Appendix

Safety and immunogenicity of chlamydia vaccine candidate CTH522 adjuvanted with CAF01 or AH: a first-in-human, randomised, double-blind, placebo-controlled, phase 1 study.

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PROTOCOL DETAILS

Inclusion criteria

1. Healthy females between 18 and 45 years of age on the day of first trial vaccination
2. Had provided signed informed consent
3. Was willing and likely to comply with the trial procedures
4. Was prepared to grant authorised persons access to their medical record
5. Was willing to use acceptable contraceptive measures during the trial (2 weeks before and 2 weeks after the trial). Heterosexually active female capable of becoming pregnant (in addition to requiring male partner to use condoms) had agreed to use hormonal contraception or complete abstinence from at least 2 weeks before the first vaccination until at least 2 weeks after the trial.

Exclusion criteria

Subjects were not included in the trial if they fulfilled any of the following criteria:

1. Had confirmed history of Pelvic Inflammatory Disease or significant gynaecological diseases
2. Was positive for *C. trachomatis* (urine, PCR)
3. Was positive for gonorrhoea (urine), HIV, Hepatitis B/C, syphilis (blood)
4. Had a positive urine pregnancy test
5. Had a significant active disease - such as cardiac, liver, immunological, neurological, psychiatric; or clinically significant abnormality of haematological or biochemical parameters
6. Had BMI of 35 kg/m^2 or greater
7. Was currently participating in another clinical trial with an investigational or non-investigational drug or device
8. Had received, or planned to receive, any immunisation within 14 days of the start of the trial or any of the immunisation visits in this trial
9. Was currently receiving treatment with immunosuppressive agents e.g. oral, inhaled, nasal or injected corticosteroids (topical steroids were allowed, unless applied to the IM injection site)
10. Was using an intrauterine device
11. Had a condition which in the opinion of the investigator was not suitable for participation in the trial
12. Had known or confirmed allergy to any of the vaccine constituents.

Unblinding

The randomisation was performed via the eCRF system. Randomisation data were kept strictly confidential, accessible only to authorised unblinded persons, until the time of unblinding. Only when the trial had been completed and the database locked, the codes were broken and made available for data analysis.

The data safety management board (DSMB) held two prescheduled meetings. Blinded safety data were uploaded to a secured website prior to the meetings. The secure website was shared between the DSMB members and Biostata only. Unblinded information was requested by the DSMB Chair at their first prescheduled meeting (treatment group for subject no 011), and at their second pre-scheduled meeting (access to unblinded data to enable evaluation of reported AEs by treatment group).

No unblinding was performed during the trial for the purpose of expedited reporting of SUSARs.

Vaccines

The multivalent vaccine antigen CTH522 is a recombinant version of the major outer membrane protein (MOMP) from *C. trachomatis*. CTH522 contains conserved T cell epitopes, which are frequently recognised by *C. trachomatis*-infected patients. Furthermore, CTH522 is linked to an optimised module that contains the main neutralising epitope from the four most frequent human genital *C. trachomatis* serovars[1,2].

The vaccine components, CTH522, CAF01, aluminium hydroxide (AH) and Tris buffer, were manufactured by the Production Department at SSI according to GMP, and a qualified person from the QA Department at SSI was responsible for the release of these components. The vaccines were mixed immediately before administration by unblinded staff. Only one batch of each of the vaccine components were used in the trial.

The compositions of vaccines are listed in the following.

CTH522:CAF01 (IM)

Ingredient	Per dose volume 0·6 ml	Function
CTH522	85 µg	Active substance
Glycerol	22 mg	Stabiliser
Tris	1·2 mg	Buffer
DDA	625 µg	Adjuvant
TDB	125 µg	Adjuvant
Water for injection	up to 0·6 ml	Diluent

CTH522:AH (IM)

Ingredient	Per dose volume 0·6 ml	Function
CTH522	85 µg	Active substance
Glycerol	22 mg	Stabiliser
Tris	1·1 mg	Buffer
Aluminium hydroxide (AH)	0·425 mg	Adjuvant
Water for injection	up to 0·6 ml	Diluent

CTH522 (IN)

Ingredient	Per dose volume 0·25 ml*	Function
CTH522	30 µg	Active substance
Glycerol	7·5 mg	Stabiliser
Tris	0·75 mg	Buffer
Water for injection	up to 0·25 ml	Diluent

Placebo (IM)
0·9% saline (NaCl) stock purchased commercially.

Collection of AEs

Solicited AEs were collected as follows:

- Local injection site reactions (recorded at any visit) after intramuscular (IM) administration (pain, erythema, tenderness, pruritus, warmth, stiffness and swelling)
- Local reactions (recorded at any visit) after IN administration (discharge, including bleeding, congestion, discomfort, sneezing and cough).
- Systemic reactions (recorded at any visit) after IM and IN administration (abnormally raised temperature (> 38·3°C), chills, myalgia, malaise, fatigue, rash, headache, nausea and vomiting, and clinically significant abnormal values among full blood count, liver function test and renal profile results)

Unsolicited AEs were sought from each vaccination, through non-leading AE questioning such as 'how have you been feeling', and through symptom-directed physical examination. Unscheduled laboratory testing and other investigations could be performed as required to investigate AE

Handling of AEs

All AEs, either solicited or unsolicited, were transferred in the source documents and entered in the electronic CRF by site staff.

Grading of AEs

The intensity of an AE was assessed by the investigator using the FDA guidance[3]. Where a specific AE was not listed in the guidance, its intensity was assessed by the investigator using the following terms:

- *Mild (Grade 1):* No interference with daily activity
- *Moderate (Grade 2):* Some interference with daily activity not requiring medical intervention
- *Severe (Grade 3):* Prevents daily activity and requires medical intervention
- *Potentially life-threatening (Grade 4):* Emergency visit or hospitalisation

Causality assessment of AEs

The causal relationship between an AE and the trial product was assessed by the investigator using the following terms:

Not related: An AE that was definitely not related to the product administered.

Possible: An AE with a reasonable time relationship to product administration, but which could also be explained by concurrent disease, other drugs or other cause.

Probable: An AE with a reasonable time relationship to product administration, and which was unlikely to be attributed to concurrent disease, other drugs or other cause.

Certain: An AE occurring in a plausible time relationship to product administration and which could not be explained by concurrent disease, other drugs or other cause.

A SAR was defined as an SAE which had been assessed as possibly, probably or certainly related to the product administered (i.e. which had a reasonable suspected causal relationship to the product administered).

Expectedness of AEs

Expectedness was assessed according to the Reference Safety Information section in the newest version of the Investigator's Brochure. Since there was no clinical experience available for CTH522, CTH522:CAF01 or CTH522:AH when the trial was conducted, no expected AEs were listed in the Reference Safety Information. Hence all related SAEs in this trial were to be reported as SUSARs.

Participant reimbursement

Subjects were paid for their time, inconvenience and travel expenses: £100 per visit, to a maximum of £1000. This included any travel expenses they might have incurred.

IMMUNOLOGICAL ANALYSES

Quantification of anti-CTH522 IgG in serum

An indirect quantitative ELISA was developed to measure the content of anti-CTH522 IgG antibodies in human sera. An in-house reference serum pool was established by combining sera from visit 7 (day 126) from participants which had a positive anti-CTH522 IgG antibody response at this time point. This reference serum pool was arbitrarily assigned a value of 3000 ELISA-Units/ml (AEU/ml). The reference serum pool was used for optimization of the ELISA assay, and to demonstrate assay suitability for quantitating anti-CTH522 IgG antibodies in human serum.

The ELISA assay was performed as follows: 96-well MaxiSorp Immunoplates (NUNC, Denmark) were coated with 50 µl/well of CTH522 (Batch DS-012-18, SSI) diluted to 0·125 µg/ml in carbonate buffer pH 9·6. The plates were incubated for 2 hrs at room temperature or overnight at 2-8°C. After incubation, the plates were washed 8 times with Wash buffer (PBS pH 7·2 with 1% Tween 20) using an automated plate washer. The reference serum pool and samples were diluted in dilution buffer (Wash buffer + 1 % BSA). The sera from the participants were tested by two-fold serial dilution in parallel with the reference serum pool and the plates were incubated for 2 hrs at room temperature or overnight at 2-8°C. The plates were washed and HRP-labelled rabbit anti-human IgG (DAKO; P0212) diluted 1:1000 in dilution buffer was added to all wells, whereafter the plates were incubated for 1 hour at room temperature. The plates were washed and substrate (OPD dissolved in a citrus buffer pH 5·5) was added. The plates were incubated in the dark for 30 min before the reaction was stopped with 100 µl 1M H_2SO_4. OD (492 nm) was read using an ELISA reader. A reference line approach on log-log transformed data was used to calculate the concentration of anti-CTH522 IgG antibodies in the serum samples using the reference serum pool as the calibrator. The samples were repeated if the dilution series had less than 3 points within the OD spectrum confined by the reference. For samples that had to be diluted <10 times and were unable to meet that criterion, concentration was calculated using only the highest OD within the limit of the reference.

Assessment of neutralising antibodies

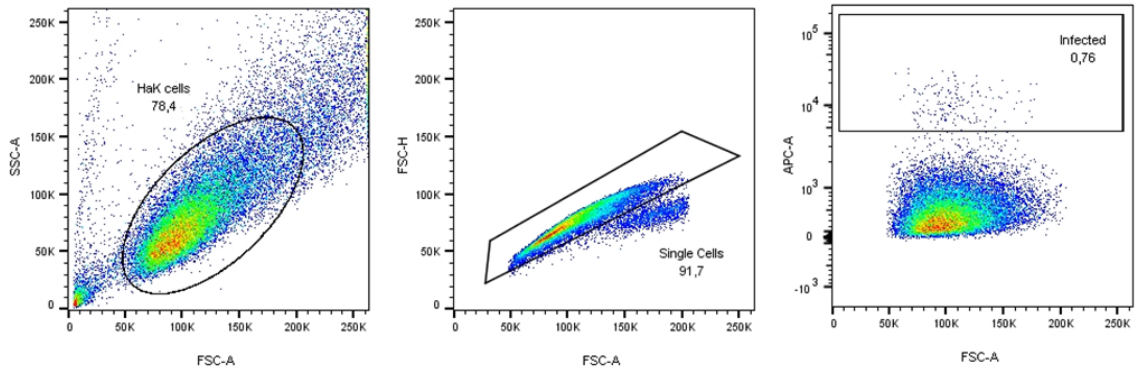
Antibody neutralising activity was assessed as previously described[1,4], however, inclusion-forming units (IFUs) were enumerated using flow cytometry (Knudsen et al., in prep). Briefly, hamster kidney cells (HaK, CCL15 from ATCC) were maintained in RPMI 1640 supplemented with 1% (vol/vol) L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 70 µM 2-mercaptoethanol, 10 µg/mL gentamicin, 1% HEPES and 5% heat-inactivated fetal bovine serum at 37°C, 5% CO_2. Cells were seeded in a 96-well flat-bottom microtiter plate at a concentration of $0{\cdot}5x10^5$ cells per well and incubated overnight.

Serum samples were heat-inactivated at 56°C for 30 min and diluted in SPG buffer (1M sucrose, 0·5M phosphate, 0·2 M glutamate) to create a 2-fold dilution series. *Chlamydia trachomatis* serovar D (strain D/UW-3/Cx) was added to serum dilutions, incubated at 37°C for 30 min, then HaK cells were inoculated with serum containing $8x10^5$ IFU, and incubated for 2 hrs at 35°C on a rocking table. After incubation, the supernatant was removed and culture media containing 0·5% glucose and cycloheximide (1:1000) was added, before incubation 22-24 hrs at 37°C 5% CO_2.

In preparation for the staining, HaK cells were washed with PBS and detached to form a single-cell suspension using 0·05% trypsin with EDTA. Cells were transferred to a 96-well U-bottom plate, fixed and permeabilised for 20 min with fixation/permeabilisation solution (Cytofix, BD Biosciences). Cells were washed twice with

Perm/Wash buffer (BD Biosciences), stained 30 min with monoclonal mouse anti-LPS 1:4000 (MAB6167, Abnova), washed twice with Perm/Wash and stained 30 min with secondary antibody (Alexa Flour 647-conjugated goat anti-mouse IgG 1:500 (A21235, Thermofischer Scientific). After two final washes in Perm/Wash the cells were resuspended in PBS and run on a FACSCanto (BD Biosciences) with a high throughput sampler. Average of 25000 cells (range 5000-45000) were recorded. Data from acquiring software BD FACSDIVA were analyzed using FlowJo version 10.3. All samples were evaluated in duplicate, run on independent days.

To exclude cell debris, gating using forward scatter (FSC) and sideward scatter (SSC) was set on a density plot. Single cells were determined using FSC-area and FSC-height to exclude doublets. Infected cells were identified in a dot plot by gating cells which were positive for the fluorescence marker[5].



All raw data values were subtracted of background (mean of blank control). If a blank value was detected higher than the limit of detecton (LOD), it was not used in subtracting the background. LOD was calculated as the mean of blanks (10 observations) plus three times the standard deviation.

All data were normalised to a no serum control to avoid the variation in infection between runs. Before normalisation, all data were corrected by removing points if duplicates had more than 40% variation.

To obtain a 50% neutralization titer, five parameter logistic (5PL) curves were generated using the package 'drc' in R to calculate the 50% neutralisation titre for each sample.

Quantification of total IgG and IgA, and isotype-specific anti-CTH522 antibodies in mucosal samples

Antigen-specific IgA/G antibody responses were measured in the mucosal compartments (vaginal and nasal) at months 0, 4·5, 5, and 6 using a supersensitive polyHRP ELISA. In brief, 96 well high-binding plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with CTH522 (Batch DF-0115-01, SSI) diluted to 0·5 µg/ml in Casein buffer (Thermo, Waltham, MA) or (for the standards) anti-human kappa and lambda light chain specific mouse antibodies (Southern Biotech, Birmingham, AL) at 1:1 ratio diluted 1:500. Casein buffer was used to block plates before addition of mucosal samples at 1:100 dilution and standards (purified human IgG or IgA, (Sigma-Aldrich, St. Louis, MO), serially diluted 1:10) for 1 hrs at 37°C. Secondary antibody, biotin-conjugated anti-human Fc IgG or IgA (Southern Biotech, Birmingham, AL), was added at 1:10000 or 1:20000, respectively, and incubated for 1 hrs at 37°C before addition of streptavidin poly-HRP40 (Fitzgerald, Acton, MA) for 40 min at room temperature. Plates were developed and read as detailed below for the conventional ELISA.

Total IgG and IgA antibodies were measured in nasal and vaginal secretions using in-house standardised conventional ELISA platforms. In brief, 96-well high-binding plates (Greiner, Kremsmünster, Austria) were coated with anti-human kappa and lambda light chain specific mouse antibodies (Southern Biotech, Birmingham, AL) at 1:1 ratio diluted 1:500 in PBS for 1 hrs at 37°C. After blocking with assay buffer (1% BSA (Sigma-Aldrich, St. Louis, MO), 0·05% Tween (Fisher, Pittsburgh, PA)) samples were initially screened at 1:100 dilution (then titrated to optimal dilutions). Serial dilutions of immunoglobulin standards (purified human IgG or IgA) were added in triplicate to kappa/lambda capture antibody-coated wells and incubated for 1 hrs at 37°C. Secondary antibody, HRP-conjugated anti-human IgG or IgA (Sigma-Aldrich, St. Louis, MO), was added at 1:20000 or 1:10000 dilution, respectively, and incubated for 1 hrs at 37°C. Plates were developed with SureBlue TMB substrate (KPL, Insight Biotechnology, London, UK). The reaction was stopped after 5 min by adding TMB stop solution (KPL, Insight Biotechnology, London, UK), and the absorbance read at 450 nm on a VersaMax 96 well microplate reader (Molecular Devices, Sunnyvale, CA). The ELISA data were expressed as positive if the blank-subtracted OD 450 nm was above the pre-determined cut-off of OD 0·2 nm and values were on the linear range of the curve. To ensure assay sensitivity, a positive control composed of positive pooled plasma samples was used. Analyses of the data were performed using SoftMax Pro GxP software (version 6.5, Molecular Devices, Sunnyvale, CA).

Interferon-γ ELISpot

Interferon-γ ELISpot assays were performed using frozen isolated peripheral blood mononuclear cells (PBMC) stimulated with CTH522 protein (Batch DF-0115-01, SSI), at sampling months 0 and 4·5. In brief, frozen PBMC were rested overnight at a concentration of $2{\cdot}5x10^6$ PBMC/ml and resuspended to a final concentration of $4x10^6$ viable PBMC/ml ($4x10^5$ PBMC/ml for positive control PHA wells). Pre-coated interferon-γ ELISpot 96 well plates (Mabtech, Stockholm, Sweden) were washed with sterile PBS and blocked with R10 media before addition of 50 µl cells/well in triplicate with 50 µl media only, stimulation media containing vaccine-specific CTH522 protein at 5 µg/ml and 2 positive controls (Phytohaemagglutinin [PHA] (Sigma-Aldrich, St. Louis, MO) and Cytomegalovirus/Epstein Barr Virus/Influenza virus/Tetanus [CEFT] (thinkpeptides, Oxford, UK) at a final concentration of 2·5 µg/ml. Plates were incubated for 24 hrs at 37°C, 5% CO_2. Plates were then washed and incubated for 2 hrs at room temperature with 1 µg/ml mouse-anti human interferon-γ (Mabtech, Stockholm, Sweden). The signal was amplified by incubating for 1 hrs with streptavidin-ALP solution (Mabtech, Stockholm, Sweden), then developed with substrate, 5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium (BCIP/NBT plus; Mabtech, Stockholm, Sweden), for 5-7 minutes. The reaction was stopped by washing with tap water and allowed to dry overnight in the dark. Plates were read with an automated AID iSPOT ELISpot plate reader (Autoimmun Diagnostika GmbH, Germany).

DATA SHARING STATEMENT

Data collected for the study, including individual participant data and a data dictionary defining each field in the set, will not be made available to others than those involved in the clinical trial conduct. The overall results and clinical trial data have been entered in the EUDRACT database, and could be made available should the data from the trial be included in a marketing authorization application at a later stage.

FIGURES AND TABLES

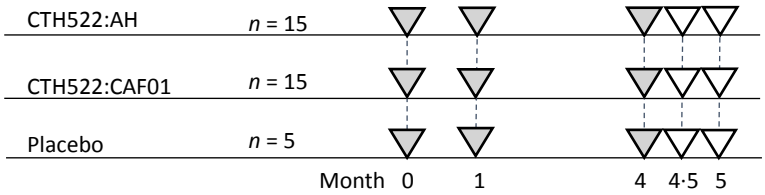


Figure S1: **Immunisation schedule**

Filled triangles indicate intramuscular immunisation with adjuvanted vaccine, open triangles denote intranasal immunisation with un-adjuvanted vaccine

	Treatment groups		
	CTH522:CAF01 (n=15)	**CTH522:AH (n=15)**	**Placebo (n=5)**
Unsolicited related adverse events			
Vessel puncture site reaction	0	1 (7%) 1	0
Nasal congestion	1 (7%) 1	0	0
Oropharyngeal pain	1 (7%) 1	0	1 (20%) 1
Headache	1 (7%) 1	0	0
Presyncope	1 (7%) 1	0	0
Musculoskeletal stiffness	0	2 (13%) 2	0
Abdominal discomfort	0	0	1 (20%) 1
Diarrhoea	0	1 (7%) 1	0
Nasopharyngitis	1 (7%) 1	1 (7%) 1	0
Flushing	0	1 (7%) 1	0

***Table S1*: All related unsolicited adverse events.**

Unsolicited events were events that occured at an unexpected time point, i.e. later than 14 days after a vaccination or after another type of vaccination (e.g. nasal congestion after IM vaccination).
All data are shown as [n (%) events], where n=number of subjects having any adverse event, %=the percentage of subjects having an adverse event, and events=total number of adverse events in that treatment group.

Months after first immunisation (days since last immunisation)	CTH522:CAF01 n (%)	CTH522:AH n (%)	Placebo n (%)
1 (28 days post 1st IM)	10 (67)	6 (40)	0 (0)
4 (84 days post 2nd IM)	15 (100)	13 (87)*	0 (0)
4·5 (14 days post 3rd IM)	15 (100)	14 (93)*	0 (0)
5 (14 days post 1st IN)	15 (100)	14 (93)*	0 (0)
5·5 (14 days post 2nd IN)	15 (100)	14 (93)*	0 (0)
6 (28 days post 2nd IN)	15 (100)	15 (100)	0 (0)

***Table S2*: Seroconversion**

n=number of seroconverted subjects. IM=intramuscular vaccination. IN=intranasal vaccination. Seroconversion was defined as a 4-fold increase in anti-CTH522 IgG compared to the baseline value. If a subject achieved seroconversion at a visit, this subject counted as seroconverted at all subsequent visits, also if the subject withdrew from the trial. *As per the statistical analysis plan, the rate of seroconversion is shown for the safety analysis set. One subject withdrew before the 3rd IM. She had not seroconverted at month 1, but had seroconverted at her end-of-trial visit, which took place 21 days after the 2nd IM. It is therefore possible that seroconversion could have been measured at the originally planned visit at month 4.

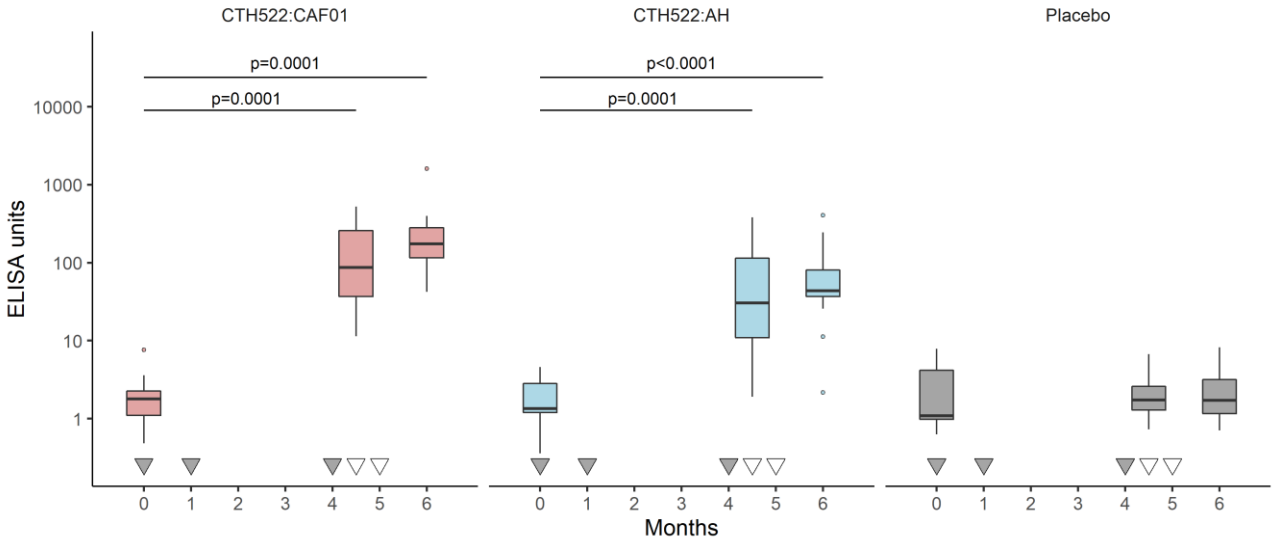


***Figure S2*: Serology IgA measurements**

Change in anti-CTH522 serum IgA ELISA units over time. The box illustrates the IQR, with a horizontal line at the median value; whiskers show 1·5×IQR, and dots represent outliers. Wilcoxon signed rank test p values are shown. The vaccine schedule is shown by the bottom axis, with filled triangles indicating intramuscular, and white triangles intranasal immunisation.

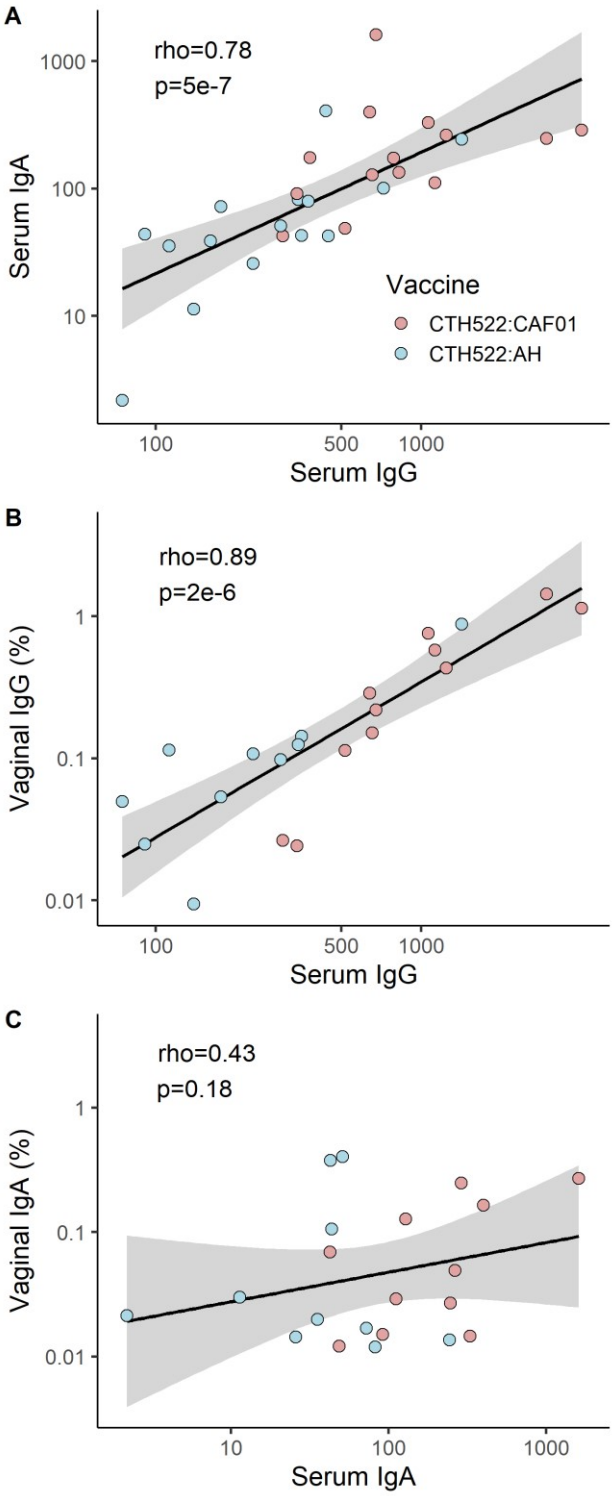


***Figure S3*: Correlations between mucosal and serum antibodies**

Linear correlation between (A) serum IgA and serum IgG, (B) vaginal IgG and serum IgG, and (C) vaginal IgA and serum IgA in the two active vaccine groups at month 6, four weeks after the second intranasal vaccination. Mucosal values are shown as CTH522-specific Ig normalised to corresponding total Ig, to minimise sampling artefacts. Each dot represents one participant, with fill colour corresponding to vaccine group. The Spearman correlation coefficient rho and p value are shown.

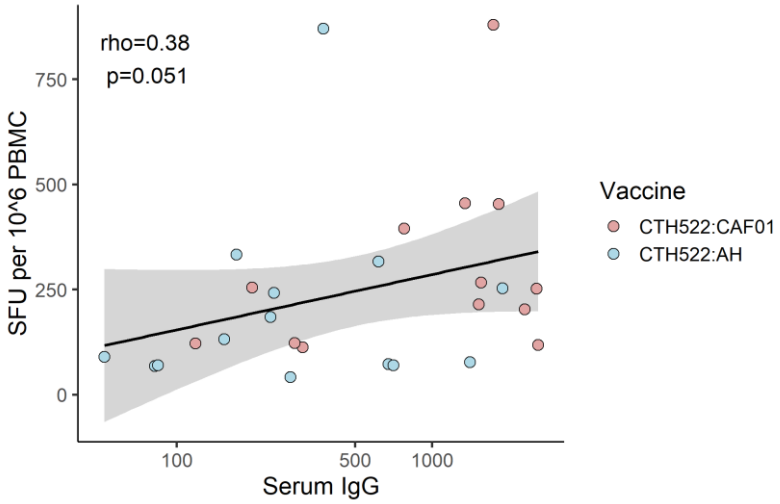


***Figure S4*: Correlation between vaccine-specific T cell responses and serum IgG antibodies**

Linear correlation between number of spot-forming units in the interferon-γ ELISpot and anti-CTH522 serum IgG ELISA units in the two active vaccine groups at month 4·5, two weeks after the third intramuscular vaccination, at the peak of the antibody response. Each dot represents one participant, with fill colour corresponding to vaccine group. The Spearman correlation coefficient rho and p value are shown.

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