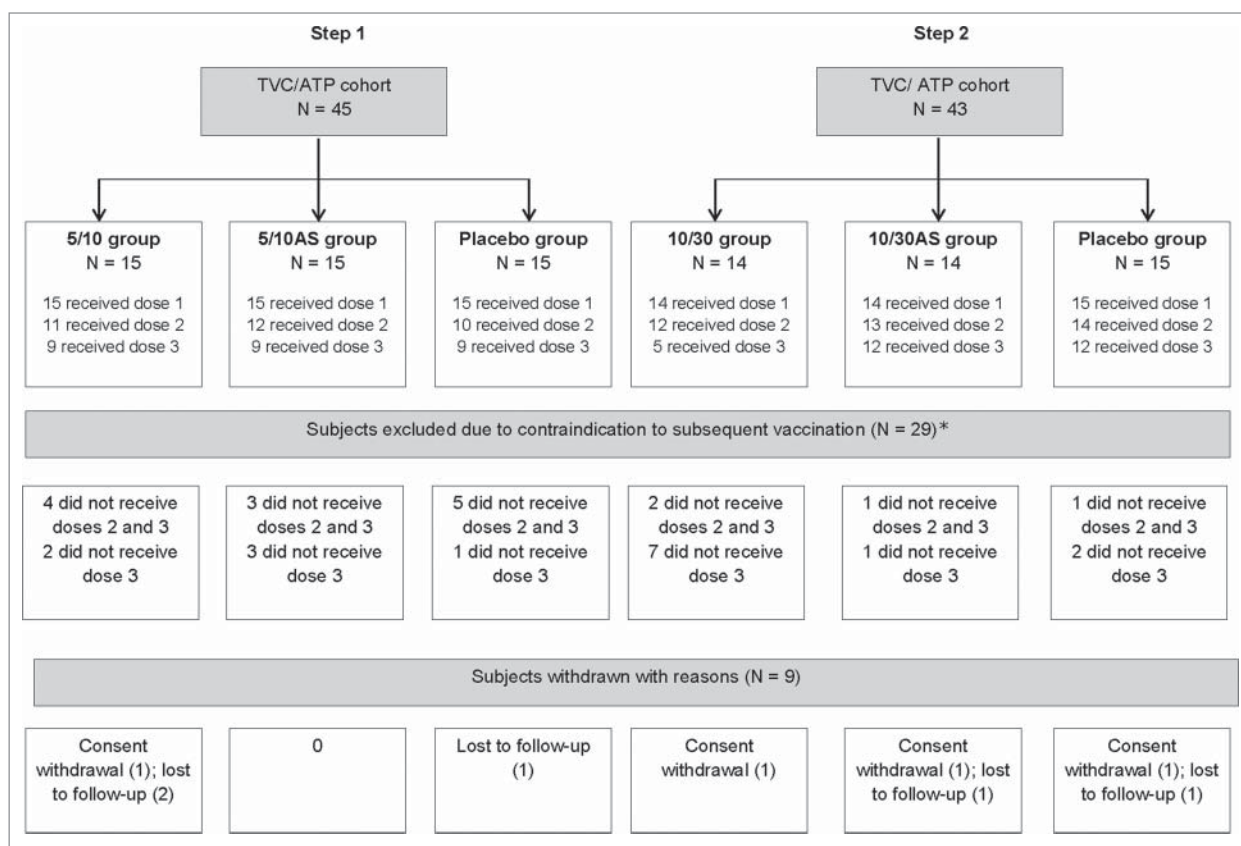
Figure 1. Participant flow diagram. Footnote to figure: 5/10 = 5 μg CPS5-TT; 5 μg CPS8-TT; 10 μgAT; 10 μg ClfA 5/10AS = 5 μg CPS5-TT; 5 μg CPS8-TT; 10 μg AT; 10 μg ClfA adjuvanted with AS03$_B$ 10/30 = 10 μg CPS5-TT; 10 μg CPS8-TT; 30 μg AT; 30 μg ClfA 10/30AS = 10 μg CPS5-TT; 10 μg CPS8-TT; 30 μg AT; 30 μg ClfA adjuvanted with AS03$_B$ TVC, total vaccinated cohort; ATP, according-to-protocol; N, number of subjects in a category. *Contraindications to subsequent vaccination: clinically relevant abnormal laboratory value (26), interval between dose 3 visit and visit before out of delay (>15 days) (2) and alcoholism (1). Laboratory values included: hematology (red blood cell count, white blood cell count, eosinophils count, neutrophils count, lymphocytes count, platelets count, reticulocytes index, hemoglobin), biochemistry (ALT, AST, creatinine, LDH, CPK, total bilirubin) and urinalysis (protein, glucose and blood).

Four serious adverse events (SAEs) (appendicitis in group 5/10, cerebral concussion in group 5/10AS, hospitalization for surgical removal of recto-vaginal nodule in group 5/10AS and hospitalization for surgical removal of cholelithiasis in placebo group) were reported, all during the follow-up period after dose 3; none were fatal or considered by investigators as vaccine-related and all resolved by study end. One case of pIMD was diagnosed in the 10/30 vaccine group (a "psoriasis-like" cutaneous eruption diagnosed at 350 d after dose 1 and considered by the investigator as not related to vaccination) and 1 in the placebo group (ulcerative colitis diagnosed in a female at 182 d after dose 3).

Few Grade 3 laboratory abnormalities were reported: elevated creatine kinase following intense physical activity (6 subjects), decrease from baseline by more than 2 g/dL for hemoglobin (but the hemoglobin level was within normal range, 1 subject), the presence of protein (4 subjects) or glucose (1 subject) in the urine. Elevated creatine kinase of Grade 4 intensity due to intense physical activity was reported for 1 subject in the 5/10AS group 330 d after dose 2.

Immunogenicity

Antibody response

Pre-vaccination seropositivity rates for anti-CPS5, -CPS8, -AT and -ClfA tested by multiplex or ELISA (IgG) and by functional assays were high (78.6%–100%), except for the ClfA functional assay (0.0%–10.0%).

As shown in **Figure 3**, geometric mean concentrations (GMCs) for anti-CPS5, -CPS8, -AT and -ClfA tested by multiplex or ELISA (IgG) sharply increased from pre-vaccination to Day-14 (4–47-fold in the low dose groups; 6–75-fold in the high dose groups). The second and third vaccine doses did not further enhance antibody concentrations. Exploratory group comparisons of GMCs at Day-14 post-dose 1 and post-dose 2, respectively, suggest a dose-response for CPS5, CPS8 and AT ($P < 0.05$) but not for ClfA (p = 0.2 and 0.8 for dose 1 and 2, respectively), with the high dose formulations eliciting higher GMCs than the low dose formulations. Exploratory group comparisons of GMCs did not show any differences between the adjuvanted and non-adjuvanted formulations ($P > 0.05$).
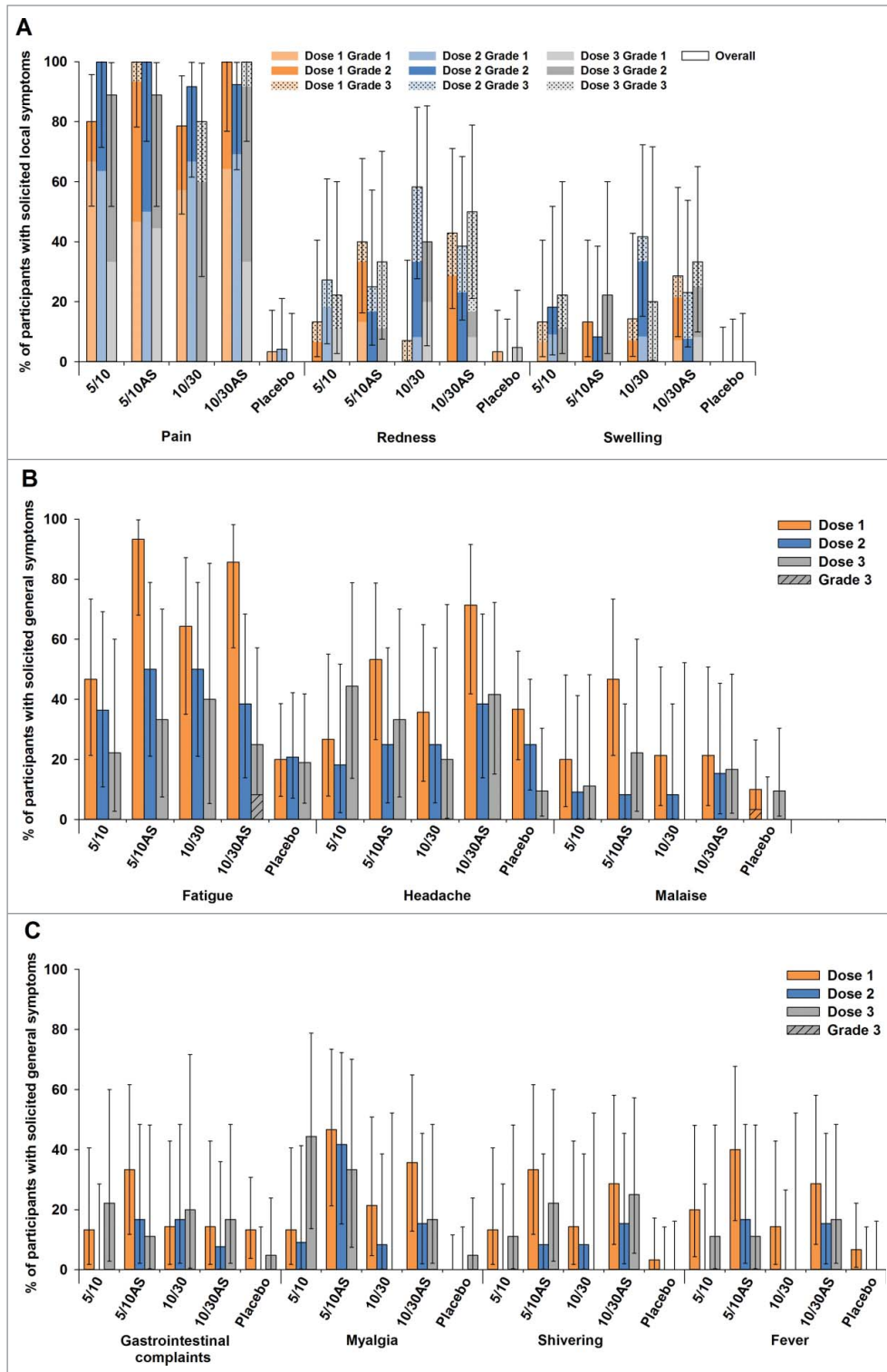
Figure 2. The incidence of solicited local (**A**) and general (**B** and **C**) adverse events reported during the 7-day post-vaccination period (total vaccinated cohort) Footnote to figure: 5/10 = 5 μg CPS5-TT; 5 μg CPS8-TT; 10 μgAT; 10 μg ClfA 5/10AS = 5 μg CPS5-TT; 5 μg CPS8-TT; 10 μg AT; 10 μg ClfA adjuvanted with AS03$_B$ 10/30 = 10 μg CPS5-TT; 10 μg CPS8-TT; 30 μgAT, 30 μg ClfA 10/30AS = 10 μg CPS5-TT; 10 μg CPS8-TT; 30 μg AT; 30 μg ClfA adjuvanted with AS03$_B$.

a dose effect for both AT (post-dose 2; p = 0.035) and ClfA (post-dose 1; p = 0.007) with higher GMTs achieved in the higher dose formulations. Adjuvant effect was seen for ClfA (p = 0.043), with higher GMT post-dose 1 in the pooled adjuvanted groups compared to the non-adjuvanted groups.

Antigen-specific CD4$^+$ and CD8$^+$ T-cells

Low levels (median < 0.05%) of AT- and ClfA-specific CD4$^+$ T-cells expressing at least 2 markers among IL-2, IFN-γ, IL-13, IL-17, TNF-α and CD40L were observed in all vaccine groups (**Fig. 4A and B**). Functional characterization of the CD4$^+$ T-cell response showed cells expressing at least IFN-γ (although at low levels) (**Fig. 4C and D**). No CD4$^+$ T-cells expressing IL-13 or IL-17 (**Fig. 4E, F, G and H**) and no CD8$^+$ T-cells were detected after vaccination with any of the formulations tested, irrespective of antigen dose and presence/absence of the AS03$_B$ adjuvant.

A robust TT-specific CD4$^+$ T-cell immune response was observed for all vaccine groups with a trend for higher CD4$^+$ T-cell frequencies in the AS03$_B$-adjuvanted vaccine groups (median after dose 1: 0.1%–0.6% for CD4$^+$ T-cells expressing at least 2 markers and 0.05%−0.3% for CD4$^+$ Th1 (**Fig. S2**). TT-specific CD8$^+$ T-cells were detected, although at low levels (median <0.01%).

Results from CPS5 opsonophagocytic assay (OPA) and AT and ClfA inhibition assays were generally consistent with IgG data. An increase of geometric mean titers (GMTs) was generally observed as of Day-7 post-dose 1 for each of the 4 vaccine groups and GMTs remained above those in the placebo group up to the study end (**Fig. S1**). Exploratory group comparisons however, suggested
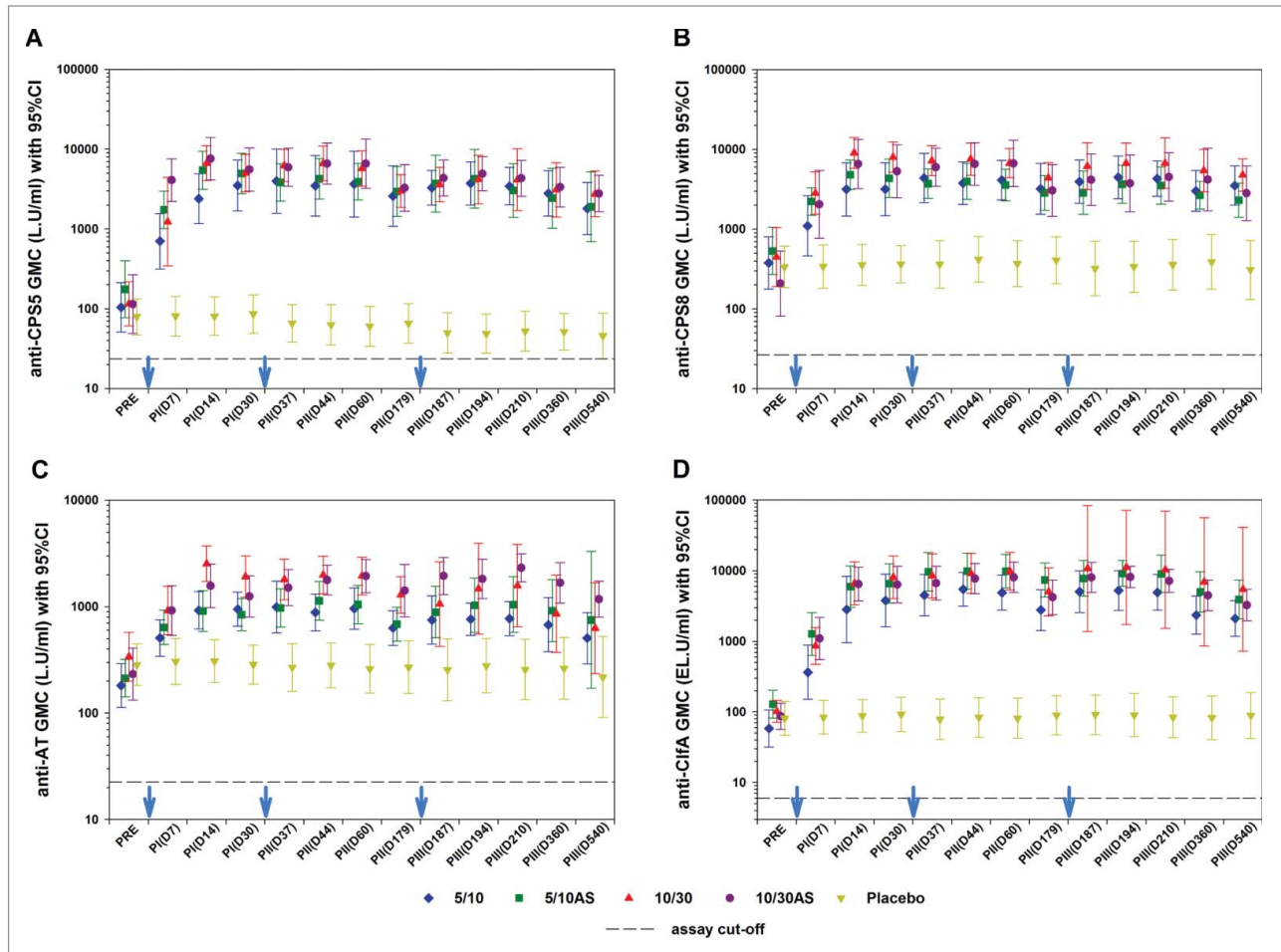
Figure 3. Geometric mean concentrations of anti-capsular polysaccharide types 5 (panel A) and 8 (panel B), anti-AT (panel C) and anti-ClfA antibodies (panel D) (according-to-protocol cohort for immunogenicity) Footnote to figure: 5/10 = 5 μg CPS5-TT; 5 μg CPS8-TT; 10 μg AT; 10 μg ClfA 5/10AS = 5 μg CPS5-TT; 5 μg CPS8-TT; 10 μg AT; 10 μg ClfA adjuvanted with AS03$_B$ 10/30 = 10 μg CPS5-TT; 10 μg CPS8-TT; 30 μg AT; 30 μg ClfA 10/30AS = 10 μg CPS5-TT; 10 μg CPS8-TT; 30 μg AT; 30 μg ClfA adjuvanted with AS03$_B$ 95% CI; 95% confidence interval; GMC, geometric mean concentration; AT; α-toxin; CPS5 and 8, capsular polysaccharides types 5 and 8; TT, tetanus toxoid; ClfA, clumping factor A; L.U, Luminex units; EL.U, ELISA units. PRE, pre-dose 1; PI(D7), 7 d post-dose 1; PtdIns(D14), 14 d post-dose 1; PI(D30), 30 d post-dose 1; PII(D37), 7 d post-dose 2; PII(D44), 14 d post-dose 2; PII(D60), 30 d post-dose 2; PII(D179), pre-dose 3; PIII(D187), 7 d post-dose 3; PIII(D194), 14 d post-dose 3; PIII(D210), 30 d post-dose 3; PIII(D360), 1 y post-dose 1 or 6 months post-dose 3; PIII(D540), 1.5 y post-dose 1 or 1 y post-dose 3 The cut-off values for these assays (dashed line) were 23.6 L.U/mL for CPS5, 26.5 L.U/mL for CPS8, 22.5 L.U/mL for AT and 6 EL.U/mL for ClfA. Blue arrows indicate days of vaccine or placebo administration. *Only 4 subjects were included in the analysis of ClfA antibody levels in the 10/30 group from PIII(D187) onwards. This explains the large 95% CI for these time points.

S. aureus carriage

No trend was observed in carriage status (**Table S3**). However, no firm conclusions can be made due to the low number of subjects included in the analysis.

Discussion

In this first-time-in-human study, no safety concerns were identified with the staphylococcal vaccine formulations tested. The most frequently reported Grade 3 solicited AEs were redness and swelling at injection site, with the highest frequencies observed in groups receiving the higher antigen dose formulation. The unsolicited Grade 3 AEs and the 4 SAEs reported during the trial were assessed by the investigator to be unrelated to the study vaccine administration and all resolved by the study end. Except for local solicited symptoms and fever, we did not discriminate between grade 1 and 2 AEs, which is a limitation in this study, as grade 1 symptoms are substantially different from grade 2.

This safety profile is similar to that reported in previous clinical trials with related antigens. *StaphVAX*[TM], containing *S. aureus* CPS5 and CPS8, each conjugated to rEPA, was shown to have a clinically acceptable safety profile when administered at up to 100 μg antigen per dose to more than 3,000 subjects.[23,24,54] Other vaccines conjugated to tetanus toxoid such as *Haemophilus influenzae type B* (Hib), meningococcal and pneumococcal polysaccharide conjugate vaccines, have been extensively administered in all age groups worldwide, including infants, and have been shown to have a clinically acceptable safety profile, with a low incidence of grade 3 solicited symptoms and SAEs.[55-57]
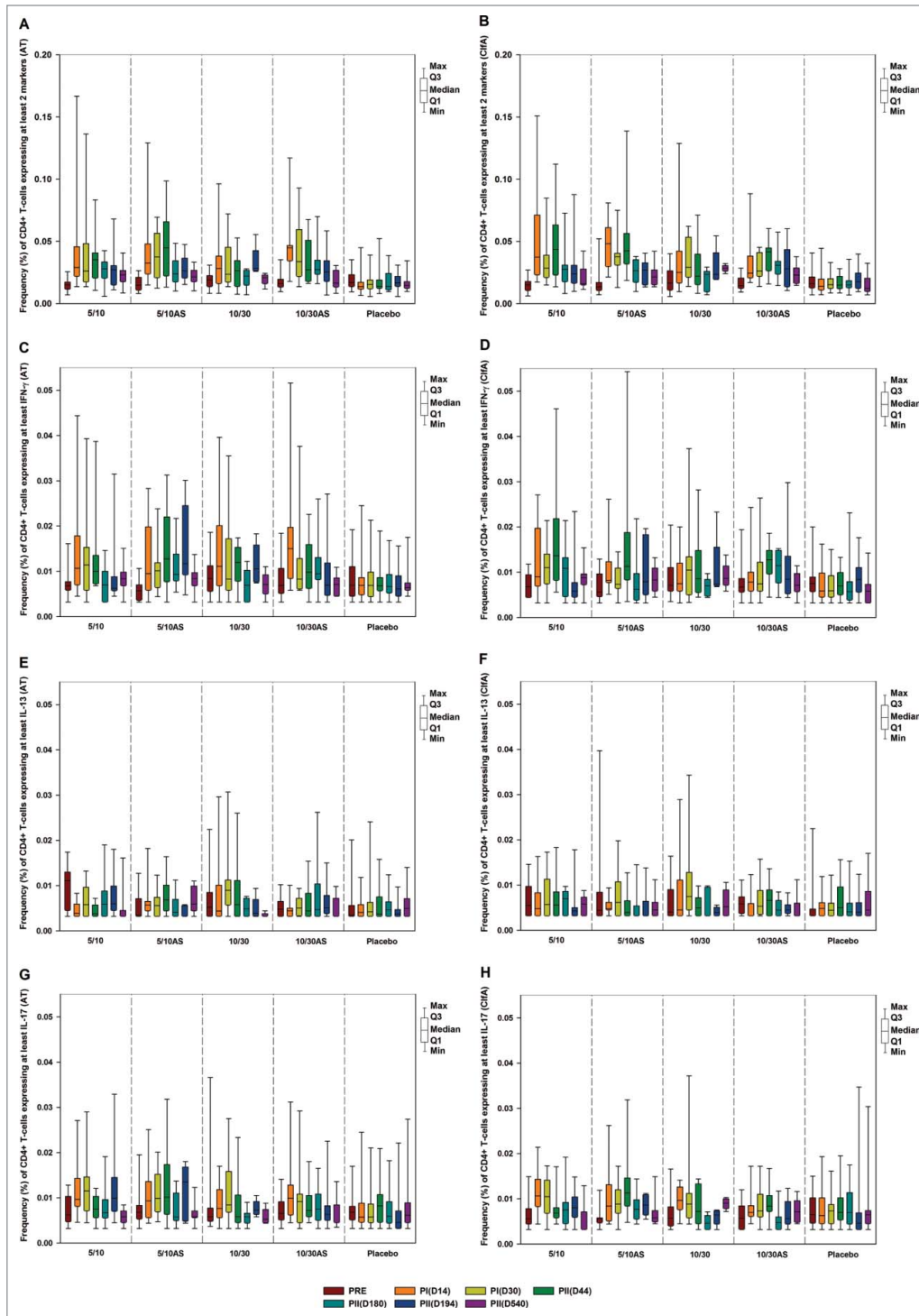
Figure 4. Frequency (%) of *S. aureus* AT-specific (panels A, C, E and G) and ClfA-specific (panels B, D, F and H) CD4$^+$ T-cells expressing at least 2 markers among IL-2, IFN-γ, IL-13, IL-17, TNF-α and CD40L (panels A and B), at least IFN-γ (CD4-Th1 profile) (panels C and D), IL-13 (panels E and F) and IL-17 (panels G and H) prior and after each vaccination (according-to-protocol cohort for immunogenicity) Footnote to figure: 5/10 = 5 μg CPS5-TT, 5 μg CPS8-TT, 10 μg AT, 10 μg ClfA 5/10AS = 5 μg CPS5-TT, 5 μg CPS8-TT, 10 μg AT, 10 μg ClfA adjuvanted with AS03$_B$ 10/30 = 10 μg CPS5-TT, 10 μg CPS8-TT, 30 μg AT, 30 μg ClfA 10/30AS = 10 μg CPS5-TT, 10 μg CPS8-TT, 30 μg AT, 30 μg ClfA adjuvanted with AS03$_B$ AT, α-toxin; ClfA, clumping factor A; PRE, pre-dose 1; PtdIns(D14), 14 d post-dose 1; PI(D30), 30 d post-dose 1; PII(D44), 14 d post-dose 2; PII(D179), pre-dose 3; PIII (D194), 14 d post-dose 3; PIII(D540), 1.5 y post-dose 1 or 1 y post-dose 3; Min/Max, Minimum/Maximum; Q1,Q3, First and third quartile.

A previous study conducted with an AS03$_A$-adjuvanted pneumococcal and non-type-able *H. influenzae* (NTHi) tri-protein vaccine was stopped due to high reactogenicity and systemic adverse events.[53] The increased reactogenicity of AS03-adjuvanted vaccines compared to non-adjuvanted vaccines[53,58,59] may be the consequence of the enhanced migration of monocytes and macrophages and the production of cytokines and chemo-kines at the injection site and in the draining lymph nodes, secondary to AS03 adjuvant administration.[60] Our study was a Phase I study enrolling a limited number of subjects, and thus no formal comparisons between vaccine groups were done to explore differences in reactogenicity profile between groups. However, we did not observe major differences in the frequency of grade 3 solicited AEs between adjuvanted and non-adjuvanted vaccine groups; nor did we observe increased reactogenicity with subsequent doses 2 or 3. In our study, we elected to use 2 relatively low antigen dosages for dose escalation, and a formulation of AS03 that contains half the dose of α-tocopherol (AS03$_B$). No safety concerns were raised during safety evaluations prior to each subsequent dose escalation.

We observed robust antibody responses to each of the 4 staphylococcal antigens as of post-dose 1, with relatively stable antibody concentrations until study end. This observation and the high pre-vaccination seropositivity rates suggest a booster-like response, potentially explaining the lack of effect of doses 2 and 3. This was also observed in clinical trials with

StaphVAX™, in subjects immunologically primed to *S. aureus* due to previous exposure.[54,61,62] In our study, the identified antibodies were functional, and persisted well above the preexisting vaccination levels up to one year after the last dose. Due to technical issues in the development of the OPA assay, we were not able to demonstrate the functionality of the CPS8 antibodies induced by our vaccine. Previously it was shown that staphylococcal polysaccharide conjugate vaccines induce opsonophagocytic antibodies against both CPS5 and CPS8.[54] The rapid increases in antibody concentrations following the first dose, with the fairly consistent trend for all antigens tested to plateau with little apparent difference by adjuvant effect, serve to lessen the impact of declining subject numbers receiving subsequent doses on the interpretation of overall immunogenicity. However, the decreased subjects number receiving dose 3 coupled with the increased variance (for anti-AT and anti-ClfA) in the high dose unadjuvanted group, limits our ability to fully assess the potential impact of the 6-month booster dose. Our data suggest that a single dose of our vaccine may suffice to induce long-lasting antibodies against each of the 4 antigens included in the vaccine.

The antibody response in our study was higher with the higher dose formulations. In other staphylococcal vaccine trials conducted in healthy subjects or end-stage renal disease patients and using vaccines with related antigens, antibody titers also increased with the antigen dose.[54,63,64] We evaluated 5 μg and 10 μg of CPS5 and CPS8, whereas *StaphVAX*™ contains 100 μg of each CPS type. In a tri-valent vaccine combining staphylococcal CPS types 5 and 8 conjugated to CRM_{197} and a mutant form of ClfA, 10, 30 or 100 μg of each CPS were previously evaluated and the dose of 30 μg was selected for further development.[64,65] The fold increases in the anti-CPS IgG response 1 month after the first dose in our study were at least as high as those observed previously with *StaphVAX*® (30–37-fold and fold7- for *StaphVAX*® vs 48-fold and 25-fold in the current study for types 5 and 8, respectively).[54] This is not unexpected as it was previously observed with other polysaccharide conjugate vaccines that a higher antigen content does not necessarily translate into a better antibody response.[66] The different carrier proteins can also play a role in the immunogenicity of conjugate vaccines.[31,67] We observed an antigen dose-effect for the AT (post-dose 2) and ClfA assays (post-dose 1), with higher functional antibody levels in the higher 30 μg dose formulation groups. In one trial testing a recombinant AT and a sub-unit of Panton-Valentine leukocidin (PVL) toxoids, Landrum *et al.* reported statistically significantly higher AT-specific antibody GMC levels in the group receiving 50 μg of AT compared with the other groups who received lower antigen doses.[63] In a tri-valent vaccine, 10, 60 and 200 μg of ClfA were evaluated and the 60 μg dose was selected for further clinical trial evaluation.[64,65] In the current study, we evaluated only 2 escalating doses for the antigen part of our vaccine and we can therefore not conclude on the optimal dosage for the AT and ClfA antigens in our vaccine; it should be noted however that the addition of a 2nd and 3rd dose did not further increase the antibody response.

No increase in the antibody response against CPS5, CPS8 and AT was observed when formulations were adjuvanted with $AS03_B$. This may be due to the high pre-vaccination seropositivity rates to CPS5, CPS8 and AT. Also, high levels of anti-ClfA IgG natural antibodies were detected by ELISA in nearly all subjects. In contrast, functional antibodies blocking ClfA binding to fibrinogen were detected in very few (less than 10%) subjects pre-vaccination, while post-vaccination, higher titers were observed in the adjuvanted groups compared to the unadjuvanted ones. High anti-ClfA antibody levels have also been observed during natural infections with *S. aureus,* but their presence did not correlate with protection against infection[21] and it has been shown that contrarily to vaccine-induced antibodies, they do not block ClfA binding to fibrinogen.[68]

Passive and active immunization strategies relying on antibodies targeting one single virulence factor to prevent *S. aureus* infection have failed to date, suggesting the need to consider multicomponent vaccines and a possible role for T-cell-mediated immune response.[22] Th17 lymphocytes play an important role in the immunity against *S. aureus* skin or lung infections in mice via IL-17 production, which plays a key role in neutrophil recruitment and in the activation of keratinocytes and mucosal cells.[69,70] High incidences of *S. aureus* skin and soft tissue infections were observed in patients with impaired Th17-cell function, such as patients with Job (hyper-IgE) syndrome,[71-73] HIV patients with low peripheral blood $CD4^+$ T cell counts,[74] patients treated with prednisone,[20,73,74] or in subjects with atopic dermatitis.[75,76] *In vivo* mouse models of *S. aureus* cutaneous infection have demonstrated the role of γδ T-cells in IL-17-dependent production of pro-inflammatory cytokines and chemokines by keratinocytes and subsequent neutrophils recruitment and bacterial clearance.[77,78] Altogether, new approaches focusing on Th1 and Th17 T-cells may be important to consider when developing staphylococcal vaccines.[20]

Several studies in humans have shown that AT stimulation may induce a combined Th1-Th17 immune response in peripheral blood mononuclear cells and isolated $CD4^+$ T-cells.[79,80] Also ClfA was shown to induce IL-17-producing cells in mice.[47] Surprisingly, in our study, all 4 vaccine formulations, whether with or without $AS03_B$ adjuvant, induced only low levels of AT- and ClfA-specific $CD4^+$ T-cells expressing at least 2 cytokine markers with a trend for IFN-γ expression; no $CD4^+$ T-cells expressing IL-13 or IL-17 and no induction of $CD8^+$ T-cells were detected. This overall weak $CD4^+$ T-cell response observed in our study was unexpected given that T-cell epitope predictions realized on various populations (Japan, USA, Europe) with the focus on more prevalent alleles revealed that AT and to a lesser extent ClfA contained $CD4^+$ T-cell epitopes. The T-cell assay showed robust TT-specific $CD4^+$ T-cell responses induced by all vaccine formulations, with a clear adjuvant effect for the higher dose formulation. This suggests that the $CD4^+$ T-cell response to ClfA and AT was impaired, but not the response to TT (used as a positive control). The absence of the Th17 response could potentially be explained by the overall weak T-cell response as well as the short-term (2 hours stimulation) T-cell read-out used for this study. Indeed, longer term T-cell cultures seem to be required to reveal antigen specific Th17 populations.[84,85] The lack of adjuvant effect is also unexpected, as previous clinical

trials have shown that AS03$_A$-adjuvanted vaccines induced stronger antigen-specific CD4$^+$ T-cell responses with a Th1 (IFN-γ) and Th17 cytokine profile than those induced by non-adjuvanted vaccine.[52,53,81,82] Those trials used AS03$_A$ while our study used AS03$_B$. However, we do not believe this could explain the lower CD4$^+$ T-cell response in our trial as AS03$_B$ was also shown to be a strong inducer of CD4$^+$T-cell response with other antigens[83] and it had marked effect upon stimulation in the TT ICS assay in our current study.

In the development of a vaccine capable of inducing an appropriate Th17 response, it is important to choose an antigen and adjuvant system that would activate the inflammasome.[84] IL-1β plays a key role in promoting IL-17 production,[85,86] as was demonstrated with pneumolysin.[87] Pneumolysin is one of the antigens included in the pneumococcal-NTHi vaccine adjuvanted with AS03$_A$ described above and for which a Th17 response was observed, suggesting the importance of the combination of both the antigen and adjuvant in eliciting an appropriate T cell response.[53] IL-6, another cytokine that plays a key role in the differentiation of the Th17 cells,[88,89] is induced by AS01$_B$-adjuvanted vaccines but to a much lower extent by AS03$_A$-adjuvanted vaccines.[60] Therefore, other adjuvants such as AS01$_B$ which has been shown to induce a potent T-cell response with different types of antigens,[90-92] could enhance the Th1/Th17 immune response. Additionally, mucosal (intranasal) administration of the vaccine may be an alternative route to induce a Th17 response.[84] Last but not least, S. aureus is a commensal microorganism that survives despite the host immune system.[1] Commensalism has been shown to occur through TLR-2-dependent induction of immune regulatory mechanisms.[93] S. aureus is also able to promote T-cells and innate cells to produce IL-10.[89,94] Immuno-downregulation may have therefore contributed to the failure of the tested vaccine formulations to induce robust antigen-specific T-cell responses.[16]

In conclusion, no safety concerns were identified with any of the staphylococcal vaccine formulations tested. Each of the vaccine formulations induced a robust antibody response post-dose 1 which then plateaued up to the study end. Vaccine-induced antibodies against CPS5, AT and ClfA were functional. Exploratory group comparisons suggested higher antibody levels in the groups receiving a formulation with higher antigen content and no adjuvant effect. Only low levels of AT and ClfA-specific CD4$^+$ T-cells were detected upon administration of the 4 vaccine formulations.

Materials and Methods

Study design and subjects

This Phase I, randomized, observer-blind, placebo-controlled, dose-escalation study (NCT01160172) across 6 vaccine groups was conducted at the ImmuneHealth center in La Louvière, Belgium, between 2010 and 2012. Healthy women and men 18–40 y of age were enrolled in 2 steps. In Step 1, 45 subjects were randomized at first dose using an internet-based randomization system and a randomization ratio of 1:1:1 to receive the 5/10 vaccine formulations (CPS5-TT/CPS8-TT/AT/ClfA; 5/5/10/ 10 μg) either with or without AS03$_B$ (5/10 and 5/10AS groups), or placebo (saline). In Step 2, 43 additional subjects were randomized (randomization ratio: 1:1:1) to receive the 10/30 formulations (CPS5-TT/CPS8-TT/AT/ClfA; 10/10/30/30 μg) with or without AS03$_B$ (10/30 and 10/30AS groups), or placebo. Subjects received 3 doses of the study vaccines or placebo according to a 0–1–6 month schedule and were followed for 1 y after the last vaccine dose administration. Dose escalation from the 5/10 to 10/30 formulations was conditioned by a favorable safety evaluation of the 5/10 formulations. The second and third vaccine doses were also only administered on the condition that a favorable outcome was obtained from the available safety data.

This study was set up in an observer-blind manner, where the vaccine recipient and staff responsible for the evaluation of any study endpoint were unaware of which vaccine was administered. As the vaccines in this study were of different appearance, vaccine preparation and administration were done by unblinded authorised medical personnel who did not participate in any of the study clinical evaluation assays.

Female subjects were not pregnant or lactating. Subjects meeting the following criteria were excluded: use of any investigational or non-registered product within 30 d preceding the first dose of study vaccine; any clinically significant acute or chronic infection proven or suspected to be caused by S. aureus and requiring antibiotic treatment within the 6 months preceding the first vaccination; and previous administration of any investigational S. aureus vaccine or antibodies. Subjects with clinically relevant out-of-range values for hematology (hemoglobin, red blood cells, white blood cells and differential count, platelets and reticulocytes index), biochemistry (ALT, AST, creatinine, LDH, CPK, total bilirubin) or urinary (blood, proteins, glucose) tests at screening were also excluded. Contraindications to subsequent administration of the study vaccines included: hypersensitivity reaction, pregnancy, pIMDs, bleeding or coagulation disorders, or any clinically relevant abnormal hematological, biochemical or urinary laboratory value following vaccine administration. All out-of-range values were considered clinically relevant contraindications, with the following exceptions: menstrual blood in urine; higher CPK levels without any other abnormal values and a plausible explanation (such as sport activity); WBC <10,800/ mm^3 (upper limit of laboratory normal range (LNR) was 10,000/mm^3), WBC >10,800 cell/mm^3 if WBC remain stable over time (i.e. maximum 10% change with respect to baseline); neutrophils >8,000 cell/mm^3 if neutrophils remain stable over time (i.e., maximum 10% change with respect to baseline); hemoglobin or RBC above the LNR; RBC below the LNR and hemoglobin normal; reticulocyte index >5%, if the hemoglobin level is within the normal range; LDH or total bilirubin <LNR, and protein concentration in urine <30 mg/dL.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The protocol and associated documents were reviewed and approved by an institutional review board. All subjects signed a written informed consent prior to any study procedures. The protocol summary was posted on ClinicalTrials.gov (NCT01160172) before study start. The

full protocol is available at http://www.gsk-clinicalstudyregister.com/ (GSK study ID 113949).

Study vaccines

The investigational *S. aureus* vaccine contained 5 or 10 μg of each CPS5-TT and CPS8-TT; 10 or 30 μg of AT and 10 or 30 μg of ClfA. Mutant AT and ClfA forms were produced using recombinant *Escherichia coli* strains. The detoxified AT H35R mutant, included in the vaccine, lacks pore-forming activity, while the ClfA mutant (ClfA_$P_{336}Y_{338}$) lacks fibrinogen-binding activity. For simplicity, they are referred to as AT and ClfA throughout this paper. AS03$_B$ is an Adjuvant System containing α-tocopherol and squalene in an o/w emulsion (5.93 mg tocopherol). Adjuvanted doses were prepared by mixing the *S. aureus* antigens and AS03$_B$ (1:1) from separate mono-dose vials. Saline placebo was used as a negative control. A volume of 0.5 mL of the assigned study vaccine or placebo was administered intramuscularly into the deltoid within 4 hours after preparation.

Assessment of safety and reactogenicity

Solicited local and general AEs were recorded for 7 d after each vaccine dose and unsolicited AEs for 30 d after each dose. The intensity of all solicited AEs was graded on a scale of 0–3. pain: (1) any pain neither interfering with nor preventing normal every day activities, (2) painful when limb was moved and interfered with every day activities, (3) pain at rest, preventing normal every day activities; redness or swelling by diameter (1) >20 mm to ≤50 mm; (2) >50 to ≤100 mm; (3) >100mm; fever by temperature (1) ≥37.5°C to ≤38.5°C, (2) >38.5°C to ≤39.5°C, (3) >39.5°C. Other AEs that prevented normal activities were considered as Grade 3 (grade 1–2 AEs were recorded, but not analyzed). Data regarding pIMDs and SAEs were collected throughout the study period.

Safety laboratory parameters (blood: ALT, AST, CPK, creatinine, LDH, total bilirubin, hemoglobin, RBC, WBC, neutrophils, eosinophils, lymphocytes, neutrophils, basophils, monocytes, platelets, reticulocyte index) were assessed. The intensity of abnormal laboratory parameters was graded according to the Food and Drug Administration Toxicity Grading Scale for laboratory abnormalities, when available.[95]

Based on clinical judgment, the investigators assessed the relationship between the study vaccine and the occurrence of each AE/SAE.

Assessment of immunogenicity

Serum samples for evaluation of anti-CPS5, anti-CPS8, anti-AT (luminex-based multiplex assay using purified native CPS5, CPS8, and recombinant AT as coating antigens) and anti-ClfA concentrations (ELISA using purified native ClfA as coating antigen) were collected prior to and at 7, 14 and 30 d after each vaccine dose, and 6 and 12 months after the last dose (i.e. Days 0, 7, 14, 30, 37, 44, 60, 179, 187, 194, 210, 360 and 540).

Anti-CPS5 opsonophagocytic activity titers were measured by an OPA. Lack of sensitivity, presumably linked to the complement used in the assay, prohibited the development of OPA for CPS8 before study conclusion and thus this assay was not performed. Functionality of anti-AT and anti-ClfA was measured by specific *in vitro* inhibition assays (hemolysis and fibrinogen-binding inhibition assay, respectively). In order to improve its sensitivity, the initial ClfA inhibition assay was adapted after the testing of post-primary samples. Samples were assessed using the initial test up to Day 210 for Step 1 (groups 5/10, 5/10AS and placebo) and up to Day 60 for Step 2 (groups 10/30, 10/30AS and placebo), after which a new test was used up to study end for both Steps.

Blood samples were collected for PBMC separation prior to and 14 d after each vaccine dose and 12 months after the last dose (i.e., Days 0, 14, 30, 44, 179, 194 and 540).The frequency of antigen-specific CD4$^+$ and CD8$^+$ T-cells elicited by AT, ClfA and TT, as characterized by cytokines secretion after short-term in vitro stimulation (IL-2, IFN-γ, TNF-α, IL-13, IL-17 and CD40L), was measured by flow cytometry using intracellular cytokine staining (ICS) as previously described.[81]

Assessment of S. aureus carriage

Swabs for the evaluation of nose, throat, armpit and groin colonization with *S. aureus* were collected at screening and prior to and 30 d after each vaccine dose and 12 months after the last dose (i.e. Days 0, 30, 60, 180, 210 and 540). Cultures for *S. aureus* were performed at the clinical laboratory of Center Hospitalier Universitaire Tivoli, La Louvière, Belgium, using standard methods.

Statistical analysis

The safety analysis was performed on the Total Vaccinated Cohort (TVC) that included all vaccinated subjects. The incidence of AEs per study group was calculated with exact 95% Confidence Intervals (CIs).

The immunogenicity analysis was performed on the According–to-Protocol (ATP) cohort for immunogenicity. This cohort included all subjects who met the eligibility criteria, complied with the protocol procedures and for whom immunogenicity data was available for at least one post-vaccination time point and for at least one antibody. Seropositivity rates, GMCs (for anti-CPS5, -CPS8, -ClfA and -AT tested by multiplex or ELISA assays) and GMTs (for functional characterization of antibodies) with their 95%CIs were calculated for each study group. Antibody response to each vaccine antigen 2 weeks after each vaccine dose administration was compared between the study groups using a 2-way ANCOVA model on the log-transformed concentration/titer with the antigen content and adjuvant as fixed effect and the pre-vaccination log-transformed concentration as regressor. Interaction (antigen content, adjuvant) was considered as statistically significant if the *P*-value was <0.10. Main factors (antigen content, adjuvant) were considered as statistically significant if the *P*-value was <0.05. These comparisons were exploratory. No adjustment for multiplicity has been performed. The frequency (%) of antigen-specific CD4$^+$/CD8$^+$ T-cells was determined by study group using descriptive statistics. Change of carriage status from baseline to each post-vaccination time point was summarized by study group.

Trademark Statement

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