UiT The Arctic University of Norway

Department of Community Medicine, Faculty of Health Sciences

Sex-steroids and social network in relation to *Staphylococcus aureus* nasal carriage

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1 Preface

This thesis is the result of my scientific work at the Department of Microbiology and Infection Control, University Hospital of North Norway, and the Department of Community Medicine, UiT The Arctic University of Norway, Tromsø, to obtain the doctor of philosophy degree in Medicine.

Infections has for long been a forgotten enemy because of the effective treatment introduced by Alexander Fleming in 1928. By a laboratory mistake he found his Petri dishes contaminated with fungi that had killed off the common, but deadly bacteria *Staphylococcus aureus.* Since Flemings discovery many have forgotten the threat of bacterial infections, but now we stand before a new antimicrobial war. WHO predicts 10 million deaths per year from antimicrobial resistance by 2050. We need a better understanding of the dynamics of the bacteria to prevent this crisis.

9 years ago, Anne-Sofie Furberg introduced me to *Staphylococcus aureus* carriage through an assignment in my second year of medical school. Since then I have been increasingly interested in the field, and I hope my research will bring some new knowledge about *Staphylococcus aureus* carriage, that may contribute to discovery of targets for prevention, to ultimately decrease transmission and risk of infection.

This thesis has given me the opportunity to investigate the relationship between hormonal exposures and *Staphylococcus aureus* carriage, as well as the effect of social network on bacterial transmission.

Dina Benedicte Berg Stensen
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2 Acknowledgements

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3 List of papers

This thesis is based on the following four papers, which are referred to in text by their Roman numerals.

Paper I
Stensen DB, Småbrekke L, Olsen K, Grimnes G, Nielsen CS, Simonsen GS, Sollid JUE, Furberg AS.
Hormonal contraceptive use and *Staphylococcus aureus* nasal and throat carriage in a Norwegian youth population. *PLoS One*. 2019;14(7):e0218511. Published 2019 Jul 5. doi:10.1371/journal.pone.0218511

Paper II
Stensen DB, Småbrekke L, Olsen K, Grimnes G, Nielsen CS, Sollid JUE, Simonsen GS, Almås B, Furberg AS.
Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general female population. Eur J Endocrinol. 2021 Feb;184(2):337-346. doi: 10.1530/EJE-20-0877. Erratum in: Eur J Endocrinol. 2021 May 13;184(6):X3. PMID: 33428587; PMCID: PMC7849480.

Paper III
Stensen DB, Småbrekke L, Olsen K, Grimnes G, Nielsen CS, Sollid JUE, Simonsen GS, Almås B, Furberg AS.
Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general male population.
(Submitted)

Paper IV
Stensen DB, Cañadas RAN, Småbrekke L, Olsen K, Nielsen CS, Svendsen K, Hanssen AM, Sollid JUE, Simonsen GS, Bongo LA, Furberg AS.
Social network analysis of *Staphylococcus aureus* carriage in a general youth population.
(Manuscript in preparation)

4 List of abbreviations

25(OH)D	25-hydroxyvitamin D
95% CI	95% confidence interval
AFLP	Amplified fragment length polymorphism
Agr	Quorum-sensing accessory gene regulator
AIC	Akaike Information Criterion
AMP	Antimicrobial peptides
BMI	Body mass index
BSI	Blood-stream infections
ClfB	Clumping factor B
Cm	Centimeters
CHIPS	Chemotaxis-inhibitory protein of *Staphylococcus aureus*
CI	Confidence interval
DAGs	Directed acyclic graphs
DHEAS	Dehydroepiandrostenedione sulfate
DHFR	Dihydrofolate reductase
DHT	Dihydrotestosterone
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estetrol
Eap	Extracellular adherence protein
ECLIA	Electrochemiluminescence immunoassay
ERGM	Exponential random graph model
FF1	Fit Futures 1 study
FF2	Fit Futures 2 study
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HbA1c	Glycated hemoglobin
HC	Hormonal contraceptives
HPLC	High performance liquid chromatography
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL17A	Interleukin-17A
IL17F	Interleukin-17F
IsdA	Iron-regulated surface determinant A
IUD	Intrauterine device
Kg	Kilogram
KI	Karyopyknotic index
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LH	Luteinizing hormone
MALDI TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MLST	Multilocus sequence typing
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing adhesive matrix molecules
NOD	Nucleotide-binding oligomerization domain
OatA	O-acetyltransferase
OR	Odds ratio
PAMP	Pattern-associated molecular patterns

PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
PRR	Pattern recognition receptors
REK	Regional Committee of Medical and Health Research Ethics
RFLP	Restriction fragment length polymorphism
SasG	*S. aureus* surface protein G
SasX	*S. aureus* surface protein X
SD	Standard deviation
SdrC	Serine aspartic acid repeat protein C
SdrD	Serine aspartic acid repeat protein D
SHBG	Sex-hormone binding globulin
SNA	Social network analysis
Spa	Staphylococcal protein A
SSSS	Staphylococcal scalded skin syndrome
SSTI	Skin and soft-tissue infection
T6	The sixth Tromsø Study
Th17	T-helper 17 cell
Th1	T-helper 1 cell
TLR	Toll-like receptor
TSS	*Staphylococcus aureus* toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
VAP	Ventilator-associated pneumonia
VNTR	Variable number of tandem repeat
WGS	Whole genome sequencing
WHO	World Health Organization
WTA	Wall teichoic acid

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5 English summary

Staphylococcus aureus (*S. aureus*) is a major human pathogen that can colonize skin and mucosa in humans. The most important anatomical areas are anterior nares and throat. Nasal carriage is common with a prevalence of 20-30% in the adult population and 40-50% in youths. Carriage of *S. aureus* is associated with increased risk of autoinfection and transmission. It is therefore important to identify possible targets for prevention of carriage and subsequently decrease disease burden.

There are several known risk factors for *S. aureus* carriage. Sex and age are the most important, where male sex and younger age are associated with increased risk. Hypotheses about sex-steroids as major host determinants have emerged. In this thesis, we investigated if hormonal contraceptives and serum concentration of endogenous sex-steroids were associated with *S. aureus* carriage. We also studied the transmission of *S. aureus* carriage in social networks and examined if known host risk factors for carriage were associated with social contact, indicating potential confounding or indirect transmission.
We tested our hypotheses using data from the population-based cross-sectional health surveys Fit Futures 1&2 in youths and the sixth Tromsø Study in adults.

Young women taking combination hormonal contraceptives (containing both estrogen and progestin) had doubled odds of nasal carriage compared to non-users. Users of progestin-only contraceptives had half the odds of nasal carriage compared to non-users.

An increase in endogenous testosterone in adult women gave reduced odds of *S. aureus* nasal carriage. The same inverse relationship was observed for other androgens in the female population though not statistically significant. We found similar associations in adult men, but data were inconclusive.

Our data prove transmission of *S. aureus* in a social network of youths, and we could substantiate this by demonstrating transmission of specific *S. aureus* genotypes. We found higher risk of bacterial transmission with female friendships, while male friendships had no influence on transmission even though men were more frequent carriers. This suggests that male sex is a true biological risk factor not influenced by social contact. Use of alcohol more than twice a month, normal BMI, and moderate/high physical activity were associated with higher risk of transmission.

The results suggest that both exogenous and endogenous sex-steroid exposures are relevant in carriage of *S. aureus*. We show for the first time that there is relatively more transmission of *S. aureus* in female social networks than in male networks. The male predominance of *S. aureus* carriage is thus largely determined by intrinsic host factors. We need more prospective studies to clarify causal relationships and targets for prevention.

6 Sammendrag

Staphylococcus aureus eller gule stafylokokker er en viktig årsak til alvorlige infeksjoner hos mennesker. Bakterien kan kolonisere hud og slimhinner, og trives spesielt godt i nese og hals. Bærere av gule stafylokokker har økt risiko for å få infeksjoner samt økt risiko for å smitte andre med bakterien. Det er derfor avgjørende å øke kunnskapen om bærerskap for å kunne identifisere mulige strategier for forebygging av infeksjon og smitte.

Om lag 20-30% av den voksne befolkningen og 40-50% av ungdommer er bærere av gule stafylokokker i nesen. Fra tidligere studier vet vi at kjønn og alder er bestemmende for bærerskap – menn og yngre mennesker har høyere forekomst. Det har derfor blitt fremsatt hypoteser om at kjønnshormoner kan være viktige determinanter for bærerskap. I denne avhandlingen undersøkte vi om hormonell prevensjon og nivå av kroppens egne kjønnshormoner har betydning for bærerskap av gule stafylokokker. Vi studerte også spredning av bakterien i sosiale nettverk og undersøkte om kjente risikofaktorer for bærerskap var assosiert med sosial kontakt. Vi testet hypotesene våre med bruk av data fra de befolkningsbaserte tverrsnittstudiene Fit Futures 1&2 og den sjette Tromsøundersøkelsen.

Resultatene viser at bruk av hormonell prevensjon som inneholder østrogen og progestin, ga dobbel odds for bærerskap sammenlignet med ikke-bruk. Bruk av hormonell prevensjon som bare inneholdt progestin halverte oddsen sammenlignet med ikke-bruk.

Høyere nivå av serum testosteron hos voksne kvinner ga redusert odds for bærerskap. Vi så samme mønster for andre androgener selv om disse ikke var statistisk signifikante. Vi fant også lignende assosiasjoner hos menn, men dataene var inkonklusive.

Analysene våre viser at bærerskap av gule stafylokokker smitter i sosiale nettverk blant ungdom. Vi fant lignende funn også for spesifikke genotyper av bakterien. Det var høyere risiko for bakteriell smitte gjennom sosial kontakt hos kvinner, mens mannlig kjønn ikke påvirket smitten selv om prevalensen av bærerskap var høyre hos menn. Dette sannsynliggjør mannlig kjønn som en biologisk risikofaktor som er upåvirket av sosial kontakt. Bruk av alkohol mer enn to ganger i måneden, normal BMI og moderat/høy fysisk aktivitet var assosiert med høyere risiko for overføring av bakterien.

Resultatene tyder på at påvirkning fra kjønnshormoner er svært relevant når det kommer til bærerskap av gule stafylokokker. For første gang viser vi at sosial kontakt er viktigere for spredning av gule stafylokokker innad blant kvinner enn blant menn. Den høyere forekomsten av bærerskap hos menn er derfor i stor grad uttrykk for iboende vertsfaktorer. Vi trenger flere prospektive studier for å avklare årsakssammenhenger og mål for forebygging.

7 Introduction

7.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium first discovered by the surgeon Sir Alexander Ogston in 1880. He observed grape-like clusters of the bacteria and named it *Staphylococcus* from the Greek expression staphyle ("A bunch of grapes") and kokkos ("berry") (1). Rosenbach was in 1884 able to grow this microbe and called it *Staphylococcus aureus* (from the latin "gold") because of the golden appearance of the bacterial colonies (2). *S. aureus* produces several hemolysins, immune-modulatory factors and extracellular enzymes that characterize the extraordinary virulence of the bacteria (3). Some of these are presented in table 1. *S. aureus* is classified as a coagulase-positive staphylococcus because of the production of the extracellular enzyme coagulase which converts plasma fibrinogen into fibrin, enabling clotting (4).

Table 1 Virulence factors of S. aureus. *Adapted from table 1 by Gnanamani et al. (5) Creative Commons License.*

Virulence factors	Function
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules. Cell surface proteins which interact with host molecules to facilitate tissue attachment.
Spa	Staphylococcal protein A. Prevents opsonization, functions as a super antigen and limits the immune response.
PVL	Panton-Valentine leukocidin. Forms porins on cell membrane of host cells, leading to leakage of cell contents and cell death.
Polysaccharide microcapsule	Resists phagocytosis
Alpha-toxin	Also called alpha hemolysin. Cell membrane pore former which causes cell leakage and cell death.
CHIPS	Chemotaxis-inhibitory protein of *S. aureus*. Extracellular protein which inhibits chemotaxis of neutrophiles and monocytes.
Extracellular adherence protein (Eap)	Exoprotein which binds to host cell matrix, plasma proteins and endothelial cell adhesion molecule ICAM-1. Functions also as an immune-modulatory factor.
Protease, lipase, nuclease, phospholipase C, coagulase, metalloprotease, staphylokinase	Extracellular enzymes that cause tissue destructions to help bacterial penetration.
Enterotoxins	Potent gastrointestinal exotoxins that can cause staphylococcal food poisoning.
Exfoliative toxins	Serine proteases which recognize and hydrolyze desmosomal proteins in the skin.
Toxic shock syndrome toxin-1 (TSST-1)	Pyrogenic toxin super antigen that causes toxic shock syndrome.

7.1.1 *S. aureus* infections

S. aureus is a major bacterial pathogen that can cause a wide range of infections in any human tissue (6). The latest national surveillance report in Norway identifies *S. aureus* as the second most common cause of BSI representing almost 14% of isolates identified in blood cultures (7). Common primary clinical foci for BSI are vascular catheter-related infections, SSTIs, pulmonary infections, osteoarticular infections and endocarditis, but in 25% of cases no primary foci can be identified (8). The annual 30-day all-cause mortality of *S. aureus* BSI is 2-10/100 000 population (9).

SSTIs range in severity from minor to life-threatening disease, and both community and healthcare acquired infections are most commonly caused by *S. aureus* (10). Estimated ambulatory care visits for SSTI range between 11.6 and 14.2 million in the United States (11). SSTIs occur in 2-5% of all surgeries (12).

S. aureus is also the leading cause of endocarditis with high mortality rate (13). The yearly incidence for prosthetic valve endocarditis ranges from 0.8-3.6% (14-16), and overall mortality of *S. aureus* endocarditis is 22 to 66% (8). The mortality is consistently higher compared to infections from other microbes.

S. aureus is the number one microbe found in osteomyelitis and septic arthritis, and the treatment has been challenging because of the anatomical and physiological characteristics of the bone (17). The risk of prosthetic joint infections is about 2% in both hip and knee arthroplasties (18, 19). A large issue with *S. aureus* infections related to medical devices, like catheters, artificial heart valves and joint prosthetics is the development of biofilms. Biofilms are bacteria contained in a polymeric matrix that adheres to the prosthetic material. Within the biofilm, the bacteria are relatively protected from antimicrobial agents and the host immune response (20).

S. aureus is the second most common microbe in nosocomial infections, leading to lengthened hospital stay for patients and large increase in costs and mortality (21). In UK, a patient with hospital acquired infection spent 2.5 times longer time in hospital and treatment cost is estimated 3000 £ more compared to uninfected patients (22). Nosocomial pneumonia, particularly ventilator-associated pneumonia (VAP) is associated with *S. aureus*. Nosocomial pneumonia is also associated with bacteremia, which is a poor prognostic indicator. One study demonstrated mortality of 50% in nosocomial *S. aureus* pneumonia (23).

7.1.2 Toxin-mediated infections

In addition to the infections mentioned, *S. aureus* can cause toxin-mediated disease. A serious toxin-mediated staphylococcal disease is *S. aureus* toxic shock syndrome (TSS). TSS is mediated by the exotoxin TSST-1 secreted by some *S. aureus* strains. TSST-1 binds to the T-cell receptor on antigen-presenting cells triggering a large-scale T-cell activation and massive cytokine release, leading to septic shock with organ failure (24). Initially TSS was linked with superabsorbent tampons, but after removal from the market, there has been a decline in incidence to approximately 1/100 000 persons in menstruating women (25). SSTI is also a common foci for *S. aureus* TSS.

Staphylococcal scalded skin syndrome (SSSS) is a spectrum of skin diseases induced by the exfoliative toxins of *S. aureus,* primarily affecting young children and infants. SSSS is

associated with diffuse skin loss and fever, and is potentially life-threatening with a mortality of 3.6-11% in children and 40-63% in adults (26).

A more common toxin-mediated *S. aureus* disease is food poisoning. It is one of the most common food-borne diseases and is a result of ingestion of staphylococcal enterotoxins produced by some *S. aureus* strains. Human contamination of food after heat treatment is the most common source of disease (27).

7.1.3 Antimicrobial resistance

There is a growing prevalence of antimicrobial resistance in *S. aureus,* with 69% of isolates resistant against penicillin, and about 1.5-2% methicillin resistant *S. aureus* (MRSA) in Norway (7). There is also an increase in the incidence of MRSA-infections acquired within Norway, while in previous years imported infections have been the most frequent. In other parts of Europe, the picture is more grim, with an overall MRSA prevalence of 28-58% among clinical *S. aureus* isolates in countries like Greece, Italy and Romania (28). Chile and India have about 80-90% MRSA prevalence (29). Development of antimicrobial resistance is linked to increasing use of antimicrobial therapy. A recent study demonstrated that the quality of antibiotic prescribing, like spectrum of activity and length of treatment, was more associated with increasing occurrence of MRSA than overall consumption. MRSA was also associated with sociocultural behaviors like lack of urgency to address risk and normalization of deviance in relation to noncompliant practices (30).

S. aureus resistance to penicillin is mediated by the gene blaZ, encoding for β-lactamase. This enzyme hydrolyzes the β-lactam ring, rendering the β-lactam antibiotic inactive. Methicillin resistance is mediated by the mecA gene which is responsible for synthesis of the penicillin-binding protein 2a (PBP2a). PBP2a has low affinity for all β-lactam antibiotics, including cephalosporins, and enables staphylococci to survive under exposure of these agents (31). In 2011, a new methicillin resistance gene was described, named mecC. This is associated with both livestock and humans suggesting zoonotic transmission. The PBP2a gene in mecC is 63% identical to the gene in mecA (32). Most mecC-MRSA isolates are susceptible to all non-beta-lactam antibiotics, thus suggesting correct identification of mecC-MRSA to prevent implementation of inappropriate therapies (33).

While the penicillin and methicillin resistance are considered most relevant, there are other mechanisms of antimicrobial resistance. Fluoroquinolone resistance is not uncommon, especially in MRSA, and is a result of mutations in topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump. For vancomycin two forms of *S. aureus* resistance have been identified. One is a result of changes in peptidoglycan biosynthesis and has been seen in vancomycin intermediate resistant *S. aureus* strains. The second is a result of conjugal transfer of the vanA operon from vancomycin-resistant *Enterococcus faecalis*, leading to complete vancomycin resistance (31). Trimethoprim/sulfamethoxazole resistance in *S. aureus* is common on the African continent. Two mechanisms of resistance have been identified. Firstly, mutations in the chromosomal dihydrofolate reductase (DHFR) gene and secondly genes that encode variant DHFRs (dfrA, dfrG and drfK). The first mechanism causes intermediate-level trimethoprim-resistance, but the second mediate high-level resistance (34).

7.2 *S. aureus* nasal and throat carriage

Human carriage of a bacteria is defined as isolation of the same bacteria from at least two surveillance samples over a period of minimum one week. Some argue that colonization must be distinguished from carriage, because colonization has been defined as the presence of a microorganism from a normally sterile organ without any host inflammatory response (35). In current literature the term colonization is often used to cover both carriage and colonization, and in this thesis they are used interchangeably.

S. aureus colonizes skin and mucosa in multiple areas of the human body. Anterior nares, pharynx, axillae and perineum are some examples (36). *S. aureus* carriage is asymptomatic and harmless, but carriers are at risk of autoinfection and transmission of the bacteria (37). In about 80% of *S. aureus* infections, the bacterial strain is already present on the skin or the mucosa of the patient (38), suggesting that carriage is the source for the infection. The increased risk of infection has also been demonstrated in neonatal intensive care units, where nasal carriage gave eight times higher odds of *S. aureus* infections (39). In patients with durable ventricular assist device, nasal colonization was a source of endogenous infections (40), and in intensive care units the daily risk for *S. aureus* ventilator associated pneumonia (VAP) was 3.6 times higher in patients colonized with *S. aureus* at admission (41).

Hands are the main vector for *S. aureus* transmission from surfaces to the nose, with contact transmission as the main transmission route (36). Horizontal transmission is also demonstrated where individuals in the same household carry genetically similar *S. aureus* strains (42). Airborne transmission is less common, but may play a role in hospital outbreaks (43). Upper respiratory infections in *S. aureus* carriers may lead to transmission and disease outbreaks (44). The extraordinary resilience and robustness of the bacteria are shown among other things, by the study on healthcare workers' mobile phones. The study confirmed identical *S. aureus* strains on the healthcare workers' phone as in their nares, making mobile phones and other contact surfaces a possible source of transmission (45).

7.2.1 Nasal carriage

Nasal carriage of *S. aureus* is considered the most clinically relevant reservoir because of the known association with autoinfection and transmission. Nasal carriage in the general adult population has a prevalence of about 25-30% and differs between countries (46, 47). In youth populations the prevalence is 40-50% (48).

Generally, researchers have classified nasal carriers in three groups because of the difference in risk of infection. Persistent carriage has been defined as multiple nasal swabs positive for *S. aureus* representing a more permanent colonization. Intermittent carriage has been defined as some positive and some negative nasal swabs, demonstrating transient colonization of the bacteria. Lastly the non-carriers that have multiple negative nasal swabs (49). It has been argued that intermittent carriers, although sometimes carrying *S. aureus*, have a similar risk of infection as non-carriers (50), and also have similar anti-staphylococcal antibody responses (51). This suggests that non-carriage could be incidental and that all humans could carry *S. aureus* transiently, or that intermittent carriers might actually be non-carriers that are colonized only under environmental pressure. Persistent carriers also differ from intermittent carriers by being colonized with the same *S. aureus* genotype over a long period, while intermittent carriers may carry different strains (52). There is also a known higher bacterial load in persistent carriers (53). Van Belkum et al. therefore argues that *S. aureus* nasal

carriage should be classified as persistent carriers and others (intermittent and non-carriers) (51).

There has also been a discussion about number of swabs required to define persistent carriage. One study proposed a "culture rule" that concludes that two nasal swabs taken with a week interval can accurately classify *S. aureus* nasal carriage, ideally combining culture results and quantity of *S. aureus* (54). The study demonstrated a positive predictive value of 0.79 and a negative predictive value of 0.99 for definition of *S. aureus* persistent nasal carriage, by culture alone. With a combination of qualitative and quantitative results the accuracy was 93.6% (54). Van Belkum et al. demonstrated a median survival of *S. aureus* of more than 154 days among persistent carriers, compared to 14 days among intermittent carriers and four days among non-carriers (51). This study defined persistent carriage as 80% of six nasal swabs culture positive. Collectively, this suggests that some intermittent carriers are misclassified when only using two swabs with an interval of one week. However, there is no clear definition on the number of swabs that should be taken and what fraction should be positive to determine carriage status (44).

7.2.2 Throat carriage

S. aureus also colonize the pharynx, and the prevalence of throat carriage has differed in previous studies (47). This is most likely due to differences in sampling techniques and microbiological detection. Throat carriage has been considered less clinically important compared to nasal carriage, and we therefore lack knowledge about this colonization niche (49). Recent studies have indicated that throat carriage is more common than nasal carriage and that subjects are often colonized in both anatomical areas (55, 56). Exclusive throat carriage is found in approximately 25% of all *S. aureus* carriers (56). Throat carriage has been associated with eradication failure, and therefore requires special attention in further studies (57).

7.3 Host immunity and *S. aureus* carriage

The knowledge on the host immune response during *S. aureus* carriage is still limited compared to invasive *S. aureus* infections (58), but colonization seems to stimulate both the innate and adaptive immune system.

The anterior nares consist of ordinary skin with nasal secretion. Nasal secretion is be bacteriostatic or bactericidal in non-carriers, indicating the importance of nasal secretions on colonization of *S. aureus (59)*. Hemoglobin in nasal secretions inhibits the quorum-sensing accessory gene regulator (agr) system and consequently promotes *S. aureus* carriage (60). The agr quorum sensing system plays a major role in regulation of virulence by increasing production of virulence factors and reduction in surface proteins (61).

The skin`s immune protection is ensured by tightly packed epidermal keratinocytes that shape the physical barrier of the skin (62). Keratinocytes and other cells at *S. aureus* colonization sites have pattern recognition receptors (PRRs) that can recognize microbial components called pathogen-associated molecular patterns (PAMPs) like Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) (63). Toll-like receptor (TLR) 2 as well as nucleotide-binding oligomerization domain (NOD)-like receptors are important examples of PRRs. PRRs trigger the production of antimicrobial peptides (AMPs), cytokines

and chemokines that initiate human immune responses by activating both the adaptive and the innate immunity (47). In addition to the antimicrobial effect of the AMPs, the AMPs can directly influence the host cells (64). Chemokines inhibit bacterial growth in vitro (65).

S. aureus carriage also induces an adaptive immune response where serum levels of immunoglobulin IgG and IgA specific for staphylococcal proteins have been reported to be higher in persistent carriers compared to non-carriers (66). In an animal study, clearance of *S. aureus* was found to be mediated through T- helper 17 (Th17) cells that produce Interleukin - 17 (IL-17) cytokines that are involved in surface pathogen clearance (67). IL-17A and IL-17F deficient mice failed to clear *S. aureus (68)*. IL-17 was not detected in a human study evaluating nasal fluid and nasal carriage of *S. aureus* (69). The authors proposed that IL-17 could be insoluble in nasal secretions. A low T-helper 1 (Th1) and Th17 ratio is also found to be associated with persistent *S. aureus* nasal carriage (70).

7.4 Nasopharyngeal microbiota

The successfulness of *S. aureus* colonization is influenced by other competing microorganisms present at the mucosal surface. The nasopharyngeal microbiota is variable, but dominated by *Corynebacterium*, *Cutibacterium* (*Priopionibacterium*) and *Staphylococci* species (71, 72). *Staphylococcus epidermidis* (*S. epidermidis*) and *Cutibacterium* acnes are associated with non-carriage of *S. aureus* (73).

The reduced colonization of *S. aureus* with the presence of *S. epidermidis* may be due to genus-specific antagonism of virulence gene expression. A study demonstrated that serine protease Esp secreted by *S. epidermidis* inhibits nasal colonization of *S. aureus* (74). This concept is well known, and appears most powerful between species of the same genus (58). *Corynebacterium pseudodiphteriticum* antagonizes *S. aureus* through *S. aureus* agr quorum sensing system that regulates expression of many virulence factors (75). *Streptococcus pneumonia* is associated with reduced colonization of *S. aureus*, but only in children and with non-consistent results (76, 77).

7.5 Determinants of *S. aureus* carriage

There is a complex interplay of multiple biological mechanisms involved in *S. aureus* carriage. Both host susceptibility factors, environmental factors and bacterial factors may play significant roles (47). The relative contribution of the different factors involved in colonization is yet to be understood, but it has been suggested that host factors play an important role (78).

7.5.1 Bacterial factors

Many bacterial factors have been identified as important during *S. aureus* carriage (figure 1). Adherence to squamous cells of *S. aureus* in persistent carriers is considerably greater than in non-carriers, demonstrating that bacterial factors that increase adherence may be of influence (79). MSCRAMMs like clumping factor B (ClfB) and iron-regulated surface determinant A (IsdA) promote adhesion (58). Wall teichoic acid (WTA) also seems to be important for *S. aureus* adhesion to mucosal surfaces (80). ClfB and IsdA adhere to cytokeratin K10 which is the human ligand in the squamous cell, as well as loricrin, a protein in the epithelial layer of

the nares (81, 82). In animal studies, lack of loricrin or ClfB has been associated with impaired colonization (83).

S. aureus secretes proteins involved in immune evasion, so called immune-modulatory factors. In combination with WTA, the enzyme O-acetyltransferase (OatA) makes *S. aureus* resistant to lysozymes (84). Staphylococcal protein A (*spa*) is known to reduce opsonization by binding to IgG and inhibit recognition by neutrophils and to bind to the complement system (85, 86). Staphylokinase binds to α-defensins and inhibits its function, thereby increasing the staphylococcal resistance to the innate immunity of the host (87). It also binds to plasminogen to form active plasmin, facilitating bacterial penetration in host tissue (88).

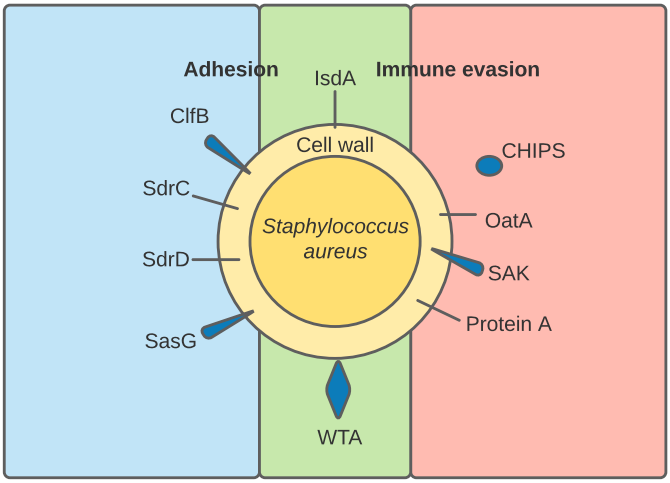

Figure 1 Staphylococcus aureus bacterial factors. *Blue background is adhesion factors, pink background is immune evasion and both is green background. Adapted from PhD thesis ISBN 978-82-7589-370-1, Jan 2013 of Sangvik M. Used with permission.*

Chemotaxis inhibitory protein of *S. aureus* (CHIPS) is an exoprotein that works as a potent inhibitor of neutrophil and monocyte chemotaxis (89). In a study of *S. aureus* isolates from persistent nasal carriers, *spa*, staphylokinase and CHIPS were expressed, suggesting these proteins importance for *S. aureus* carriage (90). Surface protein G (SasG), surface protein X (SasX) and the serine-aspartate repeat proteins SdrC and SdrD may also serve as ligands to the epithelial cells (91-93). Although their roles in humans are yet to be understood (83).

7.5.2 Host factors

Many host factors, lifestyle, environmental, biological or a combination of the former, have been identified through epidemiological research. No significant heritability for *S. aureus* nasal carriage has been detected in twins and family studies (94, 95). Age and sex have been identified as the main risk factors. *S. aureus* nasal carriage is common at a young age with a prevalence of 60% in neonates, 62-69 % in school children, 40-50% in youths and 20-30 % in the adult population (46, 48, 96, 97). The known sex-difference in prevalence is also well documented, where men has increased prevalence of carriage compared to women (98-100). Other known host factors are listed in table 2.

Table 2 Known host risk factors for S. aureus nasal carriage

Host factors
Sex
Age
Hormonal contraceptives
Vitamin D
Obesity
Diabetes
Atopic dermatitis
Tobacco use
Family size
Work in healthcare
Hygiene/living conditions
HIV-infection
Dialysis
Immunosuppression
Granulomatosis with polyangiitis
Rheumatoid arthritis

Vitamin D has a role in infections and may have immune regulatory functions (101). Vitamin D deficiency is associated with higher prevalence of nasal carriage in non-smoking men (98) and for carriage of MRSA (102). However, studies on the effect of Vitamin D3 supplements on *S. aureus* carriage have not revealed significant reduction in carriage prevalence (103).

Obesity and diabetes have been linked to an increased risk of infections, including *S. aureus* infections. Studies have shown increased risk of *S. aureus* nasal carriage in females classified as obese, and risk estimates did not change when adjusting for diabetes (104, 105). High waist circumference in men was also associated with larger carriage prevalence (105). Diabetes has also been associated with increased risk of *S. aureus* carriage in both adults and children (106, 107).

In the possible association between tobacco use and *S. aureus* carriage, smoking has been most studied. Smoking has been associated with both lower and higher probability of nasal carriage (98, 108). A recent unpublished study from Karlsen et al. showed increased risk of *S. aureus* throat carriage with use of smokeless tobacco in adolescent girls (109).

The associations between family size, work in healthcare and hygiene/living conditions and *S. aureus* carriage have been demonstrated by multiple studies and are reflecting settings with increased risk of transmission (76, 110, 111).

Atopic dermatitis has been associated with *S. aureus* nasal carriage, and the risk of carriage increased with severity as well as with allergic multimorbidity (48, 112). There is also an association between HIV-infected individuals (113), intravenous drug users (114), rheumatoid disease (115), patients with renal failure requiring dialysis (116, 117), organ-transplanted patients (118) and *S. aureus* carriage. This supports the hypothesis of compromised immune response as a possible risk factor for colonization.

Other hypothesized host factors for carriage include alcohol use and physical activity. Both heavy and moderate alcohol use have been associated with decreased immunity and host defense (119, 120). A study of risk factors for MRSA carriage in orthopedic patients found an univariable strong association with alcohol use. However, the association disappeared when adjusting for possible confounders (121). Another study of risk factors for MRSA in medical students found an increased but non-significant risk with alcohol use (122). The same study found lower prevalence of *S. aureus* colonization in participants doing physical activity >1 hour a day (122). Other research has mostly shown association in contact sports (123), that may represent increased risk of transmission. The possible relationship between alcohol use, physical activity and *S. aureus* carriage requires further studies to establish association.

Throat carriage has also been linked to ABO blood group and ABH-secretor status, where group O/non-secretor individuals had increased risk of *S. aureus* throat carriage (124).

7.5.3 Circulating sex-steroids

Sex-steroids are steroid hormones that are crucial for development and function of the human body, both by regulating sexual differentiation, secondary sex characteristics and sexual behavior. Sex-steroids are classified in three groups after molecular structure and receptor binding: estrogens, androgens and progestogens. In addition, the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have an overall function on the gonads (125). Gonadotropins are secreted from the anterior pituitary gland and are not considered sex-steroids per se. Due to the hydrophobic structure of sex-steroids, most are bound to sex-hormone binding globulin (SHBG) or albumin that function as transporter proteins. Only a small unbound fraction of the hormones is active and able to bind to the receptor.

All sex-steroid hormones are synthesized from cholesterol through a common precursor steroid pregnenolone. Androgens, progestogens and estrogens are further synthesized through various enzymes, demonstrated in figure 2.

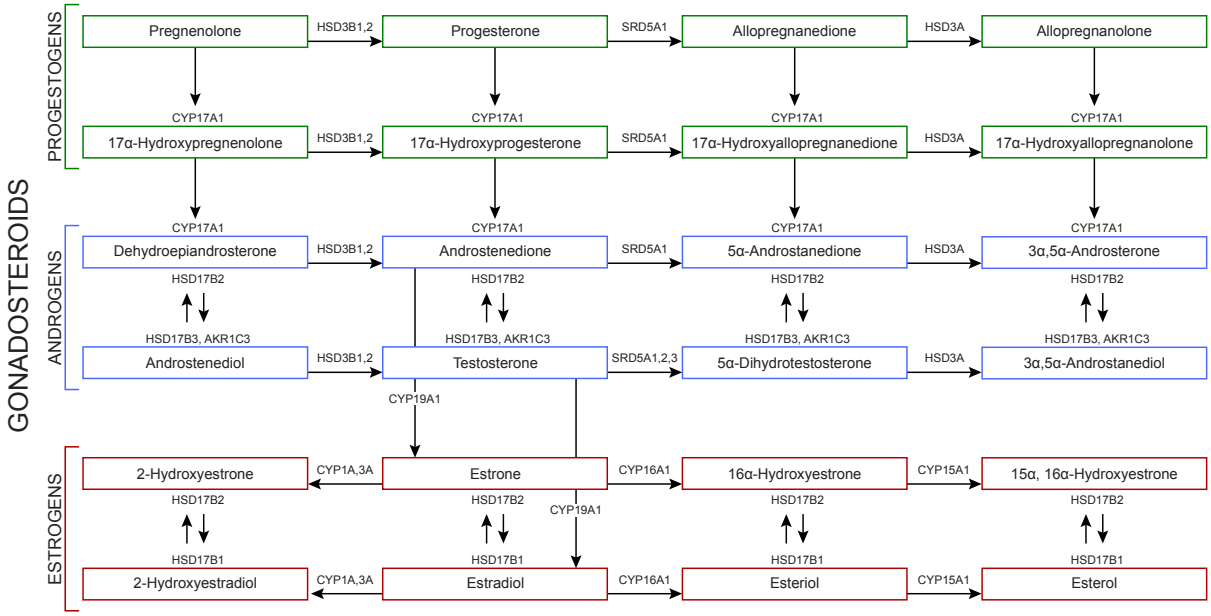

Figure 2 Synthesis of sex-steroids. Made by Rod Wolstenholme, adapted from (126). *Progestogens are highlighted with green color, androgens with blue and estrogens with red. Inside the boxes are names of the different progestogens, androgens and estrogens. Names of arrows represent enzymes converting various sex-steroids.*

Estrogens are generally considered a female reproductive hormone but is found in both men and women with different functions. There are three major estrogens with estrogenic hormonal activity; estrone (E1), estradiol (E2) and estriol (E3) where estradiol has the highest affinity for the estrogen receptor. The biosynthesis takes place in the ovaries, but are also produced in breast, placenta, and adipose tissue. Estrogens promote the development of female secondary sexual characteristics, regulate the menstrual cycle, and are important for maturation of sperm. In addition, estrogens have important functions in cognition, cardiovascular health, skeletal health and in the immune system (127). The production of estradiol is reduced in postmenopausal women and are comparable to the level in men.

Androgens are generally considered a male reproductive hormone, though found in both sexes. The major androgens are testosterone, dihydrotestosterone (DHT) and androstenedione. Androgens bind to androgen receptors and are important for development and maintenance of the male phenotype. Androgens are synthesized from cholesterol and produced in the gonads and adrenal glands. Transformation from testosterone to DHT occurs in prostate gland, liver, brain, and skin.

Progestogens bind to the progesterone receptor and are important for maintaining pregnancy, regulation of menstrual cycle and ovulation, spermiogenesis and testosterone synthesis. Unlike estrogen, progestogens have no effect on feminization. The most important progestogen is progesterone, but also 17α-hydroxyprogesterone (17-OH progesterone) have a degree of progestogenic activity. Progesterone is commonly produced by the adrenal cortex and the ovaries and testes. It is also secreted by the corpus luteum in the first ten weeks of pregnancy, and the placenta in later phases.

7.5.3.1 Sex-steroid function in females

Estrogens are responsible for development of female secondary sexual characteristics during puberty. Androgens are responsible for body hair growth, acne, and axillary odor. In premenopausal women, the menstrual cycle is tightly regulated by the hypothalamus-pituitary-gonadal axis. The hypothalamus releases gonadotropin-releasing hormone (GnRH) in pulses that affect the anterior pituitary gland to produce FSH and LH. The average menstrual cycle consists of the follicular phase (day 1-13), ovulation (day 14) and luteal phase (day 15-28). In the first days of the menstrual cycle a rise in FSH stimulates a few ovarian follicles where the dominant follicle continues to maturity. LH stimulates further development of the ovarian follicle that secretes large amounts of androstenedione that converts to estradiol by the enzyme aromatase. Estradiol inhibits production of FSH and LH by negative feedback, but the dominant follicle continues to secrete estradiol that finally gives a positive feedback signal which in turn give a FSH and LH surge. This surge stimulates the ovulation. If there is no fertilization, the luteal phase starts when FSH and LH cause the remaining parts of the dominant follicle to transform to the corpus luteum which produces progesterone and induces estradiol production. FSH and LH levels then fall quickly and the corpus luteum wastes. Falling levels of progesterone triggers menstruation and the beginning of the next cycle (128). The physiology of the menstrual cycle is demonstrated in figure 3.

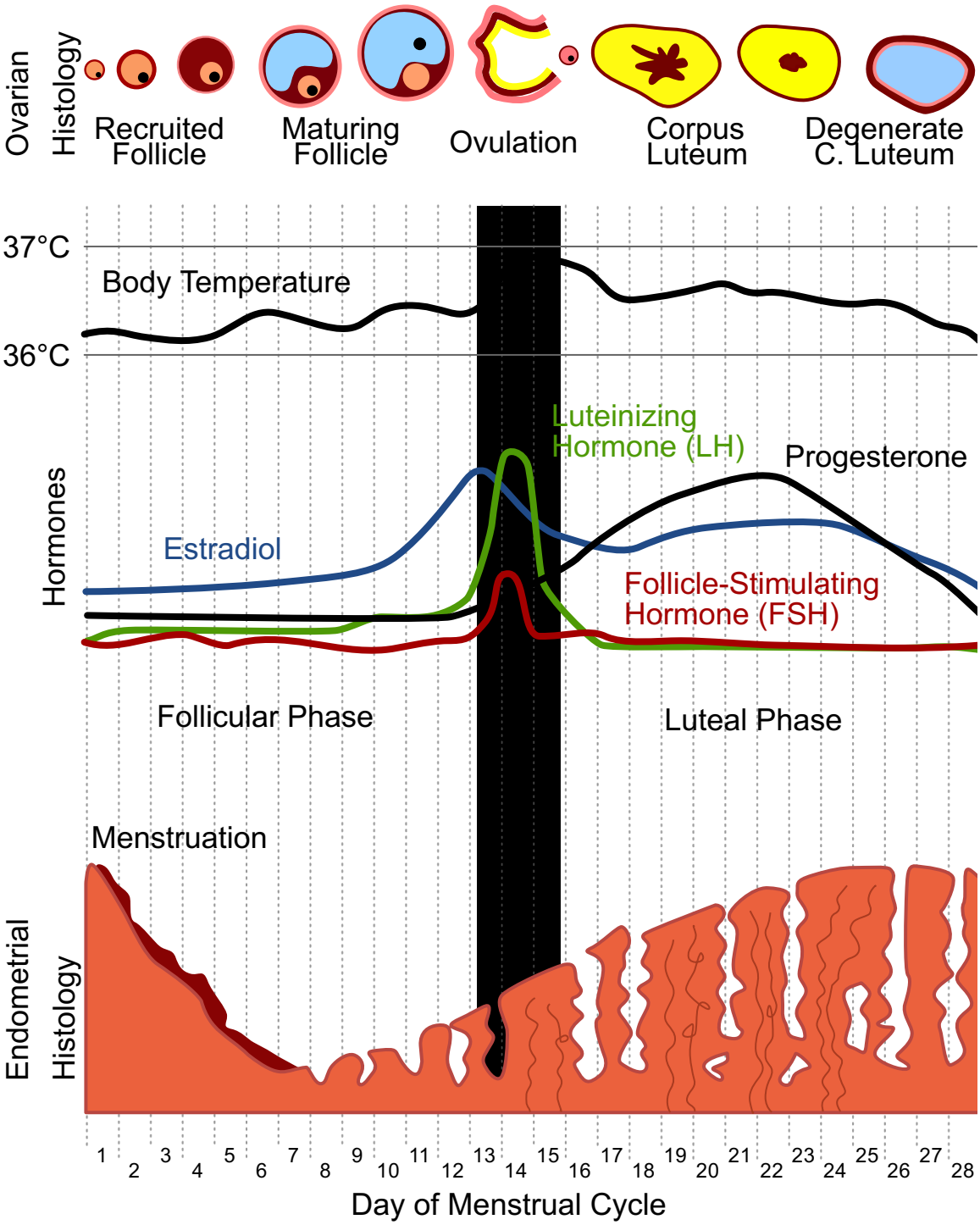

Figure 3 Female reproductive cycle. (129)

The menopause typically occurs between 49-52 years and is mainly caused by limited number of oocytes. The depletion of oocytes causes an increase in FSH and LH because of the reduced production of estradiol by the oocytes. Estradiol continues to be produced in fat tissues and in small amounts in ovaries, bone, blood vessels and brain. In contrast to the sudden decrease in estradiol, the androgens decline more steadily with age (130).

7.5.3.2 Sex-steroid function in males

In the first part of life, androgens regulate the development of the testes and later, secondary male characteristics in puberty. In adult men androgens and FSH cooperatively act on cells in the testes to support spermatogenesis and the production of estrogen and testosterone. High levels of testosterone give negative feedback to the hypothalamus, reducing GnRH production and consequently FSH and LH production. Testosterone in men is therefore diurnal, but the reason for testosterone's circadian rhythm is unknown. In elderly men the mean level of testosterone declines (131).

7.5.3.3 Sex-steroids and immunity and infection

Male sex is associated with greater susceptibility, prevalence and severity of infection compared to women. This is demonstrated across a wide variety of pathogens, including parasites, fungi, bacteria and viruses (132). Multiple large studies report a higher prevalence of sepsis in men compared to women (133, 134), and *S. aureus* bacteremia is more frequent in males (135, 136). A recent study in a Norwegian population-based cohort demonstrated that men had 41% higher risk of any first-time BSI compared to women, and that the BSI related mortality also was higher in men (137). This forms the basis for the hypothesis that sex hormones may be determinants for bacterial colonization and infection.

Estrogens, androgens, and glucocorticoids influence a large proportion of the cell transcriptome and interact with specific receptors on immune cells (138). Women have higher number of CD4+ T cells compared to men, and the number of regulatory T cells varies during the menstrual cycle with increasing levels in follicular phase where estrogen is high (139, 140). Estrogens also affect antibody production by decreasing negative selection of immature B cells and increase in autoreactive B cells and polyclonal activation of B cells (141). Androgens decrease the proliferation of T- and B-cells and the production of immunoglobulins and cytokines, thereby working as immunosuppressants on the adaptive immune response (142).

In relation to the innate immune system, there have been reports of higher number of circulating monocytes in men and in women after menopause, compared to premenopausal women (143). Estrogen most likely acts by downregulating the CD16 expression and decrease the proinflammatory cytokines (144). Neutrophils and natural killer cells are suppressed by estrogens (142). Testosterone has a suppressive effect on monocytes and macrophages by decreasing the expression of TLR-4 (145).

Carriage of *S. aureus* induces responses in both the innate and adaptive immune system, but the bacteria overcome host defense mechanisms to establish carrier status (47). There have been studies reporting a positive association of *S. aureus* nasal carriage with biomarkers of endogenous estrogen levels (146). There is also in vitro evidence of increased staphylococcal binding to HeLa cells in the presence of estrogen (147). One study among premenopausal women found higher prevalence of persistent throat carriage of *S. aureus* with increasing levels of free testosterone (99). Sangvik et al. also demonstrated an association between specific *spa*-types and sex, where male sex was associated with *spa*-type t084 and inversely associated with *spa*-type t012 (46). The lack of knowledge about the effects of circulating sex-steroids on immunity and *S. aureus* carriage, increases the importance of further research.

7.5.4 Hormonal contraceptives

Hormonal contraceptives (HC) were introduced in 1960 as a combined oral contraceptive pill containing synthetic estrogen and progestin. The combined oral contraceptive pill is still the most used contraceptive method all over the world and considered highly effective in preventing pregnancy (148). More recently developed administration routes for the combined contraceptives have been introduced with the contraceptive patch and vaginal ring. Progestin-only methods have also been introduced with drugs only containing progestin. Progestin-only contraceptives also have different administration routes with both oral tablets, intrauterine device (IUD), injections and subcutaneous implants. In addition to drug contents and administration routes, classification of oral hormonal contraceptives can be done by contents of progestin. To mimic the changes in the female menstrual cycle, biphasic, triphasic and quadriphasic oral contraceptives have been developed (149). These contraceptives contain different amounts of progestin in different phases of the menstrual cycle. Overview of classifications of different hormonal contraceptives can be found in table 3.

Table 3 Classification of different hormonal contraceptives

Contents	Progestin-only contraceptives Combination contraceptives
Cycle	Monophasic Bipasic Triphasic Quadriphasic
Administration	Pill Patch Vaginal ring Intrauterine device Subcutaneous implant Injection

Ethinyl estradiol is the most common synthetic estrogen used in combined contraceptives. Compared to estrogen, ethinyl estradiol has greater bioavailability and is more potent because of the long half-life and slow metabolism (150). Other estrogens, more rarely used in combined contraceptives, are estradiol valerate and estradiol as hemihydrate.
Progestins are synthetic steroids with progestogen activity and have a considerable variation in chemical structure (151). List of commonly used progestins is shown in table 4. In addition to the binding to the progesterone receptor, many progestins also have an androgenic activity by binding to the androgen receptor. This can cause unwanted androgenic side effects like acne or hirsutism (152). Newer progestins such as drospirenone and cyproterone acetate are classified as antiandrogenic and are used to treat disorders like polycystic ovary syndrome (153).

Table 4 Commonly used progestins in hormonal contraceptives*. Adapted from table 5 in Allen et al (154).*

First generation	Second generation	Third generation	Unclassified
Norethindrone acetate Ethynodiol diacetate Lynestrenol Norethynodrel	dl-Norgestrel Levonogestrel	Desogestrel Gestodene Norgestimate	Drospirenone Cyproterone acetate

Progestin and ethinylestradiol inhibit ovulation by suppressing FSH and LH. Low levels of FSH and LH inhibit the evolvement of follicles. Progestin also functions on the endometrium by changing it to be less receptive for implantation and prevents sperm penetration by increasing the viscosity of the cervical mucus (155).

7.5.4.1 Hormonal contraceptives, immunity and infection

A recent study examined infection rates in women aged 16-40 over a period of 16 years. The authors showed increased overall infection risk in women using hormonal contraceptives compared to non-users (156). The same research group also found an association between use of hormonal contraceptives and neutropenia (157).

The effect of progestins on mucosal sites reduces inflammation. Progestins inhibits activation and inflammatory pathways in dendritic cells, as well as promoting anti-inflammatory phenotypes of T-cells (158). There have been conflicting results of hormonal contraceptive effects on the production of immunoglobulins (159-161). The full effect of hormonal contraceptives on the immune system is not completely understood.

Hormonal contraceptive use has been associated with *S. aureus* nasal carriage. Zanger et al found tripled odds of persistent nasal carriage (OR= 3.2;95%CI=1.4-7.3) in users of combined hormonal contraceptives in an adult population (162). As the prevalence of *S. aureus* carriage is high in adolescents, the effect of hormonal contraceptives in a general youth population is of particular interest.

7.6 Social network and *S. aureus* carriage

Social network analysis (SNA) is a broad strategy for investigating social structures (163). Study participants represent nodes and relationships or interactions between them represents edges connecting nodes. SNAs is often visualized in sociograms where the nodes are represented as dots and edges as lines. After constructing the social network from information, usually self-reported from the study participants, statistical methods can be used to evaluate the effect of different traits or factors in the network. SNA has been used in infectious disease research both for investigating transmission, distribution, treatment, and prevention. This statistical method has been very useful in epidemics and disease outbreaks (164). HIV infections, tuberculosis and sexually transmitted diseases have been strongly connected to social networks (165-167). *S. aureus* is mostly transmitted through physical contact (168), and is therefore an ideal candidate for SNA of bacterial carriage. A few studies have investigated transmission of *S. aureus* through SNA. One study demonstrated the transmission of *S. aureus* including MRSA in an illicit drug user population which was identified as a reservoir for the bacteria with linkages to the general population (169). A recent case-control study used SNA to reveal transmission of MRSA through a social network in a health care facility (170). There is a lack of SNA-studies on *S. aureus* carriage in larger general populations.

Another advantage of SNA analysis in epidemiological research of *S. aureus* carriage is the reduction of the possible confounding by social contact. The known host factors for *S. aureus* carriage have been attained from studies not adjusting for social contact. Host risk factors are known to affect the risk of *S. aureus* carriage, but may also be determinants of friendship,

therefore producing an association by confounding. Figure 3 demonstrates an example of this confounding. Lifestyle factors like tobacco use and hormonal contraceptives may also spread through friendship (171).

This lack of adjustment for social contact in epidemiological studies may give bias of unknown size and are of importance for prevention of carriage. If no host risk factor can be identified without the effect of social contact, interventions like hygiene and infection control might be indicated. If some host factors can be identified, adjusted for social network, this can give important information on which host factors that can be the basis for new preventative research.

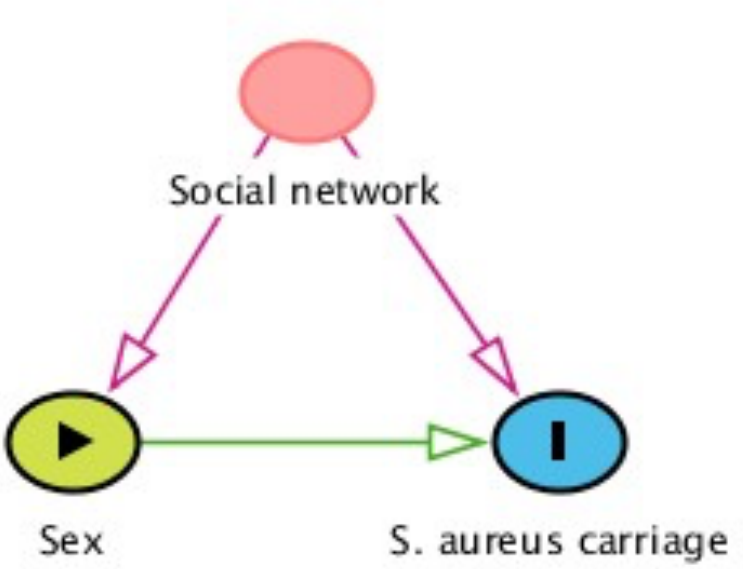

Figure 4 DAG model(172) of the possible confounding of social networks. Green: Exposure, Blue: Outcome, Red: Confounder. There is a known association between sex and *S. aureus* carriage, where male sex is linked to higher prevalence of *S. aureus* carriage. This association could be confounded by social networks. If male sex is a determinant for friendship (male participants in a study have social contact mostly with other male participants), the effect of sex on carriage could be a result of increased social contact between male participants and therefore a greater transmission of the bacteria. If the effect of sex on *S. aureus* carriage is adjusted for social network and there is still an association, we have to believe that this is a result of biological, lifestyle or environmental effects of sex.

7.7 Decolonization of *S. aureus* carriage

Due to the risk of autoinfection and transmission among carriers, studies have investigated the possibility of *S. aureus* eradication. Nasal decolonization is most studied in both surgical and non-surgical patients (173). Decolonization has also been evaluated for carriers of MRSA (174). An often-used approach is treatment with mupirocin nasal ointment which is considered the gold standard for eradication treatment. Mupirocin is a polyketide antibiotic naturally produced by *Pseudomonas fluorescens* that inhibits the bacterial isoleucyl t-RNA synthetase of the bacteria (175).

In studies of mupirocin nasal ointment, the estimated success of elimination of nasal carriage was 90-94% after three days to one week (176), and approximately 60% in one year follow up

(174). It is hypothesized that the reduction in long-term efficacy is due to recolonization or acquisition of exogenous *S. aureus* strains after treatment (177). Throat carriage has also been associated with failure of eradication of nasal colonization (57).

Two large studies demonstrated significant reduction in the incidence of nosocomial *S. aureus* infections after decolonization (178, 179). One of the studies also showed reduction in deep surgical site infections (179). The World Health Organization (WHO) recommends decolonization with intranasal mupirocin ointment to all *S. aureus* nasal carriers undergoing cardiothoracic and orthopedic surgery, and that decolonization is considered for all carriers undergoing other types of surgery (180).

There is less convincing evidence for the effect of decolonization on infection risk among non-surgical patients. A 2016 meta-analysis concluded that mupirocin reduced the risk of *S. aureus* infections by 59% in patients receiving dialysis (181). However, a review from 2017 found no definite evidence for a reduced risk of infections by decolonization in peritoneal dialysis patients (182).

In addition to the limitation of reduced long-term success rate of decolonization, there has been development of antimicrobial resistance against mupirocin. Plasmid-mediated genes like mupA and mupB have been reported and are associated with treatment failure (183). Mupirocin was first used in 1985 and resistance was already described in 1987 (184). In Norway, the prevalence of mupirocin resistance is low and observed in only 0.6% of MRSA isolates (7). In a Swiss hospital the prevalence reached 64% (185). The WHO recommendation for eradication has therefore been targeted only to confirmed *S. aureus* carriers. There are limitations to this regime in clinical practice especially for emergency surgery, where both testing and treatment length are not achievable pre-operatively.

There is a need for fast-acting, single dosage, safe molecules that do not select resistant strains. A few newer drugs have shown promise, like LTX-109 (186), NP108 (187), XF-73 (188), lysostaphin (189) and squalamine (190) among others, but these are still in the early phase of development.

7.8 Microbiological diagnostics

The identification of *S. aureus* has no uniform laboratory protocol and differs between studies. Nasal samples are taken from the anterior nares with a sterile dry or wet swab, often cotton or rayon tipped (54, 110). The swab is rotated three or more times for best sampling. Throat swabs are taken from both tonsillar surfaces. The throat sampling is a more difficult method and requires both trained personnel and a compliant participant compared to sampling from nares.

After sampling the swabs are immediately placed in transport medium (Amies or Stuarts) and stored at room temperature before culturing within 6-72 hours (96, 191), or at 2-8 °C for longer storage (162). Before plating, enrichment broths and incubation at 37°C can be used to facilitate the growth of *S. aureus* (94). This will give a higher prevalence of *S. aureus* because also low bacterial loads will be detected (192). In research, direct culture without enrichment broth is mostly used mainly because of the unknown clinical significance of low bacterial load carriage. Culture swabs of persistent carriers, considered as the most relevant phenotype, contained higher bacterial loads compared to intermittent and non-carriers (44).

S. aureus culture can be directly streaked on growth media plates or enriched samples can be streaked, both after incubation at 37 °C. Mannitol salt agars (193), blood agars (177) and chromogenic media (194) are used, with chromogenic agars as the most expensive but with higher sensitivity and specificity. After a 24-48 hours incubation period at 37°, *S. aureus* is identified based on colony morphology and color. Conformation of *S. aureus* isolates are done by confirmation test, usually based on the presence of coagulase (either free coagulase or clumping factor), or detection of protein A and/or clumping factor (195). A newer method Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has also been showed to be rapid, accurate and cost-effective (196).

7.8.1 Molecular typing

Molecular typing of *S. aureus* is used for resolving transmission routes in outbreaks and for infection control. There are many techniques for staphylococcal typing, and one of the methods is identification of different *spa* genes that code for protein A. This method uses the region X of the *spa* gene containing a variable number of 24-27 base pair tandem repeats. Repeats are assigned a code according to sequence and the *spa* type is deduced from the repeat succession (197). The *spa* types are registered in a localized internet server by Ridom SeqSphere+ software (198). The principle of *spa*-typing is presented in figure 4.

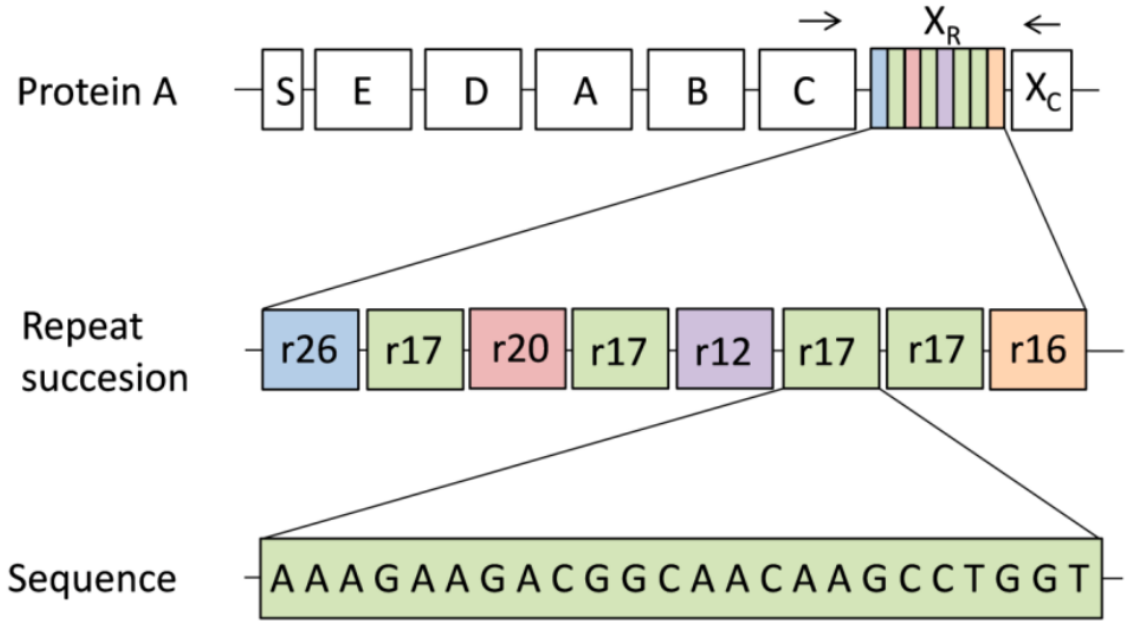

Figure 5 The principle of spa-typing. *The variable number tandem repeat repeat region X_R of Protein A is the basis for spa-typing. The figure shows the repeat succession in spa type t003 (PhD thesis, ISBN 978-82-7589-370-1, Jan 2013 of Sangvik M). Used with permission.*

Another method, pulsed-field gel electrophoresis (PFGE), has high sensitivity towards rapid genomic variation and are therefore a valuable tool in disease outbreaks. The technique involves creating large DNA fragments from bacterial chromosomes using rare cutting restriction endonucleases. These DNA fragments are separated in gel by alternating the direction of the electrical fields, resulting in “DNA fingerprints” that can be used to discriminate clonal relatedness (199).

Multilocus sequence typing (MLST) is a method that determines allelic variation by sequencing internal fragments of encoding genes for housekeeping enzymes. MLST involves polymerase chain reaction (PCR) amplification and sequencing of internal fragments of seven housekeeping genes. Different sequences are assigned allele numbers and the seven assigned numbers form an allelic profile, or sequence type (200).

Other more rarely used methods include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and variable number of tandem repeat (VNTR) (201-203). The MALDI-TOF MS is a relatively new method used for both identification of *S. aureus* and *S. aureus* typing. The advantages of MALDI-TOF MS include its rapidness, low cost and high specificity. Currently, MALDI-TOF MS is used for identification and distinction of bacterial strains in clinical microbiological laboratories.

Whole genome sequencing (WGS) is now the "goldstandard" for typing *S. aureus* and MRSA in research, but is currently being introduced to clinical microbiology (204). WGS determines the complete DNA of the bacteria and two main types of WGS is used; short-read and long-read sequencers. Short-read WGS uses DNA polymerase to incorporate nucleotides in order to determine the DNA sequence with short reads length (50-300 base pairs). One limitation of short-read WGS is the short read length, because of the high complexity of bacterial genomes, and the need for amplification which is time consuming and could introduce bias. Long-read WGS span complex regions with a single continuous read and therefore solves some of the issues with short-read WGS. The error rates are however higher and total number of reads is lower.

7.9 Clinical significance

Because of *S. aureus'* extraordinary virulence (3), its increasing antibiotic resistance (7, 28, 29), high prevalence of carriage in the general population (47) and strong association with hospital-acquired infections (21), it is a significant threat to modern medicine and public health. In the WHO action plan against antimicrobial resistance, *S. aureus* is on the high priority pathogens list for which new antibiotics and better infection prevention are urgently needed (205). In about 80% of *S. aureus* infections the infecting strain is already present on the skin or the mucosa of the patient (38). Thus, reduction in prevalence of carriage would be very beneficial to decrease the disease burden. Decolonization is effective, but mostly short-term and there is an increasing resistance against the most used agent, mupirocin (173).

Predisposing host factors for *S. aureus* carriage have been hypothesized to be potential key targets for prevention of colonization and subsequent infection (78). The two most important risk factors for carriage are sex and age (46). However, the hypothesis about sex-steroid related mechanisms has not yet been fully explored. Moreover, research on *S. aureus* carriage has generally not considered whether host risk factors might be confounded by social contacts.

Thus, we do not know whether the distribution of *S. aureus* in populations reflects direct spread of the microbe among contacts (i.e., hygiene measures would be relevant) or contacts sharing the same host risk factors for colonization (i.e., lifestyle interventions would be relevant). There is a crucial need of more research on *S. aureus* carriage to reduce transmission, infection and increase infection control.

8 Aims of the thesis

The overall aim was to investigate whether exogenous and endogenous sex-steroids are associated with *S. aureus* nasal carriage, to investigate the effect of social networks on transmission of *S. aureus*, as well as to estimate the effect of the social network on host risk factors for *S. aureus* nasal carriage.

The aims of this thesis were:

- To examine the possible relationship between exogenous sex-steroids in the form of hormonal contraceptives and *S. aureus* nasal and throat carriage in a general female youth population.
- To investigate whether endogenous sex-steroids are associated with *S. aureus* nasal carriage in a general female population.
- To investigate whether endogenous sex-steroids are associated with *S. aureus* nasal carriage in a general male population.
- To examine the transmission of *S. aureus* carriage among contacts in a general youth population and identify host risk factors for *S. aureus* carriage and differentiate between the risk attributable to social contact between similar individuals compared to biological or lifestyle related risk per se.

9 Materials and methods

9.1 The study populations

9.1.1 The Tromsø Study

The Tromsø Study is a longitudinal health study conducted in seven waves from 1974 until 2016 focusing on life-style related diseases. Each wave included clinical examinations, biological samples, questionnaires, and interviews. This is Norway's most comprehensive population study with more than 45 000 individuals attending at least once. The sixth Tromsø Study (T6) was conducted in 2007-2008 with a total of 12 984 participants and attendance of 65.7%. All residents of Tromsø aged 40-42 and 60-87 years (n=12 578) were invited as well as a 10% random sample aged 30-39 (n=1056) and a 40% random sample of individuals aged 43-59 years (n=5787). Individuals who had attended the second visit in the fourth Tromsø Study but were not included in the three groups mentioned above were also invited (n=341). Women constituted 53.4% of the participants (206, 207).

Nasal swab cultures were collected in T6, from October 2007 to June 2008. All participants aged 30-49 years (n=1730) and a random cohort of participants aged 50-87 years (n=2629) were invited for nasal swab sampling at their first visit at the screening center. The number of participants with nasal swab culture were 1597 and 2429 in the two age groups, respectively. Altogether 4026 participated; 1741 males and 2285 females. Among these, 2997 participants had a repeated nasal swab culture taken at a second visit with a median interval of 28 days (98).

9.1.2 Fit Futures

Fit Futures is a longitudinal youth health study conducted in two waves in 2010-2011 and 2012-2013. Each wave included clinical examinations, biological samples, questionnaires, and interviews. In Fit Futures 1 (FF1), all first-year of high school students in the municipalities of Tromsø and Balsfjord, North Norway, were invited (n=1117). A total of 1038 students attended (92.9%); 508 girls and 530 boys (208). In Fit Futures 2 (FF2), all third-year high school students (n=775) and all participants from FF1 not attending school (n=464) were eligible to attend the study. FF1 participants that could not be successfully contacted were not invited to FF2 (n=31). A total of 868 students participated with an attendance of 71.9% (48). All participants in both waves of Fit Futures were invited to provide nasal and throat swab samples. All participants in FF1 were also invited to have a second set of microbiological samples taken at school after a mean interval of 17 days.

9.1.3 Ethics

Each participant in T6 signed a declaration of consent. Participants that had their declaration of consent withdrawn after participation were excluded from all analysis. The T6 study was approved by the Regional Committee for Medical and Health Research Ethics (REK) and the Norwegian Data Protection Authority.

A declaration of consent was signed by each participant in both FF1 and FF2. Participants younger than 16 years had to bring written consent from a parent or guardian. FF1 and 2 were approved by the Regional Committee of Medical and Health Research Ethics (REK) and the Norwegian Data Protection Authority.

Paper I and IV was approved by REK North with reference 2011/1710, while **paper II-III** was approved by REK North with reference number 2018/1975.

9.1.4 Study population – Paper I

In the analysis of hormonal contraceptives in relation to *S. aureus* nasal carriage in **paper I**, the study population included participants in FF2. Of the 868 students who attended; participants with no or invalid nasal or throat swab (n=46 or 43), male sex (n=366), age exceeding 21 years (n=17) and females with missing data on hormonal contraceptives use (n=3) were excluded. This resulted in 436 women for the analysis of *S. aureus* nasal carriage and 439 for the analysis of *S. aureus* throat carriage. A visual presentation of the study populations in all the papers are demonstrated in Figure 5.

9.1.5 Study population – Paper II

In the analysis of circulating sex-steroids in relation to *S. aureus* nasal carriage in women in **paper II**, the study population included female participants with nasal swab sample in T6 (N = 2285). All women without data on serum hormones (n=1320), participants reporting antibiotic use or missing data on antibiotic use the last 24 hours (n=20), ongoing pregnancy (n=6), use of hormonal contraceptives, use of hormonal replacement therapy, endocrine breast cancer therapy and IVF treatment (n=208 for all drugs) were excluded from the analysis. A total of 724 female participants had one valid nasal sample (n=7 invalid samples) and 700 females had two consecutive valid samples (n=31 invalid or missing samples).

9.1.6 Study population – Paper III

In the analysis of circulating sex-steroids in relation to *S. aureus* nasal carriage in men in **paper III**, the study population included male participants with nasal swab sample in T6 (n=1741). Participants without data on serum hormones (n=853), use of antibiotics the last 24 hours (n=19) or only one nasal sample (n=117) were excluded from the analysis. The final cohort included 752 males with two consecutive nasal samples.

9.1.7 Study population – Paper IV

In the analysis of social network in relation to *S. aureus* carriage in **paper IV,** the FF1 cohort was used. No participants were excluded from the main analysis. A total of 746 participants had a valid *spa*-typing of a positive throat culture and are included in the analysis of *spa*-types in the social network.

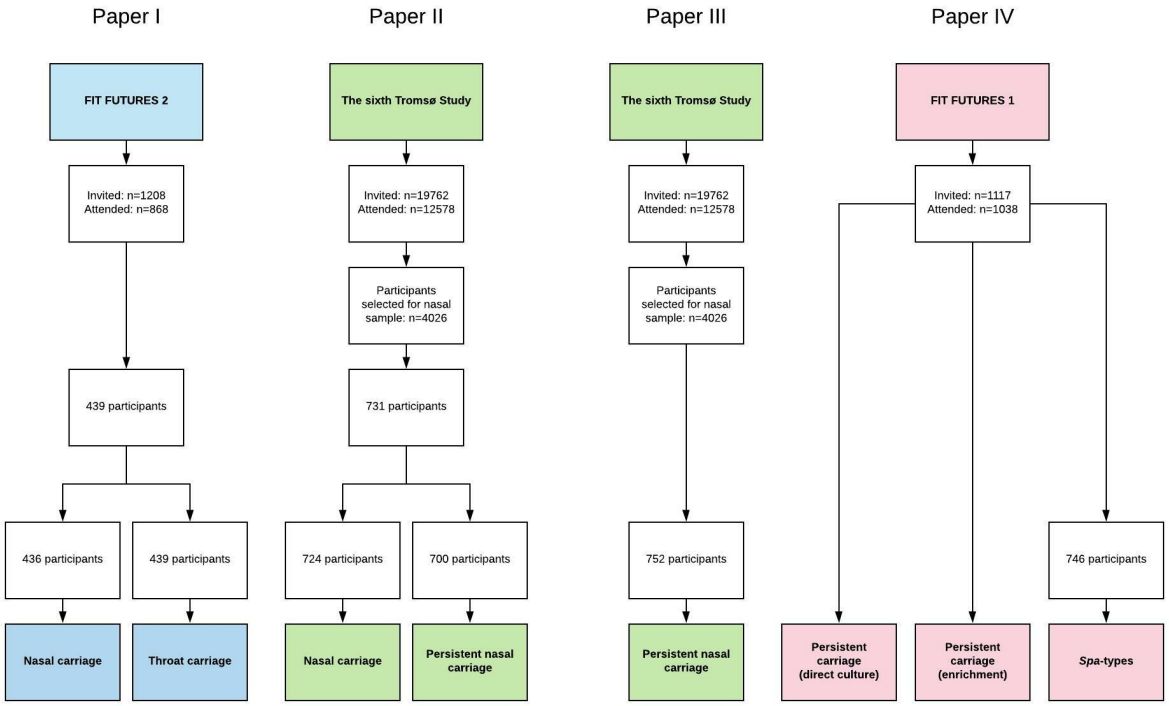

Figure 6 Overview of the study population, paper I-IV. *Exclusion criteria described in text. The study populations in paper I and II include only women, while the study population in paper III includes only men. Nasal carriage is defined as one positive microbiological nasal swab for S. aureus. Throat carriage is defined as one positive microbiological throat swab for S. aureus. Persistent nasal carriage is defined as two positive nasal swabs for S. aureus. Persistent carriage (direct culture) is defined as two positive nasal swabs for S. aureus using direct culture. Persistent carriage (enrichment) is defined as two positive nasal swabs for S. aureus using enrichment broth before culture. Spa-types is defined as typable S. aureus spa-types in throat samples.*

9.2 Measurements

9.2.1 Assessment of *S. aureus* carriage

9.2.1.1 The Fit Futures cohort (Paper I and IV)

Nasal and throat swabs were collected by research nurses at the Clinical Research Department at the University Hospital of North Norway (UNN), Tromsø. A NaCl (0.9%)-moistened sterile rayon-tipped swab rotated three times with gentle pressure was used to sample both vestibule nasi. A second swab was used to sample both tonsillar regions with moderate pressure. The swabs were placed in transport medium (Amies Copan, Brescia, Italy) and stored at 4°C for a maximum of 3 days in FF1 and 3-7 days in FF2.

The analysis was performed by trained personnel at the Department of Medical Biology, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø. Swabs were enriched in Bacto Staphylococcus medium broth (Difco laboratories, Sparks, MD, USA) and incubated for 18-24 hours at 37°C. One drop of enrichment broth was streaked on blood agar (Oxoid, UK) for growth control and CROMagar-plates for *S. aureus* detection (SmithMed AS/Microbiological media production). The agar plates were incubated for 42-48 hours at 37 °C. The most dominating colony type on the SAID plate was then plated on blood agar and incubated for 20-24 hours at 37°C before confirming the presence of *S. aureus* by the Staphaurex plus agglutination test (Remel, USA). Growth of any bacterial colonies on any of

the agar plates was registered as a valid culture. All confirmed isolates were frozen at -70°C in glycerol-containing liquid media. In FF1 used in **paper IV**, an additional set of samples was taken at the participants' school after a mean interval of 17 days. In addition to the enrichment culture, FF1 also included direct culture of the microbiological samples. The direct culture method is different only by the swabs not being placed in enrichment broth.

For **paper I** only one sample from nose and throat were available in FF2. Outcome is therefore defined as nasal carriage (versus non-carriage), meaning one positive nasal swab using enrichment, and throat carriage (versus non-carriage), meaning one positive throat swab using enrichment. For **paper IV** two samples from both locations and results from two different culturing methods were available in FF1. Outcome is therefore defined as persistent nasal carriage using direct culture (versus others), meaning two positive nasal swabs using direct culture, and persistent nasal carriage using enrichment culture (versus others) meaning two positive nasal swabs using the enrichment step. Throat persistent carriage was not used in the main analysis for **paper IV**.

9.2.1.2 The sixth Tromsø study cohort (Paper II and III)

Nurses at the sixth Tromsø study screening center collected nasal swab samples with a NaCl (0.9%) moistened sterile rayon-tipped swab rotated three times in each nasal vestibule. A second nasal swab was collected with a median interval of 28 days. The samples were placed in transport medium (Amies Copan, Bescia, Italy) and stored at 4°C for a maximum of 3 days.

Trained personnel at the Department of Microbiology and Infection Control, UNN Tromsø, did the microbiological analysis. Specimens were cultured on blood agar (Oxoid, Cambridge, UK) for growth control and chromID-plates for *S. aureus* detection (bioMérieux, Marcy I'Etoile, France). Agar plates were incubated for 42-48 hours at 37°C before selecting the most dominating colony to confirm presence of *S. aureus* by the Staphaurex plus agglutination test (Murex Diagnostics Ltd, Dartford, UK). Growth of any bacterial colonies on any of the agar plates was registered as a valid culture. All confirmed isolates were frozen at -70°C in glycerol-containing liquid media.

Nasal carriage (versus non-carriage) was defined based on baseline sample status, requiring first swab positive for *S. aureus*. Persistent nasal carriage was defined as both samples positive for *S. aureus* (versus others). An overview of methods and definitions for all papers are presented in table 5.

Table 5 Overview of S. aureus carriage definitions in paper I-IV. *The alternative value for persistent nasal carriage was "others", including both intermittent and non-carriers. The alternative value for carriage (nasal and throat) is non-carriage.*

	OUTCOME	DEFINITION	MICROBIOLOGICAL METHOD
PAPER I	Nasal carriage	Single nasal swab positive for *S. aureus*	Enrichment culture
	Throat carriage	Single throat swab positive for *S. aureus*	Enrichment culture
PAPER II	Nasal carriage	First nasal swab positive for *S. aureus*	Direct culture
	Persistent nasal carriage	First and second nasal swab positive for *S. aureus*	Direct culture
PAPER III	Persistent nasal carriage	First and second nasal swab positive for *S. aureus*	Direct culture
PAPER IV	Persistent nasal carriage	First and second nasal swab positive for *S. aureus*	Direct culture
	Persistent nasal carriage	First and second nasal swab positive for *S. aureus*	Enrichment culture
	Spa-type	Molecular typing of *S. aureus* from throat isolate	Frozen isolate from any culture method

9.2.2 *Spa*-typing

In **paper IV** (FF1) *spa*-typing of *S. aureus* throat isolates defined different *S. aureus* genotypes. All *S. aureus* isolates from the first throat swab samples were s*pa*-typed. The frozen (-70°C) *S. aureus* isolates were inoculated on blood agar (Oxoid) and incubated overnight at 37°C. Two or three colonies were transferred to 200 µl sterile H_2O and vortexed. The isolates were *spa*-typed using primers spa-1113f and spa-1514r (209) with the following conditions for cycling: 95°C for 10 minute; 35 cycles for 30 seconds, 60°C for 15 seconds, and 72°C for 1 minute; and 72°C for 10 minutes and then kept at 4°C. *Spa*-types were determined using Ridom StaphType softwere (Ridom GmbH, Würzburg, Germany) (197) and the Ridom SpaServer website (http://www.SeqNet.org/). We excluded isolates that were not typable for *spa* and isolates that were not confirmed as *S. aureus* by coagulase test and Staphaurex plus (Remel).

9.2.3 Questionnaires and interviews

9.2.3.1 Hormonal contraceptives

In **paper I and IV**, information on current hormonal contraceptive use from interview in FF1 and FF2 was used. Trained nurses asked female participants the questions: "If you have started menstruating; do you use any kind of contraceptives?" (yes/no), and "If you use any kind of contraceptives: what type?" (Tablets/Injections/Implants/Condom/Transdermal contraceptive patch/Vaginal contraceptive ring/Intrauterine device (IUD)/Other). Two

participants reported no menstruation and were not asked further questions. Condom and other were defined as non-hormonal contraceptives. HC users were asked about brand name of tablets, implants, transdermal contraceptive patch, vaginal ring or IUD and were shown photos of different brands to help correct reporting. In the analysis, HC types and brands were categorized into combination HC and progestin-only HC. Combination HCs were further divided into HC containing high dosage estradiol (combination contraceptives containing ≥ 30 µg ethinylestradiol), and low dosage estradiol (combination contraceptives containing ≤ 20 µg ethinylestradiol). No combination contraceptives contained ethinylestradiol dosages between 20 and 30 µg. Definitions of hormonal contraceptive use are presented in table 6.

Table 6 Definitions of hormonal contraceptive use in paper I and IV

	Estradiol dosages		Brand name
Combination contraceptives	Low	≤ 20 µg ethinylestradiol	Mercilon, Yasminelle, Loette 28, Nuvaring
	High	≥ 30 µg ethinylestradiol	Marvelon, Yasmin, Microgynon, Oralcon, Diane, Synfase, Evra, Zyrona
Progestin-only contraceptives	None		Cerazette, Nexplanon, Depo-provera, Implanon.

9.2.3.2 Social network

In **paper IV** the social network was constructed based on questions from the interview in FF1. The participants were asked: "Which first level high school students have you had most contact with the last week? Name up to five students at your own school or other schools in Tromsø and Balsfjord". Reciprocity in nominations was not mandatory. For each nomination, five "yes/no" questions assessed type of contact: "Did you have physical contact?", "Have you been together at school?", "Have you been together at sports?", "Have you been together at home?", "Have you been together at other places?". This resulted in five social networks: "physical contact", "school", "sport", "home" and "other". Adding all relationships together formed a sixth network: overall network.

9.2.3.3 Other lifestyle and health variables

In **paper I and IV**, FF1 and FF2 questionnaire data on lifestyle (smoking, snuff, alcohol, physical activity) was used in the analysis. Smoking status and snuff use were assessed with the questions "Do you smoke?" and "Do you use snuff?" with the response categories "No, never/Sometimes/Daily". Information on alcohol use was obtained with the question "How often do you drink alcohol?" (Never/Once per month or less/2-4 times per month/2-3 times per week/4 or more times per week). Level of physical activity was assessed with the question "Which description of your exercise and exertion in leisure time fits best? If your activity varies much, for example between summer and winter, then give an average. The question refers only to the last twelve months." (Reading, watching TV, or other sedentary activity/Walking, cycling or other forms of exercise at least 4 hours a week/Participation in recreational sport, heavy outdoor activities, snow clearing etc/Participation in hard training or sports competitions, regularly several times a week). In **Paper I**, we used multiple variables from the FF2 questionnaire to assess current atopic eczema; dry skin or itchy rashes with age-specific location (antecubital or popliteal fossae, wrists, ancles, neck or face) for 2 weeks or more in the past 12 months, or self-reported eczema combined with use of topical corticosteroids in the past 12 months (48).

In **paper I**, FF2 interview data on antibiotic use the past 3 months was included in the analysis: "Have you taken any antibiotics (tablets or oral suspensions, nasal ointments, eye drops or eye ointment applicated in the nose/eye) the last 3 months?" (Yes/No). Questionnaires and interview guide from FF1 and FF2 can be found in appendices B and C.

Paper II and III used questionnaire data on lifestyle from the T6 study (Appendix A). Smoking status were assessed through the question "Do you/did you smoke daily?" (Yes, now/Yes, previously/Never). Information on alcohol use was obtained through the question "How often do you drink alcohol? (Never/Monthly or more infrequently/2-4 times a month/2-3 times a week/4 or more times a week). Hospital admissions was evaluated though the question "Have you during the last 12 months been to a hospital?" (Yes/No).

9.2.4 Clinical examination

All **papers (I-IV)** included information on measured body mass index (BMI) in the analysis. In T6, FF1 and FF2 body height in centimeters (cm) and weight in kilograms (kg) were measured with electronic scale to the nearest 0.1 unit with participants wearing light clothing and no shoes. BMI was calculated as weight divided by height squared (kg/m^2).

9.2.5 Blood samples

In **paper II and III,** non-fasting blood samples were drawn from an antecubital vein. After 30 minutes at room temperature, the coagulated samples were centrifuged at 2000 g for 10 minutes, and the sera were transferred within 1 hour to plastic tubes and kept between 1°C and 10°C. The blood samples were sent twice daily to the Department of Laboratory Medicine, University Hospital North Norway, Tromsø (206). The Hormone laboratory at Haukeland University Hospital, Bergen, Norway, used serum samples from T6 participants to analyze a panel of serum sex-steroids as a part of the laboratory's work on establishing reference values within subgroups of the general population (210). The frozen serum samples were stored in the Tromsø Study biobank at minus 80°C from 2008 until 2013 when they were transported on dry ice to Haukeland for analysis.

9.2.5.1 Sex-steroids

Testosterone, androstenedione, 17α-hydroxyprogesterone (17-OH progesterone) and progesterone were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS, SCIEX API 5500 triple-quadrupole mass spectrometer, Applied Biosystems/MDS with an Aglient 1920 UPLC system). Serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), as well as the binding proteins sex-hormone binding globulin (SHBG), dehydroepiandrostenedione sulfate (DHEAS), and albumin were measured using DPC immulite 200 XPi (Siemens Healthcare Diagnostics). Analytical coefficients of variations are reported in table 7. Estimated bioavailable testosterone, which includes free and albumin-bound testosterone, was obtained by the equation "(testosterone/SHBG) x 10" (211).

Table 7 Analytical coefficients for sex-steroids

Sex-steroid	Analytical coefficient of variation
Testosterone	6% by 1.7 nmol/L 7% by 5.7 nmol/L 6% by 35 nmol/L
Androstenedione	7% by 2.2 nmol/L 7% by 9.1 nmol/L

	7% by 48 nmol/L
DHEAS	9% by 3.8 µmol/L 9% by 12.6 µmol/L
17-OHprogesterone	8% by 1.9 nmol/L 6% by 8.9 nmol/L 6% by 68 nmol/L
Progesterone	10% by 4.8 nmol/L 6% by 25 nmol/L 10% by 53 nmol/L
LH	7% by 5 IE/L 8% by 10.1 IE/L 8% by 28 IE/L
FSH	6% by 5 IE/L 6% by 17.8 IE/L 6% by 32 IE/L
SHBG	7% by 4.7 nmol/L 7% by 54.9 nmol/L
Albumin	3%

9.2.5.2 25-OH vitamin D

In **paper I**, serum 25-hydroxyvitamin D (25(OH)D) was analyzed using liquid chromatography mass spectroscopy (LC-MS/MS) at The Hormone Laboratory, Haukeland University Hospital. The total coefficients of variation were <6%.

In **paper II and III**, serum 25-hydroxyvitamin D (25(OH)D) was analyzed by electrochemiluminescence immunoassay (ECLIA) using an automated clinical chemistry analyzer (Modular E170, Roche Diagnostics). The total analytical coefficient of variation was 7.3%. There was a known overestimation of 25(OH)D levels in smokers when using ECLIA (Roche) method (212).

9.2.5.3 Glycated hemoglobin

For **paper I, II and III**, glycated hemoglobin (HbA1c) was determined in EDTA-blood by high performance liquid chromatography (HPLC) using an automated analyzer (Variant II, Bio-Rad Laboratories INC., Hercules, CA, USA). Total analytical coefficient of variation was < 3.0%.

9.2.6 Statistical analysis

9.2.6.1 Paper I

Statistical analysis was performed using SPSS version 23 for Mac OS X. Univariable associations were analyzed in contingency tables and by calculating means and standard deviations (SD) using chi-square and t-test to quantify the potential role of chance. Multivariable logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) to describe the association between hormonal contraceptive use and *S. aureus* nasal and throat carriage adjusting for potential confounders. The R package DAGitty 2.3 for directed acyclic graphs (213) and Akaike Information Criterion (AIC) was

used for model selection. Testing for potential interaction between explanatory variables was done by including multiplicative terms of two predictor variables in the model and comparing models using AIC. The final model included adjustment for age, BMI, HbA1c, Vitamin D, atopic eczema, smoking, daily snuff use, alcohol use, recreational physical activity and antibiotic use the past 3 months.

9.2.6.2 Paper II

Statistical analysis was performed using Stata/MP 15.1 for Mac OS X, Univariable associations were analyzed in contingency tables and by calculating means and standard deviations (SD) using chi-square and t-test to quantify the potential role of chance. Independent samples t-test was used to test possible association between sex-steroid hormones and *S. aureus* nasal carriage and persistent nasal carriage. Multivariable logistic regression analysis was used to describe possible association between sex-steroid hormones and *S. aureus* nasal carriage and persistent nasal carriage while adjusting for potential confounders, using odds ratios (OR) and 95% confidence intervals (CI). DAGitty 3.0 was used for model selection. Testing for potential interaction between explanatory variables was done by including the multiplicative terms of two predictor variables in the model. The final model included adjustment for age and BMI. Female participants in luteal phase was excluded from the analysis of testosterone and androgens to increase accuracy (214).

9.2.6.3 Paper III

The statistical analysis was performed using R version 3.6.3 and R Studio 1.3.1093.Univariable associations were analyzed by chi-square test, Student´s t-test, or Mann-Whitney U test (Wilcoxon rank-sum test). Multivariable logistic regression models were used to assess the possible association between *S. aureus* and persistent nasal carriage while adjusting for potential confounders. DAGitty 3.0 was used for model selection, and possible interactions were assessed in the final model. A sensitivity analysis on an age-stratified population (cut-off 55 years, median age) was performed as both concentration of serum androgens and *S. aureus* persistent nasal carriage were inversely related to age.

9.2.6.4 Paper IV

Univariable associations were assessed by t-test and chi-square tests, with Yates`s correction for 2x2 tables and Fisher's exact test was used when applicable. The social network was constructed with each participant represented by a node and each relationship between students represented by an undirected line in the network. The connection between nodes was analyzed using Exponential Random Graph Model (ERGM) or Additive and Multiplicative Effects Models. Patterns of connections(non-carriers connected to non-carriers, non-carriers connected to carriers, carriers connected to carriers) were analyzed using Simulation Investigation for Empirical Network Analysis (Autocorrelation model) (215). Further analysis was done with bootstrapping simulated networks against the observed network with descriptive analysis and logistic regression. The mathematical background for the analysis is presented in supplementary materials for **paper IV.**

10 Summary of main results

10.1 Paper I

Hormonal contraceptive use and *Staphylococcus aureus* nasal and throat carriage in a Norwegian youth population

In this female youth cohort (median age 18 years), prevalence of *S. aureus* nasal carriage was 50.0% (218/436) and prevalence of throat carriage was 59.7% (263/439) (carriage for both sites determined by a single swab and use of enrichment culture). The prevalence of nasal carriage was 64.2% in women using combination HC with high estrogen and 55.6% in women using HC with low estrogen. Women using progestin only contraceptives had a prevalence of nasal carriage of 34.3% and non-users a prevalence of 41.8%. For throat carriage combination HC users with high and low estrogen had a prevalence of 62.4% and 65.4%, respectively. Progestin-only users had a prevalence of throat carriage of 52.6% and non-users 57.6%. In the multivariable logistic regression analysis, adjusted for potential confounders, users of combination HC had more than doubled odds for nasal carriage compared to non-users, and the odds increased with increasing estrogen dosage (high estrogen OR=2.44;95%CI=1.39-4.28, low estrogen OR=2.14;95%CI=1.17-3.91). Users of progestin-only contraceptives had an adjusted OR of 0.29 (95%CI=0.12-0.67) compared to users of combination contraceptives. Multivariable logistic regression analysis of *S. aureus* throat carriage showed a trend towards modestly higher odds of participants using high and low dosage of estradiol.

10.2 Paper II

Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general female population

In this adult female cohort (mean age=53.5 years, SD=13.6), prevalence of *S. aureus* nasal carriage was 24% (180/724) and prevalence of *S. aureus* persistent nasal carriage was 22% (151/700) (results from direct culture). In postmenopausal women, mean level of testosterone was significantly lower in persistent nasal carriers compared to others (mean difference=0.10 nmol/L; 95%CI=0.00-0.20). In postmenopausal women, serum levels of androstenedione, DHEAS and 17α-hydroxyprogesterone were lower in nasal carriers compared to others but not statistically significant. In a multivariable logistic regression analysis adjusted for age and BMI, an increase of 1 SD of testosterone lowered the odds of nasal carriage of *S. aureus* (OR=0.60; 95%CI=0.39-0.94, SD=0.81 nmol/L) as well as persistent nasal carriage (OR=0.57; 95% CI=0.35-0.92, SD=0.82 nmol/L) in the total female population. Analysis stratified on menopausal status showed a decrease in odds of persistent carriage by higher testosterone for postmenopausal women separately (OR=0.74; 95% CI=0.55-0.99, SD=0.43 nmol/L). Multivariable logistic regression analysis showed lower odds of nasal carriage (OR=0.53; 95% CI=0.32- 0.90) and persistent nasal carriage (OR=0.52;95% CI=0.30–0.92) for an increase also in bioavailable testosterone by one standard deviation (SD=0.19 nmol/L) in the total female population. As for testosterone a decrease in odds of persistent nasal carriage was also found for postmenopausal women (OR=0.72; 95%CI=0.52-0.99; SD=0.09 nmol/L). In premenopausal women, the OR estimates for testosterone and bioavailable testosterone were 0.35 and 0.38, respectively, but broad confidence intervals gave inconclusive results.

10.3 Paper III

Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general male population

In this adult male population (mean age=54.8 years, SD=13.2) the prevalence of *S. aureus* persistent nasal carriage was 32% (results from direct culture). Among men aged 55 years and above (median split), there was an inverse dose-response relationship between serum concentration of testosterone and persistent nasal carriage, and persistent carriers had significantly lower mean levels of testosterone (p = 0.028). This association was attenuated when adjusting for BMI and age (OR=0.96 per nmol/L change in testosterone; 95% CI=0.91-1.01). There was no association between any circulating sex-steroid and *S. aureus* persistent nasal carriage in the total male population adjusted for BMI and age.

10.4 Paper IV

Social network analysis of *Staphylococcus aureus* carriage in a general youth population

In this general youth population (mean age=16.4 years, SD=1.24), the prevalence of persistent nasal carriage of *S. aureus* determined by direct culture was 30.3%. The prevalence of persistent nasal carriage of *S. aureus* determined by enrichment culture was 42.6%. Prevalence of persistent carriage was higher in male participants compared to female (Prevalence: 36·4% and 48·1%, men direct culture and enrichment culture; Prevalence: 24·0% and 36·8%, women direct culture and enrichment culture). We found no other significant differences between groups according to population characteristics.

To evaluate if there was a significant influence of social network on the transmission of *S. aureus*, a simulation of 1000 networks with the same topology was performed and the distribution of same-to-same relationships (non-carriers connected to non-carriers, carriers connected to carriers) in the simulation was compared with the observed social network. For persistent nasal carriage defined by enrichment culture, transmission of *S. aureus* could be demonstrated for the overall network (p=0.02), for the physical network (p=0.04) and for the school network (p=0.01). For persistent nasal carriage defined by direct culture there was similar results for school network (p=0.02) and for the overall network (p=0.07) and physical network (p=0.06) with near significant p-values. To further demonstrate the transmission of *S. aureus* nasal carriage through the social network, we did the same analysis with specific *S. aureus* genotypes (*spa*-types). For the overall network, a transmission of *S. aureus spa*-types could be demonstrated with a p-value of 0.01. In a univariable logistic regression model, the chance of being a persistent carrier increased by 3.7% (95%CI=3.52-3.94) when increasing your friend circle by one friend who was defined as a persistent carrier by direct culture. Similarly, 3.4% (95% CI=3.33-3.45) for persistent carriage defined by enrichment culture. In an adapted linear autocorrelation analysis adjusted for potential host risk factors there was an increase of 4.8% (p<0.001) in risk of being a persistent nasal carrier for each friend defined as a persistent nasal carrier by direct culture, and an increase of 6% (p<0.001) for persistent nasal carriers defined by enrichment culture.

In the univariable logistic regression model, female participants had the highest risk of transmitting *S. aureus* through social interaction (p <0.001 for direct culture and p=0.03 for enrichment culture). The relative risk of *S. aureus* transmission within the male social network was 0.85 (95% CI = 0.80-0.88) compared to the female network using direct culture data, and similarly 0.94 (95% CI=0.90-0.97) for enrichment culture data. Also, participants using alcohol two times or more per month had a higher risk of transmission of *S. aureus* compared to non-users and participants using alcohol once per month or less (p-value of 0.04;

direct culture). For direct culture, participants doing medium physical activity had a higher probability of transmission ($p = 0.008$) compared to those not physically active. The autocorrelation model also measured which of the host risk factors that made the participants´ friends significantly more contagious. For direct culture there was an increase in contagiousness for sex, BMI and physical activity ($p = 0.001$-0.008), and for enrichment culture; study program, BMI, alcohol and physical activity ($p = 0.001$-0.002 for all). Females tended to have a more relationships than males, which is also true for participants with normal BMI and participants being both medium and hard level physically active.

11 Discussion

11.1 Internal validity

Observational studies have limitations regarding the validity of the findings. Internal validity is defined as to what extent the observed results represent the population studied, and are not due to methodological errors (216).
The four papers in this thesis, are all based on studies with a cross-sectional design. Cross-sectional studies have the limitation of the exposure and the outcome being assessed simultaneously, and generally cannot give evidence of a temporal relationship between exposure and outcome.

11.1.1 Selection bias

Selection bias is an important source of error in epidemiology. It is a bias based on improper selection of participants resulting in a sample not representative for the population. In the T6 study and Fit Futures studies used in the four papers, the attendance ranged from 66% to 93%. This contributes to reduced selection bias and increase of both internal and external validity. In FF1 and FF2, used in **paper I and IV**, the age groups were homogenous. In T6, **paper II and III**, some of the youngest and the oldest age groups were underrepresented with 47% attendance in age group 30-39 years and 40% in the age group 80-87 years (206). Participants in T6 selected for nasal swabs were more evenly distributed across age groups, although the population under 30 years of age were not invited.

A study demonstrated that higher BMI in FF1 was associated with non-attendance in FF2 in the female population (217). This may suggest that individuals with known risk factors for disease do not attend health studies to the same extent as individuals with less risk factors, representing non-response bias. Among those invited to the second, third and fourth wave of Tromsø study, there was higher mortality in participants only attending the last survey compared to participants attending all three surveys (207). Individuals with serious illness, residents of nursing homes, homeless or illicit drug users could be less likely to attend health surveys. Thus, participants in both FF, particularly FF2, and T6 may represent a healthier population compared to non-participants and may therefore have a lower rate of *S. aureus* carriage.

11.1.2 Information bias

Information bias occurs when the means of obtaining information about participants are inadequate so that as a result, some of the information gathered regarding exposures and/or outcome is incorrect. This inaccuracy may introduce misclassification bias. Misclassification can be differential and non-differential (218). Differential misclassification is where the misclassification differs between study groups whereas non-differential misclassification results from the degree of inaccuracy that characterizes how the information is obtained and is not related to exposure or outcome status.

11.1.2.1 Outcome

The primary outcome in the four papers is carriage of *S. aureus.* There is no uniform laboratory method for identification of *S. aureus*, and there is no clear definition on the

number of swabs that should be taken and what fraction should be positive to determine carriage status. There is a difference in both methods for identification and number of swabs in all four papers as demonstrated in table 5.

The methods used for identification are similar in all four papers with the difference of direct culturing and enrichment culturing. In all papers, swab sampling and laboratory analysis were performed by trained personnel and lab technicians according to procedures that were specified in a protocol. A few agar plates had no growth representing inadequate sampling or malfunction in equipment used. Lack of growth on agar plates was identified as invalid and excluded from analysis.

In all papers we used Staphaurex plus agglutination test for the confirmation of *S. aureus*. This test is based on latex particles sensitized with human fibrinogen and monoclonal antibodies for the simultaneous detection of clumping factor, staphylococcal protein A and group-specific antigens of the *S. aureus* cell surface. The Staphaurex plus agglutination test could give false positive reactions for *Staphylococcus lugdunensis, Staphylococcus capitis, Staphylococcus hyicus, Staphylococcus intermedius and Staphylococcus schleiferi* (219). In the studies we used the Staphaurex plus agglutination test in combination with the *S. aureus* selective CHROMagars, and we assume that the sensitivity and specificity are high. However, newer methods like MALDI-TOF MS could have given even more accurate detection.

In **paper I** we used enrichment culture for definition of both throat and nasal carriage. When enrichment broth is used also low bacterial loads will be detected, and therefore give increased prevalence of *S. aureus* positive tests (192). In studies of decolonization, it is recommended to use enrichment broth in identification of *S. aureus* to prevent possible elimination failure (220). The relevance of low bacterial load carriage in *S. aureus* epidemiology is not known as most studies have used direct culturing only. Persistent carriage is generally thought to be the most relevant phenotype; importantly, swab cultures from persistent carriers contained higher bacterial loads compared to intermittent and non-carriers (44). In **paper IV** we compared the results of direct culture and enrichment culture for identification of nasal persistent carrier without observing substantial differences. **Paper II** and **III** used direct culture only and some participants may have been misclassified because of the lack of enrichment step. However, as enrichment culture also detects low bacterial loads more associated with the less clinically relevant intermittent carrier state, the specificity of direct culture might increase.

The number of swab samples and time interval between samples differ in the four papers. The FF2 participants included in **paper I** provided only a single sample, which renders it impossible to distinguish between persistent and intermittent carriers and this may represent a bias of unknown effect. The T6 participants included in **Paper II** and **III** had two separate swab samples taken with an interval of 0-124 days (median 28 days). There was an interval of ≥ 12 days for 90% of the samples (105). The T6 data shows that only 3.9% of the participants were misclassified as carriers from the first nasal swab, thus giving an insight to the degree of bias also in **paper I**. Studies have shown that intermittent nasal carriers may be colonized with the organism for a median of 14 days (51). In **paper IV**, two swabs were provided by the FF1 participants with a mean interval of 17 days. Thus, it is likely that some participants in **paper II**, **III** and **IV** had swabs taken in the interval of possible transient carriage and may have been misclassified. Although the "culture rule" defined by Nouwen et al. is widely used (54), some studies argue that ≥ 3 samples are needed to correctly classify *S. aureus* carriers as

persistent carriers and other definitions are still used in recent literature (221). A possible limitation of all papers is therefore the number of repeated swab samples taken.

Paper I also used throat carriage of *S. aureus* as a main outcome. A possible source of bias with throat samples compared to nasal samples, is the more difficult sampling method with lower participant compliance due to discomfort. Although all samples were performed by trained research nurses, some samples may be inadequate because of reduced compliance and more difficult sampling.

Several agar plates (15%, n=68) from the throat samples had poor or no growth. The enrichment broth promotes growth of staphylococci, while other members of the upper respiratory microbiota are inhibited by the relatively high concentration of sodium chloride. Therefore, agar plates with no bacterial growth were included in the study as valid negative throat samples. For nasal samples agar plates without bacterial growth were excluded from the analysis because of the risk of inadequate sampling or equipment malfunction.

Paper IV also had *spa*-types as outcome. *Spa*-typing is considered a rapid and accurate method to discriminate *S. aureus* isolates but there are also certain limitations. The method is based on a single locus typing that can misclassify particular types due to recombination and/or sequencing errors (222). Another limitation related to this paper is the use of only throat isolates for molecular typing, while nasal carriage is the primary outcome variable that is most strongly associated with transmission. A validation study among 100 observations in FF1 with *S. aureus* isolated from both nasal and throat swab cultures showed that nasal *spa*-types and throat *spa*-types coincided in 82% of cases. The same effect has been demonstrated in other studies, where Nurjadi et al. showed identical genotypes in nose and throat in 72% of the participants (124). Based on this, it is likely that the FF1 data give conservative estimates for the effect of social network on transmission of *S. aureus*.

In **paper IV** we had 10 invalid nasal samples for the first swab, and 51 invalid samples for the second swab. These were reclassified as negative for *S. aureus*. Because of the analysis of social network, we believe that it would have introduced a larger bias excluding parts of the social network compared to the bias of including possible misclassified samples.

All potential sources of bias mentioned for the outcome variables are sources of non-differential bias, with no obvious link to the exposure variables. Non-differential bias is more likely to give an underestimation of the results (223).

11.1.2.2 Exposure

In **paper I** the main exposure variable was hormonal contraceptive use. Females that had missing data on hormonal contraceptive use were excluded from the analysis. The information was assessed in photo-assisted interviews by trained nurses to reduce risk of information bias.

In **paper II** and **III** the exposure variable consisted of a panel of serum sex-steroids and binding proteins. Serum concentrations of testosterone, androstenedione, 17-OH progesterone and progesterone were analyzed by liquid chromatography tandem mass spectrometry. LC-MS/MS is considered the gold standard method for steroid profiling due to very high sensitivity and specificity (224). The frozen serum samples were stored at minus 80°C with no light exposure from 2008 until 2013 when they were transported on dry ice for analysis at

the Haukeland Hormone laboratory. Preparation of samples were done by trained personnel at laboratory, and this would ensure little to no degrading of samples. Serum concentrations of LH, FSH, DHEAS as well as SHBG and albumin were measured using DPC immulite 200 XPi. Analytical coefficients of variation are low for all sex-steroids, gonadotropins and binding proteins, ensuring high precision.

In premenopausal women, sex-steroid hormones are cyclic and vary across the menstrual cycle (figure 3). In the T6 study, serum sample was taken at only one time point and the measured concentration may therefore not be representative for the participants´ hormonal status. In postmenopausal women, sex-steroids vary less, but decline with age. Adjustment of age may therefore be considered sufficient in postmenopausal women. To classify premenopausal and postmenopausal women both medical history and circulating FSH-levels were used reducing possible misclassification bias.
Premenopausal women were classified as being in follicular or luteal phase according to progesterone level (cut off 5.5 nmol/L). Ideally, menstrual phase should be classified by both medical history and circulating progesterone levels (225), but we lacked accurate information on last menstruation for most of the participants. Some premenstrual women in luteal phase could therefore be misclassified as follicular phase and vice versa.

Male sex-steroid hormones are diurnal, but less so compared to women and this may result in a more representative value with only one measurement. Testosterone in men has a circadian rhythm with optimal sampling from 8 to 10 am. The blood samples were taken from 8 am to 8 pm in T6, thus representing a suboptimal sampling for an unknown proportion of samples in our study.

Testosterone is mostly bound to SHBG and albumin in serum because of the hydrophobic structure of the molecule. The free fraction of testosterone is low, about 1-3% in serum. The largest proportion of testosterone is tightly bound to SHBG and physiologically unavailable. Testosterone is loosely bound to albumin, and both the albumin-bound fraction and free fraction are physiologically available to body tissues (226). Bioavailable testosterone, that includes free and albumin-bound testosterone, is therefore most applicable to measure possible testosterone effects. Bioavailable testosterone was in **paper II and III** obtained through the equation "(testosterone/SHBG) x 10" (211). Some claim that this equation is not reliable when SHBG concentration is low (227). A sensitivity analysis using the equation by Morris et al. was therefore performed (226). This did not alter the results of the analysis and the simpler equation is therefore used in the papers.

Paper IV used social network based on information from the participants as exposure. The information was collected through questions in the interview by the research nurses where participants could nominate up to five friends. The nominations were limited to the students with whom the participants had have most contact with the last week. One obvious bias in the gathering of social network data is the representativeness of the nominations. Some participants may have a large social network, but could have been prevented from recent social contact, because of for example illness or travel, the week in question. The interview therefore stated a final question about the representativeness of the information gathered: "To what degree does this table of friends give an overview of your social network? Please indicate on a scale from 0 (small degree) to 10 (high degree)". About 76% of the participants indicated representativeness of five or above. Only 5% claimed a score of zero to two.

The overall social network was further divided into five social networks based on reported type or place of contact - physical network, school network, sport network, home network and other network. One limitation in the physical network is the lack of information on the amount of physical contact, which is highly relevant for the risk of transmission of *S. aureus*. Also, the sports network is not specific in type of physical activity and if contact sport is involved.

The social network was for statistical reasons modeled using one time point, while the interviews were conducted at multiple timepoints over a period of seven months. This may give a misclassification of friends. A sensitivity analysis from the same population showed that most participants nominated friends with whom they attended the survey. Furthermore, we used persistent nasal carriage as main outcome variable, characterized by relatively long survival of the nasal *S. aureus* strain, e.g. median survival of about 150 days in a study by van Belkum et al. (51). Therefore, the analysis should be minimally affected by time, as the survey lasted only 7 months.

11.1.3 Confounding

Confounding is a statistical concept where the effect of the exposure under study on a given outcome is mixed with the effect of an additional factor resulting in a distortion of the true relationship (228). All papers identified confounders from Directed acyclic graphs (DAGs), using DAGitty software (172). DAGs have become an established framework in the analysis of causal interference in epidemiological research for minimizing confounding bias (213).

11.1.3.1 Paper I

The DAG in **paper I** identified age, BMI, HbA1c, vitamin D, atopic eczema, smoking, daily snuff use, alcohol use, recreational physical activity, and antibiotic use past 3 months as possible confounders. The epidemiological evidence for the possible associations of *S. aureus* carriage with alcohol use and physical activity is limited and considering that the study population was young healthy adolescents, the value of HbA1c can be debated. The exclusion of these possible confounders did not alter the main results and were kept as covariates in the model.

Importantly, atopic eczema, smoking, daily snuff use, alcohol use, recreational physical activity and antibiotic use past three months were self-reported. It is known from epidemiological research that participants underreport health risk factors like smoking and snuff use (229), while overreporting physical activity that can give health gain (230).

Two possible unobserved confounders were identified, both related to the transmission of *S. aureus*. Firstly, sexual behavior is relevant both for use of hormonal contraceptives and transmission of *S. aureus* as contact transmission is the most common route of spread of the bacteria. Secondly, social network has been associated with *S. aureus* carriage and with decision-making related to hormonal contraceptives (169, 170, 231).

Nevertheless, our study found a lower risk of nasal carriage in progestin-only than non-users, while combination contraceptives gave a higher risk. This suggests direct exogenous hormonal effects, and not confounding by environmental factors such as differences in human contact between the non-user and the user group. Yet, unknown risk factors may account for some of the observed associations.

11.1.3.2 Paper II/III

In **paper II and III**, the analysis of an adult female and male population respectively, only BMI, age and HbA1c were identified as possible confounders among the available variables. Inclusion of HbA1c as a confounder did not alter the main results and was not included as covariate in the final analysis. As described in 9.1.5 above, female participants using hormonal contraceptives were excluded from the analysis.

Unlike in **paper I**, sexual behavior will here function as a mediator, not as a possible confounder. Sex-steroids have an effect on sexual behavior (232), and sexual behavior could have an effect on transmission of *S. aureus*. To assess the direct effect of sex-steroids on *S. aureus* carriage, you would need to adjust for sexual behavior. Because we lack information of sexual behaviors of the participants we can therefore only report the total effect of sex-steroids on *S. aureus* carriage.

Because of limitation in volume of available serum samples, the laboratory was unable to analyze estrogens in addition to the other sex-steroids. We can therefore not evaluate the possible effects of endogenous estrogens on *S. aureus* carriage in our study. Androgens are known precursors of estrogens (Figure 2). High levels of androgens like testosterone may represent higher levels of estrogen, or an unknown ratio between testosterone and estrogen, and therefore produce an association by confounding.

11.1.3.3 Paper IV

In **paper IV**, the primary aim was to investigate the transmission of *S. aureus* in social networks. Because the use of SNA in evaluating the effect of different host factors is a novel investigation, all known host factors with available data were included in the model. The final analysis by adapted linear autocorrelation model investigated the effect of each of the host factors, adjusted for the other host factors. The study has no main exposure. One important limitation is the lack of adjustment for family and household contacts. The study population is based on a youth population with mean age 16.4 years, most of them living with family. Former studies have shown transmission of *S. aureus* in households (233) and association with larger family size (234) and healthcare workers living with children (110). In infants, *S. aureus* carriage has also been associated with number of siblings (235). The lack of adjustment for family, introduces a bias of unknown size.

11.2 External validity

External validity refers to the extent that the results of the study are generalizable to the population (216). All papers are based on health studies inviting a general population in the Tromsø area. Tromsø is the largest city in North Norway and includes the UiT The Arctic University of Norway and the University Hospital of North Norway. It inhabits 77 000 residents in the year 2020. The population is generally of high socioeconomic status, with few residents under the poverty threshold. Tromsø also harbors some of Norway's indigenous community, the Sami people. The Tromsø Study cohorts are regarded as a nationally representative sample of the population. **Paper I** includes the youth population attending third year of upper secondary education (at high school or in apprenticeship training) in the more urban municipality of Tromsø and more rural municipality of Balsfjord with an overall participation rate of 72%. **Paper II/III** investigated adults (30-87 years) in the municipality of Tromsø with a participation of 66%. **Paper IV** invited students attending first year of high

school in Tromsø and Balsfjord and had participation rate of 93%. Because of the high attendance in all four studies, one may assume that the results are representative for the same age-groups in similar modern societies.

11.3 Main results

In this thesis, we assessed the associations of sex-steroids and social network with *S. aureus* carriage in a general youth and adult population. We also developed a statistical method for evaluation of the transmission of *S. aureus*.
With the use of cross-sectional study design, we investigated association and not causality although Bradford-Hills criteria's for causation (236) might apply for some of the demonstrated associations.
The prevalence of *S. aureus* nasal and throat carriage were similar to former studies in a general population (47). We also observed a higher prevalence when using enrichment broth as a part of the culturing methods, also demonstrated in literature (192).

11.3.1 Hormonal contraceptive use and *S. aureus* carriage

As both use of hormonal contraceptives and *S. aureus* carriage are prevalent among youths, insight into a possible relationship would be beneficial for further research on prevention of carriage and subsequent infection and transmission of the microbe.
In our study we demonstrated strong association between the use of hormonal contraceptives and nasal carriage. For combination contraceptives, the positive association was stronger with higher estrogen dosage, suggesting a dose-response relationship. Interestingly, we found the opposite relationship with progestin-only contraceptives which reduced the risk of nasal carriage compared to non-use. Zanger et al. did the first study on hormonal contraceptives and *S. aureus* nasal carriage in a larger population of young adult women in Germany (237). This study demonstrated 60% higher risk of nasal carriage at baseline and tripled risk after three months follow-up for women using combined hormonal contraceptives compared to non-users. Our results lie between their baseline and follow-up risk estimates, and this could partly be explained by the difference in microbiological method where our study included only one swab sample analyzed with enrichment culture. Choi et al. investigated potential risk factors for *S. aureus* carriage in a Malaysian cohort (mean age 25 years) and identified oral contraceptives as a substantial factor with five times higher odds for carriage in users (238). A recent study from 2020 also observed a similar association between hormonal contraceptives and *S. aureus* carriage (239). Adult women using hormonal contraceptives were found to have a prevalence of 48% of *S. aureus* nasal carriage compared to 20% in the non-user group.

Zanger et al. did not find any association with the estrogen dosage or progestin component in combined preparations, however, this may be due to limited number of users in different subgroups (237). To our knowledge, we are the first to demonstrate the association with progestin-only use and reduced odds of *S. aureus* carriage. A recent experimental study demonstrated that desogestrel, a common progestin, was a potent growth inhibitor of drug resistant strains of *S. aureus* (240). This supports our findings in progestin-only users.

As hormonal contraceptives have been linked to both autoimmune diseases, increased risk of infections and neutropenia, the potential effect on the immune system is well known (156, 157, 241). Nasal carriage of *S. aureus* is considered a subclinical inflammatory process, due to suppression of the innate immune system (242). One recent study can give some insight to

the biological mechanisms for our findings. This study reported an association between higher Toll-like receptor 9 (TLR9) transcription levels and non-carrier status of *S. aureus* (243). They also demonstrated that both the association with TLR9 genotype and transcription levels were modified by sex, suggesting that reproductive hormones play a role in *S. aureus* immunity.

Another factor that may influence the association is microbial interference. Acne is an inflammatory skin disease and common in young adults. Combination contraceptives are frequently used to treat acne, while progestin-only contraceptives may exacerbate the condition (244). Acne is associated with the bacteria *C. acnes* that are known to suppress growth of other bacteria like *S. aureus* (245) and is associated with non-carriage of *S. aureus* (73). If progestin-only contraceptives increase the risk of acne, by it being a common side-effect of use, this could explain the lower rates of carriage by *S. aureus* being suppressed by *C. acnes*. This can most likely only partly explain the findings, as acne is only reported in about 11% of women using progestin-only contraceptives and the evidence of hormonal contraceptives' effect on acne is conflicting (246).

Due to our methodological limitations, as described previously, the results on throat carriage should be interpreted with caution. We found no association with hormonal contraceptives and throat carriage in this youth population. We observed high rates of co-colonization of throat and nasal carriage in this population (n=178), but throat carriage had a higher prevalence (60%) compared to nasal carriage (50%). A sensitivity study was performed on co-colonized participants, as these could represent more heavy colonization. We found no association between hormonal contraceptives and co-colonization (colonization of both nasal and throat) of *S. aureus* (results not presented in **paper I**). Studies on host risk factors for both throat carriage and co-colonization are largely missing. Our study may demonstrate a true difference in host-microbe relationship between nose and throat.

11.3.2 Circulating sex-steroids and *S. aureus* carriage

In **paper II** and **III**, we investigated the relationship between circulating sex-steroids and *S. aureus* nasal carriage, in a general female and male population. In the female population, we found a decreased odds of *S. aureus* nasal carriage and persistent nasal carriage with increasing total testosterone and bioavailable testosterone. This association was also apparent when stratifying by menopausal status, though only statistically significant for postmenopausal women. In men, our data did not confirm any associations, although a sensitivity analysis suggested that testosterone levels may be inversely related to *S. aureus* nasal carriage in older men above 55 years of age.

In this study we defined carriage both by one and two swabs positive for *S. aureus,* where persistent carriage was defined by two swabs. As persistent carriage is considered most clinically relevant (179), this was the primary outcome. Similar results were shown both for *S. aureus* carriage and *S. aureus* persistent carriage. This may be explained by the low misclassification rate of only 3.9% when using only the first nasal swab sample compared to both samples to assess carriage.

A few studies have evaluated endogenous sex-steroids and *S. aureus* carriage. One smaller study among women found a positive association of nasal carriage with biomarkers of endogenous estrogen levels (146). This study found higher carriage rates in women with high karyopyknotic index (KI). KI, sometimes also known as cornification index, is the proportion

of estrogenized superficial cells to intermediate and parabasal cells expressed as percentage of the total cell count (247). This gives a measure of estrogen activity, although estrogen status has shown to be indeterminate in non-atrophic smears (248). Another small study among premenopausal women found higher prevalence of persistent throat carriage of *S. aureus* with increasing levels of free testosterone (99). They found no similar relationship in men. Our results contradict this study, but this may be explained by differences in the outcome variable between the studies. In **paper I**, we investigated the association of hormonal contraceptives with both throat and nasal carriage and found no relationship with throat carriage. We hypothesized that this could be due to more difficult sampling procedure or also a true biological difference between throat and nasal carriage. The methods in the study by Nowak et al. also differs methodologically where they present univariable associations in a much smaller study population of younger women and men (99).

Paper I demonstrated lower odds of carriage in women using progestin only contraceptives. Progestins have a large variation in structure, with the common feature of binding to the progesterone receptor. Most of the progestins also bind to androgen receptors to varying degrees (249). Because progestin-only contraceptives lack the estrogen component, there has been demonstrated higher prevalence of androgenic side-effects (250). The lower odds of carriage in women using progestin only contraceptives therefore supports our results of lower carriage rates in women with higher testosterone.

In women, testosterone has been characterized as immunosuppressive, but newer research suggests that testosterone's immunomodulatory effects depend on the reproductive context and menopausal status. There is also inconsistency of results for testosterone in both postmenopausal and premenopausal women where testosterone has been characterized as both pro-inflammatory and anti-inflammatory (251). In our study, we lack measurements of estrogen levels. Androgens are precursors of estrogen production (252), and higher levels of testosterone may therefore represent higher level of estrogen. Estrogen is more commonly associated with increased risk of autoimmune disease and decreased risk of infection. Because we lack information on estrogen levels we do not know if the effect of testosterone is dependent or independent of estrogen.

A recent study on sex-differences in *S. aureus* skin infections concluded that because of the complexity of hormone signaling in vivo, protection against invading pathogens cannot be attributed to a single hormone or receptor (253). One study found that high testosterone and estradiol, and low SHBG levels were associated with conditions that represent a low-grade inflammation process, comparable to the immune response in *S. aureus* colonization (254). The results could therefore also represent the net effect of an unknown ratio between testosterone and estrogen.

The non-significant and thus inconclusive findings in men could partly be explained by limitation in the methods. Testosterone in men has an optimal sampling from 8 to 10 am, and blood samples were taken from 8 am to 4 pm in our study. We found an association between testosterone and *S. aureus* carriage in older men, although the effect was attenuated when adjusting for age. Former studies have shown a reduced circadian rhythm by ageing in men (131). We therefore believe that the stratified model of men above 55 years of age could be more representative.

Also in **paper II**, microbial interference could be part in the effect demonstrated. High androgen and testosterone levels stimulate the sebaceous glands to increased sebum

production and play and important role in development of acne (255). A study from Goodarzi et al. demonstrated that 50-70% of women with polycystic ovary syndrome, where diagnostic criteria are androgenic features like acne, have high values of bioavailable testosterone (256). Another study also demonstrated that late onset and persistent acne was associated with higher levels of free testosterone, dihydrotestosterone and DHEAS (257). As acne is associated with *C. acnes*, known to suppress growth of *S. aureus* (245) this could explain a possible biological mechanism for female participants in our study.

11.3.3 Social network and *S. aureus* carriage

Paper IV had two primary aims. Firstly, we wanted to demonstrate transmission of *S. aureus* carriage in a general youth population by using SNA. Secondly, we wanted to utilize one of the advantages with SNA analysis and investigate the effect of known host factors adjusted for social contact.

We found evidence for both transmission of *S. aureus* carriage per se and transmission of specific *spa*-types in the cohort. When investigating known host risk factors for *S. aureus* we used two statistical methods, a univariable logistic regression model and an adapted linear regression model. For each additional *S. aureus* positive friend, your probability of being a persistent nasal carrier increased by 3.4-3.7% in the logistic regression model and 4.8-6.0% in the autocorrelation model. The difference between the models may represent the effect of the host factors because the autocorrelation model uses a multivariable statistical approach while the logistic regression analysis is univariable.

The transmission of *S. aureus* in the general population is obvious, although this has not been statistically demonstrated before, and validates the social network. SNA has been used in other infectious diseases like tuberculosis, HIV, and sexually transmitted diseases (165-167). These studies aim to identify methods for investigating infectious diseases in outbreaks and identify targets for disease control. A few studies have investigated transmission of MRSA in various populations. One study demonstrated that introduction of MRSA into a social network of active drug users created an identifiable reservoir for the bacteria with linkages to the general population (169). A case-control study used SNA to reveal transmission of MRSA through a social network in a tertiary health care facility (170). Social group effects also occur in humans as unrelated individuals living in the same household were found to have a more similar microbiota than relatives living in different households (258).

In addition to the overall network of youth we evaluated the transmission in subnetworks. We demonstrate statistical evidence for transmission in the physical network and school network. In the physical network, participants claimed they had physical contact with their friends, although we lack exact information on the amount and type of physical contact. As contact transmission is the main source of spread of *S. aureus* (259), this is the most likely justification for this observed association. The association with the school network and transmission of *S. aureus* is not as obvious. In a study of MRSA transmission among household contacts, prolonged exposure time to MRSA was a significant risk factor for transmission (234). A Norwegian school day is estimated 6-8 hours, which may suggest prolonged exposure. Although, we lack information about amount of physical contact and estimated time together. We did not find any significant spread of *S. aureus* in the home and sports network, also supporting time as an important risk factor for transmission.

The logistic regression model identified sex, alcohol use and physical activity as host factors that were associated with social transmission. The autocorrelation model demonstrated similar results with sex, BMI and physical activity significantly related to transmission because of social contact.

In this and other studies, the prevalence of *S. aureus* nasal carriage is considerably higher in males compared to females (98). Former studies have not been able to distinguish the effect of male sex per se, compared to the possible confounding of social network. This possible confounding effect is demonstrated in figure 3. In this study, male sex was associated with a 15% lower risk of transmission compared to women, although the prevalence of carriage was higher for men (36% versus 24%). Female participants also had a higher average number of friends than male participants. This could demonstrate that the male predominance in carriage is determined by sex-specific predisposing host characteristics as S. aureus social transmission is less frequent than in females.

In this youth study we could not find the association between BMI and *S. aureus* carriage unadjusted for social network, as other studies have demonstrated (105). Use of BMI as a reference method for body fat in children and adolescents is generally considered not suitable, although some support the use also in children (260). We found an association between BMI and transmission of *S. aureus* in this social network of youths. This could be partly explained by the higher number of friends for participants with normal BMI compared to the other BMI groups, particularly the obese BMI group. This could suggest BMI as a "social risk factor" in adolescents, and this may represent a different relationship compared to the association between BMI and *S. aureus* carriage in adults.

The associations of alcohol use and physical activity with *S. aureus* carriage are not as well established as other host risk factors (120-122). We did not find any association between these host factors and carriage unadjusted for social network in this youth study. We could demonstrate an association between medium physical activity and carriage of *S. aureus* because of social contact. In the cohort, there was a higher prevalence in women of medium physical activity compared to men (women = 27%, men = 23%). Some of the effect demonstrated could be attributed to the known sex-difference in *S. aureus* carriage and not represent a true increased risk of transmission. One study demonstrated higher prevalence of *S. aureus* nasal carriage in athletes doing contact sport (123). We have no information on the type of physical activity, or relatedness to increased physical contact during medium physical activity. We can therefore not exclude increased physical contact as the biological mechanism for the observed increased transmission. We also found an association between drinking alcohol more than twice a month and increased risk of transmission of *S. aureus.* Again, participants consuming more alcohol had a larger average number of friends compared to those reporting less use. The effect may represent increased physical contact and with multiple friends at the same time at parties and social gatherings.

We did not find a statistical association between hormonal contraceptives and *S. aureus* carriage, not adjusting for the social network. In the FF1 survey we had a lower number of hormonal contraceptive users compared to the FF2 survey that included the same population, two years later. The low number of hormonal contraceptive users could explain the lack of association between hormonal contraceptive use and *S. aureus* carriage that have been presented in **paper I** and by other authors (162). In the logistic regression analysis, we found no statistical evidence of transmission of *S. aureus* for any of the hormonal contraceptive groups. This could suggest that the effect of hormonal contraceptives on *S. aureus* is not

confounded by the social network, but we cannot conclude because of the lack of association between hormonal contraceptives and *S. aureus* carriage in this population.

Hormonal contraceptives could only be analyzed in the univariable logistic regression model, because of the assumptions of the adapted linear autocorrelation model that requires analysis of the total social network of both female and male participants.

In this study we defined outcome as persistent nasal carriage determined by two swabs, and with both the use of enrichment and direct culturing. We observed a higher prevalence of *S. aureus* persistent nasal carriage when using enrichment culture (prevalence = 43%) compared to direct culture (prevalence = 30%). The results of the SNA were similar for either definition. This may demonstrate the robustness of the findings.

11.3.4 Sex-differences in *S. aureus* carriage

The sex-difference in *S. aureus* carriage is widely reported (36, 98). In all four papers we observed a higher prevalence of *S. aureus* carriage in men. The known sex-difference was the basis for all aims. We have demonstrated a relationship between both exogenous and endogenous sex-steroids and *S. aureus* carriage in women. We were unable to distinguish the effects of age and the effects of endogenous sex-steroids in men. Nevertheless, we show that male sex is an independent risk factor for carriage, not confounded by social networks.

The sex-difference in carriage is most likely multifactorial, including differences in both behavioral and biological factors. Studies of MRSA in healthcare workers and the general population have found associations between hand-hygiene and colonization and infection rates (261, 262). In observational studies in restrooms, males had significantly lower frequency of hand hygiene, soap use and hand-hygiene duration (263, 264). Wertheim et al. reported an association between nose picking and *S. aureus* nasal carriage (265). There was an association of self-reported nose picking both for positive culture results and bacterial load. The habit of nose picking has been associated with male sex (266). One study investigated social contact to try to understand the age and sex dependence of respiratory infections. They found a tendency, especially among children and adolescents, of social contact mostly with other people their own sex and age. Also, women were more in contact with children than men (267). Another study observed children, public-sector and healthcare workers to have the highest number of total contact hours and therefore most likely to catch and transmit infectious disease (268). The impact of these behavior differences is not completely understood, and if this translates into general populations including all age-groups is not known.

Because of the large number of studies reporting sex-differences in both autoimmune diseases (269), bacterial infections (135) and bacterial colonization (98) and evidence of sex-steroid hormones' effect on the immune system (142), behavior alone is most likely not the cause of the observed sex-difference in *S. aureus* colonization.

To further investigate the relationship between sex-steroids and *S. aureus* carriage, we need both experimental and observational studies. A study with the evaluation of both behavior and social network could enlighten the size of the sex-difference in *S. aureus* colonization. To assess the role of hormonal contraceptives, prospective studies need to assess the *S. aureus* nasal carrier state of women before starting on hormonal contraceptives and do follow-up assessments to compare effects between progestin-only contraceptives and combination

contraceptives cohorts and compare the results with a non-user control cohort. Endogenous testosterone levels have been linked to alcohol use and obesity in women (270, 271). This could suggest that circulating sex-steroids are modifiable to some extent, and that lifestyle changes could affect the probability of *S. aureus* carriage. Studies with longitudinal design could evaluate both the effect of alcohol use and obesity on testosterone levels, as well as the probability of *S. aureus* carriage.

Further studies on the effect of endogenous and exogenous hormones and social networks on the nasal microbiome could give insight on the possible effect of bacterial competition, and colonization resistance of *S. aureus*.

12 Main conclusions

In summary, the studies of this thesis illustrate the complex relationship between hormonal exposures and *S. aureus* carriage. The known difference in carriage between men and women, and in different age-groups are most likely linked to some unknown biological influence on the innate immunity.

- There is an increased risk of *S. aureus* nasal carriage for young women using combination hormonal contraceptives compared to non-users. There is also a decreased risk for *S. aureus* nasal carriage in women using progestin-only contraceptives.

- There is an inverse relationship between the risk of *S. aureus* nasal carriage and *S. aureu*s persistent nasal carriage in adult women with higher levels of testosterone and bioavailable testosterone.

- Our data on the relationship between sex-steroids and *S. aureus* persistent nasal carriage in adult men are inconclusive.

- We have developed a statistical method to assess transmission of *S. aureus* carriage in a social network, and we can demonstrate transmission of both *S. aureus* nasal carriage and specific *S. aureus* genotypes in a general youth population.

- Male sex increases the risk of *S. aureus* carriage independently of social network, indicating a biological predisposition to carriage in men.

- Alcohol use, BMI and physical activity are host risk factors that may represent increased social contact, and therefore increased transmission of *S. aureus*. A biological or lifestyle related component of the effect cannot be rejected.

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Paper I

PLOS ONE

RESEARCH ARTICLE

Hormonal contraceptive use and *Staphylococcus aureus* nasal and throat carriage in a Norwegian youth population

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Abstract

Background

Use of hormonal contraceptives has been associated with *Staphylococcus aureus* nasal carriage in adult women. However, the role of hormonal contraceptives in *S. aureus* colonization among adolescents and associations with progestin only contraceptives are unknown.

Methods

We obtained nasal and throat swab samples from 439 girls aged 17–21 years in the population-based Tromsø study Fit Futures, 2012–2013, Norway, with information on lifestyle, health and biomarkers. We used multivariable logistic regression to study the association between use of hormonal contraceptives and *Staphylococcus aureus* carriage while adjusting for potential confounding factors.

Results

Staphylococcus aureus nasal carriage prevalence were 34%, 42%, and 61% among progestin-only users, non-users, and progestin-estrogen combination contraceptive users, respectively (P<0.001). Use of combination contraceptives doubled the odds of nasal carriage (non-users reference; OR = 2.31, 95%CI = 1.43–3.74). The OR of nasal carriage was 0.29 among progestin-only users compared to combination contraceptives users (95% CI = 0.12–0.67).

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Discussion

In this study, use of combination hormonal contraceptives was associated with higher risk of *Staphylococcus aureus* nasal carriage in adolescent girls. Experimental design studies are needed to establish the role of exogenous sex steroids in *Staphylococcus aureus* colonization in women.

Introduction

Staphylococcus aureus colonizes the skin and mucosal surfaces including nose and throat, and may cause a wide range of clinically important infections [1–3]. The nasal mucosa is the major *S. aureus* reservoir associated with transmission to other body sites and auto-infections, as well as transmission to others [1, 3–4]. The prevalence of nasal carriage increases from 20–30% in young children to 40–50% in older children and adolescents, after which the prevalence drops to 20–30% in the adult population [1, 3, 5–6]. Men have higher *S. aureus* nasal carrier rates than women [7]. Exclusive throat carriage is increasingly identified as an additional *S. aureus* reservoir particularly in young populations, but is considered less important in transmission and infection [8–11].

Prevention and eradication of *S. aureus* carriage may reduce the *S. aureus* disease burden [1, 3, 12]. In the carrier state, *S. aureus* is not successfully cleared by the host innate immune system, which function is determined by genes and environment. No significant heritability of *S. aureus* nasal carriage was found in a twin study [13], and evidence for host genetic determinants from observational studies is scarce [14–18]. This motivates the search for modifiable host lifestyle and environmental determinants as potential targets for prevention and infection control [4]. The strong associations of *S. aureus* carriage with age and sex, suggest that reproductive hormones may be key factors in regulating the immune response. Both endogenous and exogenous sex steroid exposure have been associated with *S. aureus* nasal carriage in women [19–21]. The initial hypothesis was partly based on in vitro evidence of increased staphylococcal binding to HeLa cells in the presence of estrogen [22]. The first epidemiological study showed an association between high circulating estrogen levels and staphylococcal nasal carriage among women [21]. In a cohort study among 694 healthy female volunteers in their third decade visiting a travel clinic, hormonal contraceptive (HC) users had an increased risk (OR 1.6, 95% CI 1.1–2.3) of *S. aureus* carriage at baseline and an increased risk (OR 3.2, 95% CI 1.4–7.3) of persistent *S. aureus* carriage after a median follow-up of 70 days, when compared to non-users [19]. However, it was impossible to separate the effect of progestin and estrogen as 96% used combination contraceptives. Another study based on nine HC users supports an association between oral hormonal contraceptives and nasal carriage [20]. Despite the high prevalence of *S. aureus* nasal carriage among adolescents, the association with HC use in women below 20 years has not been addressed.

In general, few host risk factors for *S. aureus* carriage have been investigated in adolescents, besides the link with atopic dermatitis [23] and low vitamin D as a predictor of methicillin resistant staphylococcus aureus (MRSA) nasal carriage [24]. Additional associations observed among adults, include higher *S. aureus* nasal carriage rates with obesity and type 2 diabetes [25], and lower rates in smokers [5].

The main aim of this study was to assess the association between overall use of HC and different types of HC, and *S. aureus* nasal and throat carriage in a population-based sample of

healthy girls aged 17–21 years, and to test whether an association is independent of other known risk factors for *S. aureus* carriage.

Methods

Population and study design

The Tromsø Study Fit Futures 1 and 2 (TFF1 and TFF2) comprise two waves of large population-based studies of lifestyle and health among upper-secondary school students in the Norwegian municipalities of Tromsø and Balsfjord [23, 26]. TFF2 was conducted in 2012–2013 and invited all third year students (n = 775), as well as TFF1 participants not attending school in 2012–2013 (n = 464). A total of 31 individuals (27 participants in TFF1 and 4 new students) could not be successfully contacted, and were not invited in TFF2. Among all students invited to TFF2 (n = 1208), 868 participated (71.9%). All males, participants with no or invalid nasal or throat swab, age exceeding 21 years, and females with missing data on HC use were excluded. The final study population included 436 and 439 women for the analysis of *S. aureus* nasal and throat carriage, respectively (Fig 1).

The participants had a half-day visit at The Clinical Research Unit, University Hospital of North Norway (UNN). A web-based questionnaire was used to collect data on lifestyle, health and disease. Nasal and throat swab cultures, clinical examinations, blood sampling, and interviews were performed by trained research nurses according to standardised procedures. Height and weight were measured on an electronic scale with lightweight clothing, and body mass index was calculated (kg/m^2). Non-fasting blood samples were drawn from an antecubital vein. Methods for the assessment of EDTA-blood glycated hemoglobin (HbA1c) and serum 25-hydroxyvitamin D [25(OH)D] concentrations in TFF have been described previously [27].

Assessment of *S. aureus* carriage

Nasal and throat swab samples were collected by research nurses. A NaCl (0.9%)-moistened sterile rayon-tipped swab rotated three times with a gentle pressure was used to sample both vestibule nasi. A second swab was used to sample both tonsillar regions with moderate pressure on the tonsillar surfaces. The swabs were immediately placed in transport medium (Amies Copan, Brescia, Italy) and stored at 4˚C for 3–7 days (dx.doi.org/10.17504/protocols.io.2j5gcq6). The microbiological analysis was done by trained personnel at the Department of Medical Biology, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø. First, the swabs were enriched in Bacto Staphylococcus medium broth (Difco laboratories, Sparks, MD, USA) and incubated for 18–24 hours at 37 ˚C. After enrichment, one drop of enrichment broth were streaked on blood agar (Oxoid, UK), CROMagar-plates for *S. aureus* detection (SmithMed AS/Microbiological media production and MRSA agar plates SmithMed AS/Microbiological media production, Department of Microbiology and Infection Control, UNN). In a pilot study among 10 adult volunteers, we demonstrated the validity of the method. One nasal swab sample from each nasal vestibule was taken and stored at 4˚C for 9 days before enrichment and plating, gave the same culturing result as beginning the culturing with enrichment on the day of sampling. In order to increase statistical power and test for differences between agar plates, each nasal swab was streaked on blood agar and two different *S. aureus* growth media; the one used in the present study and chromId *S. aureus* agar (bioMérieux, France). The agar plates were incubated for 42–48 hours at 37˚C. To retain high specificity, colony morphology was examined and the most dominating colony type on the SAID or MRSA plate was plated on blood agar and incubated for 20–24 hours at 37˚C before confirmation as *S. aureus* by the Staphaurex plus agglutination test (Remel, USA). All confirmed *S. aureus* isolates were frozen at– 70 ˚C in glycerol-containing liquid media for molecular

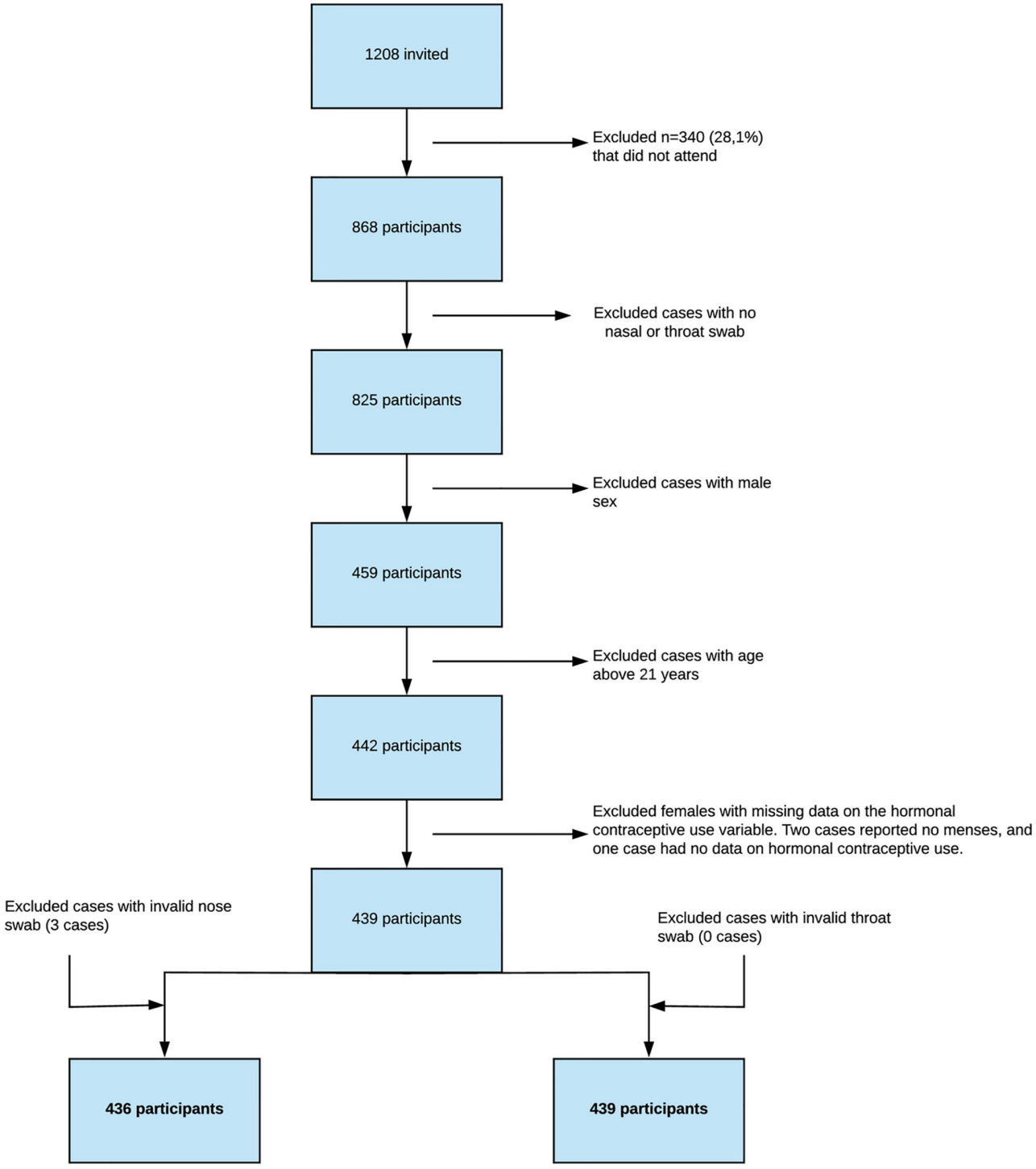

Fig 1. The study population. The Tromsø Study, Fit Futures 2. *68 throat cultures with no bacterial growth were recoded as valid swabs negative for *S. aureus*.

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analysis (dx.doi.org/10.17504/protocols.io.2j6gcre). Growth of any bacterial colonies on any of the agar plates was registered as a valid culture. *S. aureus* carriage state was determined by nasal or throat swab positive for *S. aureus*. For throat cultures, all samples with no growth on neither plate, were recoded as valid cultures negative for *S. aureus* (n = 68). One MRSA isolate was confirmed from the nasal swabs. Two MRSA isolates from two different participants was confirmed from throat swabs. The verification of MRSA was done by detection of the thermostable nuclease of *S. aureus* and the *mecA* gene with the use of an in house real time PCR.

Assessment of hormonal contraceptive use

Self-reported information on current contraceptive use was obtained by trained nurses asking female participants the interview questions: "If you have started menstruating; do you use any kind of contraceptives?" (yes/no), and "If you use any kind of contraceptives; what type?" (Tablets/Injections/Implants/Condom/Transdermal contraceptive patch/Vaginal contraceptive ring/Intrauterine device (IUD)/Other). Condom and other were defined as non-hormonal contraceptives. None used IUD as contraception, and the variable was excluded from further analysis.

HC users were asked about brand name of tablets, implants, transdermal contraceptive patch, or vaginal contraceptive ring, and all were shown photos of different brands sold in Norway to help correct reporting. In the analysis, HC types and brands were categorised into combination HC and progestin-only HC. The combination HCs were further divided into two groups according to the ethinylestradiol daily dosage (high and low). High dosage was defined as HC containing $\geq$ 30 μg ethinylestradiol. Low dosage was defined as contraceptives containing $\leq$ 20 μg ethinylestradiol. No HC brand contained between 20 and 30 μg ethinylestradiol.

Statistical analysis

Univariable associations and differences between comparison groups were analyzed in contingency tables and by calculating means and standard deviations, using chi-square and t-tests to quantify the potential role of chance. Univariable and multivariable logistic regression were fitted to estimate odds ratios (ORs) and 95% confidence intervals (CIs) to describe the association between HC use and *S. aureus* nasal and throat carriage while adjusting for potential confounders. We used DAGitty 2.3 and Akaike Information Criterion (AIC) for model selection. Testing for potential interaction between explanatory variables was done by including the multiplicative terms of two predictor variables in the model and comparing models using AIC. We analyzed our data using SPSS version 23 and considered p-values < 0.05 as statistical evidence and $0.1 > p \geq 0.05$ as weak statistical evidence.

Ethics

A declaration of consent was signed by each participant in TFF2. TFF2 was approved by the Regional Committee for Medical and Health Research Ethics (REK) and the Norwegian Data Protection Authority. The present study was approved by REK North, reference 2011/1710.

Results

In this population of healthy female adolescents with mean age 18 years (range 17–21), the prevalence of *S. aureus* nasal carriage was 50.0% (218/436) while the prevalence of throat carriage was 49.7% (263/439). Simultaneous nasal and throat carriage were found in 178 participants. Table 1 shows selected population characteristics by *S. aureus* nasal and throat carriage

Table 1. Characteristics of the study population by *S. aureus* nasal and throat carriage. The Tromsø Study Fit Futures 2.

	Nasal carriage (n = 436)				Throat carriage (n = 439)			
	Non-carrier (n = 218)	Carrier (n = 218)	*P*[a]	OR (95%CI)[b]	Non-carrier (n = 176)	Carrier (n = 263)	*P*[a]	OR (95% CI)[b]
Age, years (mean, SD)	18.2 (0.7)	18.3 (0.7)	.37	1.13 (0.86–1.48)	18.3 (0.7)	18.3 (0.7)	.65	0.86 (0.63–1.18)
BMI, kg/m^2 (mean, SD)	23.2 (4.3)	23.1 (4.3)	.74	0.99 (0.95–1.04)	23.3 (4.4)	23.0 (4.1)	.53	0.97 (0.93–1.02)
BMI-category			.66				.36	
< 18.5 kg/m2	12 (57.1)	9 (42.9)		0.7 (0.29–1.70)	10 (47.6)	11 (52.4)		0.48 (0.19–1.24)
18.5-<25 kg/m2	154 (48.3)	165 (51.7)		1.0 (ref)	121 (37.6)	201 (62.4)		1.0 (ref)
25-<30 kg/m2	35 (55.6)	28 (44.4)		0.75 (0.43–1.29)	29 (46.0)	34 (54.0)		0.74 (0.39–1.42)
30 + kg/m2	17 (51.5)	16 (48.5)		0.88 (0.43–1.80)	16 (48.5)	17 (51.5)		0.42 (0.20–0.90)
HbA1c (mean, SD)	5.4 (0.4)	5.3(0.3)	.05	0.49 (0.24–1.02)	5.4 (0.4)	5.3 (0.3)	.03	0.64 (0.26–1.58)
Vitamin D (mean, SD)	49.5 (22.9)	51.1 (22.9)	.15	1.01 (0.99–1.02)	51.3 (23.7)	50.9 (22.4)	.83	1.01 (0.99–1.01)
Vitamin D group[c]			.09				.64	
Deficiency	25 (53.2)	22 (46.8)		0.77 (0.40–1.45)	18 (37.5)	30 (62.5)		0.99 (0.47–2.11)
Insufficiency	95 (58.3)	68 (41.7)		0.62 (0.40–0.95)	73 (44.5)	91 (55.5)		0.80 (0.49–1.3)
Normal	87 (46.5)	100 (53.5)		1.0 (ref)	77 (41.0)	111 (59.0)		1.0 (ref)
Smoking			.17				.71	
Yes	50 (56.8)	38 (43.2)		0.7 (0.44–1.12)	37 (42.0)	51 (58.0)		0.82 (0.47–1.42)
No	164 (48.0)	178 (52.0)		1.0 (ref)	135 (39.1)	210 (60.9)		1.0 (ref)
Daily snuff use			.03				.11	
Yes	47 (40.9)	68 (59.1)		1.63 (1.06–2.51)	38 (33.0)	77 (67.0)		1.50 (0.88–2.56)
No	168 (53.0)	149 (47.0)		1.0 (ref)	135 (42.2)	185 (57.8)		1.0 (ref)
Alcohol use			.03				.009	
> 4 times/month	11 (57.9)	8 (42.1)		2.09 (0.62–7.05)	8 (42.1)	11 (57.9)		2.75 (0.77–9.86)
2–4 times/month	111 (48.9)	116 (51.1)		3.01 (1.29–6.99)	88 (38.8)	139 (61.2)		4.63 (1.95–11.04)
≤ 1 time/month	70 (45.2)	85 (54.8)		3.49 (1.47–8.28)	56 (35.4)	102 (64.6)		3.83 (1.59–9.22)
Never	23 (74.2)	8 (25.8)		1.0 (ref)	21 (67.7)	10 (32.3)		1.0 (ref)
Physical activity[d]			.02				.05	
Low level	21 (34.4)	40 (65.6)		2.35 (1.29–4.30)	20 (32.3)	42 (67.7)		1.83 (0.91–3.71)
Medium level	100 (55.2)	81 (44.8)		1.0 (ref)	84 (46.4)	97 (53.6)		1.0 (ref)
High level	92 (48.9)	96 (51.1)		1.29 (0.86–1.94)	68 (35.8)	122 (64.2)		1.87 (1.14–3.07)
Atopic eczema			.007				.37	
Yes	19 (32.8)	39 (67.2)		2.28 (1.27–4.09)	20 (33.9)	39 (66.1)		1.71 (0.82–3.56)
No	199 (52.6)	179 (47.4)		1.0 (ref)	156 (41.1)	224 (58.9)		1.0 (ref)
Antibiotic use past 3 months			.36				.01	
Yes	39 (55.7)	31 (44.3)		0.76 (0.45–1.27)	38 (54.3)	32 (45.7)		0.54 (0.30–0.98)
No	179 (48.9)	187 (51.1)		1.0	138 (37.4)	231 (62.6)		1.0 (ref)

Values are number of subjects (%) if not otherwise stated.

BMI = body mass index; SD = standard deviation; HbA1c, glycated haemoglobin.

[a] Chi-square test for categorical and t-tests for continuous variables.

[b] Univariable logistic regression analysis. OR = Odd ratio CI = 95% confidence intervals

[c] Serum 25-hydroxyvitamin D: Deficiency = < 25 nmol/l; Insufficiency = 25–50 nmol/l; Normal = >50 nmol/l.

[d] Recreational physical activity: Low level = reading, watching TV, or other sedentary activity; Medium level = walking, cycling, or other forms of exercise at least 4 hours a week; High level = participation in recreational sports, heavy outdoor activities with minimum duration of 4 hours a week, or participation in heavy training or sports competitions regularly several times a week.

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Table 2. Prevalence of *S. aureus* nasal and throat carriage by group of hormonal contraceptive use. The Tromsø Study Fit Futures 2.

	Nasal carriage (n = 436)			Throat carriage (n = 439)		
	Non-carrier (n = 218) n (%)	Carrier (n = 218) n (%)	P-value[a]	Non-carrier (n = 176) n (%)	Carrier (n = 263) n (%)	P-value[a]
Non-user	113 (58.2)	81 (41.8)	<.001	84 (42.4)	114 (57.6)	.460
Combination with Low estrogen[b]	36 (44.4)	45 (55.6)		27 (34.6)	51 (65.4)	
Combination with High estrogen [c]	44 (35.8)	79 (64.2)		47 (37.6)	78 (62.4)	
Progestin-only[d]	25 (65.8)	13 (34.2)		18 (47.4)	20 (52.6)	

[a] Chi-square test

[b] Combination contraceptive with ethinyl estradiol dosage less than or equal to 20μg. Mercilon, Yasminelle, Loette 28, Nuvaring.

[c] Combination contraceptive with ethinyl estradiol dosage greater than or equal to 30μg. Marvelon, Yasmin, Microgynon, Oralcon, Diane, Synfase, Evra

[d] Progestin-only contraceptives. Cerazette, Nexplanon, Depo-provera.

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types. *S. aureus* nasal carriage was positively associated with self-reported doctor diagnosed atopic eczema, low recreational physical activity, daily snuff use, and use of alcohol (all $p < 0.05$). *S. aureus* throat carriage was associated with low recreational physical activity and use of alcohol, while use of antibiotics the past three months was negatively associated with throat carriage (all $p < 0.05$).

A higher proportion of women using combination HC were nasal carriers compared to non-users, and there was a positive association with higher oestrogen dosage. Progestin-only contraceptives were negatively associated with nasal carriage ($p < 0.001$) (Table 2). There was no evidence for differences in prevalence of throat carriage across categories of HC use.

In a multivariable logistic regression model, users of high dosage estrogen HC had a 2.4 fold higher odds of *S. aureus* nasal carriage as compared with non-users (OR = 2.44; 95% CI = 1.39–4.28, adjusted for age, BMI, smoking, snuff- and alcohol use, recreational physical activity, HbA1c and 25-OH-vitamin D levels, atopic eczema and the use of antibiotics in the past three months) (Table 3). Users of low dosage estrogen HC had an adjusted OR of 2.14 compared to non-users (95%CI = 1.17–3.91). Users of progestin-only contraceptives had an adjusted OR of 0.29 (95%CI = 0.12–0.67) compared to users of combination contraceptives (results not presented in tables). In the same model, higher odds of *S. aureus* nasal carriage was also observed for atopic eczema (OR = 2.50; 95%CI = 1.28–4.89), low physical activity (OR = 2.12; 95%CI = 1.06–4.23), use of alcohol once a month or less (OR = 3.81; 95% CI = 1.42–10.23), and use of alcohol 2–4 times a month (OR = 3.48; 95%CI = 1.32–9.21). Weak statistical evidence was found for a negative association with increasing HbA1c (OR = 0.52; 95%CI = 0.23–1.20).

Multivariable logistic regression analysis of *S. aureus* throat carriage showed a trend towards modestly higher odds for participants using high (OR = 2.06; 95%CI = 0.96–4.44), and low dosage (OR = 1.79; 95%CI = 0.89–3.60), combination HC versus non-users, but this finding could also be due to chance (Table 3). There was a statistically significant higher odds of *S. aureus* throat carriage for the use of alcohol once a month or less (OR = 3.39; 95% CI = 1.29–8.86), for alcohol use 2–4 times a month (OR = 3.85; 95%-CI = 1.49–9.95), and for high level physical activity (OR = 1.83; 95%CI = 1.04–3.21).

Test for interaction was performed by including multiplicative terms of two and two predictors in the logistic regression model. In the analysis for throat carriage, four interactions were detected. There was an interaction between atopic eczema and HbA1c ($p = 0.01$), between antibiotic use and alcohol use ($p = 0.02$), between age and smoking status ($p = 0.04$) and between smoking and HbA1c ($p = 0.05$). In the analysis for nasal carriage, there was an

Table 3. Associations between hormonal contraceptive use and *S. aureus* nasal and throat carriage. Odd ratios (OR) and 95% confidence intervals (95%CI) from multivariable logistic regression analysis.[a] The Tromsø Study Fit Futures 2.

	Nasal carriage (n = 436)	Throat carriage (n = 439)
	OR (95%CI)	OR (95%CI)
Hormonal contraceptive use		
Non-user	1.0 (ref)	1.0 (ref)
Progestin-only[b]	0.63 (0.27–1.45)	0.90 (0.37–2.19)
Combination with low estrogen[c]	2.14 (1.17–3.91)	1.79 (0.89–3.60)
Combination with high estrogen[d]	2.44 (1.39–4.28)	2.06 (0.96–4.44)
Age continuous	1.15 (0.84–1.58)	0.93 (0.65–1.32)
BMI continuous	0.99 (0.94–1.05)	0.96 (0.90–1.02)
HbA1c continuous	0.52 (0.23–1.20)	0.72 (0.26–1.96)
Vitamin D continuous	1.00 (0.99–1.01)	0.99 (0.98–1.00)
Atopic eczema		
Yes	2.50 (1.28–4.89)	1.49 (0.68–3.31)
No	1.0 (ref)	1.0 (ref)
Smoking		
Yes	0.53 (0.30–0.95)	0.75 (0.39–1.47)
No	1.0 (ref)	1.0 (ref)
Daily snuff use		
Yes	1.39 (0.82–2.37)	1.55 (0.82–2.94)
No	1.0 (ref)	1.0 (ref)
Alcohol use		
More than 4 times a month	2.12 (0.50–8.94)	1.98 (0.45–8.72)
2–4 times a month	3.48 (1.32–9.21)	3.85 (1.49–9.95)
Once a month or less	3.81 (1.42–10.23)	3.39 (1.29–8.86)
Never	1.0 (ref)	1.0 (ref)
Recreational physical activity[e]		
Low level	2.12 (1.06–4.23)	1.35 (0.62–2.93)
Medium level	1.0 (ref)	1.0 (ref)
High level	1.30 (0.80–2.11)	1.83 (1.04–3.21)
Antibiotic use past 3 months		
Yes	0.88 (0.49–1.57)	0.63 (0.32–1.24)
No	1.0 (ref)	1.0 (ref)

BMI = body mass index; HbA1c, glycated haemoglobin.

[a] All variables in the table are mutually adjusted for

[b] Progestin-only = Cerazette, Nexplanon, Depo-provera.

[c] Combination contraceptive with ethinyl estradiol dosage less than or equal to 20μg. Mercilon, Yasminelle, Loette 28, Nuvaring.

[d] Combination contraceptive with ethinyl estradiol dosage greater than or equal to 30μg. Marvelon, Yasmin, Microgynon, Oralcon, Diane, Synfase, Evra

[e] Recreational physical activity: Low level = reading, watching TV, or other sedentary activity; Medium level = Walking, cycling, or other forms of exercise at least 4 hours a week; High level = Participation in recreational sports, heavy outdoor activities with minimum duration of 4 hours a week, or participation in heavy training or sports competitions regularly several times a week.

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interaction between physical activity and HbA1c ($p<0.00$) and between use of antibiotics and smoking status ($p = 0.04$). None of the interactions affected the main result and where therefore not included in the final analysis. We performed sensitivity analysis to check the robustness of our results. The results presented in tables were generated from multivariable logistic regression analysis in which observations with missing values were excluded. A multiple imputation analysis was used to evaluate the effect of differently handled data analysis, but the estimates were not significantly changed.

Discussion

This is the first report on the association between use of HC and *S. aureus* carriage based on a representative sample of healthy women aged 17–21 years. We have demonstrated a strong association between use of combination HC and nasal carriage, where the association is strengthen with higher dosage of estrogen. Women using combination HC had more than doubled odds for nasal carriage compared with non-users, suggesting that exogenous estrogen is a major predictor of *S. aureus* nasal carriage. Among users of combination HC containing both progestin and estrogen, the *S. aureus* nasal carriage prevalence was 64.2% (79/123) in the high-estrogen group and 55.6% (45/81) in the low-estrogen group. This may suggest a dose-response relationship. In contrast, users of progestin-only HC had an OR of 0.29 for nasal carriage compared to women using combination therapy. This substantial difference in risk between the HC-user groups suggests that estrogen and progestin have opposite immune-modulatory effects on *S. aureus* colonization, and that the risk associated with exogenous estrogen alone is considerably higher than that of combination HC.

Our findings are supported by three epidemiological studies in adults, one on endogenous estrogen [21] and two on HC use [19–21]. Zanger *et al* did the first study of HC use in a larger population of 694 women in Germany [19]. The doubled odds of *S. aureus* nasal carriage in our study lies between their observed 60% higher risk of *S. aureus* nasal carriage at baseline and tripled risk of being persistent carrier after two months follow-up. These authors took two nasal swabs at each time point, and defined only those with both samples positive for *S. aureus* after direct culturing as carriers, while in the TTF2 we used one nasal swab cultured with enrichment broth. This could explain some of the difference in risk estimates between the studies. Also, there were differences in age, type of source population (visitors to travel clinic versus students in upper-secondary school) and access to information on possible confounding factors. The other study on HC use and *S. aureus* has limited validity as the sample size was small [20]. Our study provides the first evidence for differences in the effect of progestin-only and combination HC on *S. aureus* colonization, as hypothesised by Zanger's group, who were unable to explore this due to the low prevalence of progestin-only users [19].

Nasal carriage of *S. aureus* is generally considered as a subclinical inflammatory process due to suppression of the innate immune system [28]. Immune responses vary with gender and the reproductive phase, suggesting that factors associated with reproduction regulate immune response [29]. There have been conflicting results on the association between HC use and immunoglobulin production [30–32]. One study showed decreased immunoglobulin levels in HC users [32]. A recent study demonstrated an association between higher Toll-like receptor 9 (TLR9) transcription levels and non-carrier status of *S. aureus* [33]. This study also showed that both the association with TLR9 genotype and transcription level were modified by sex, suggesting a role of reproductive hormones in *S. aureus* immunity. This may be underlying biological mechanisms that can explain why users of combination HC are more likely to be nasal carriers of *S. aureus*.

Though not statistically significant, our analysis shows a lower risk of *S. aureus* nasal carriage in progestin-only users compared to non-users, contrasting the higher risk among users of combination HC. These differences point to direct exogenous hormonal effects, and that the observed associations are not due to environmental factors such as differences in human contact between non-user group and user group. Nevertheless, we cannot rule out that unknown risk factors may account for some of the observed associations.

The study demonstrate a high overall prevalence in *S. aureus* carriage with a prevalence of nasal carriage of 50% (218/436) and a prevalence for throat carriage of 60% (263/439). In a previous validation study (unpublished), the prevalence of enriched samples were 70% for throat samples and 49% for nasal samples. When using direct cultivation of the swabs we detected a prevalence of 36% for throat samples and 36% for nasal samples. The overall high prevalence in our study is the result of the method used and must be taken in to consideration when comparing to other studies.

The main strengths in our study include a population-based design with high attendance and thereby reduced risk of selection bias. Exogenous hormone exposure was assessed in photo-assisted interviews by trained nurses to reduce the risk of information bias. Thus, we may assume that the TFF2 data are representative for Norwegian adolescents and youth populations from similar modern societies. As TFF2 includes detailed information on lifestyle and health, we were able to adjust for possible confounding. DAGitty 2.3 and Akaike Information Criterion (AIC) were used for selecting the optimal regression model.

We included both smoking status and snuff use as covariates in the model as smoking has been consistently associated with lower *S. aureus* nasal carriage in adults [1, 5–6]. Considering that this is a healthy, young population the value of adjusting for HbA1c can be debated. Very few subjects reported no alcohol use or use more than 4 times per month. The association between alcohol use and carriage should therefore be interpreted with caution.

A weakness in our study is that only one sample from throat and nose was taken from each participant. This renders it impossible to distinguish between persistent and intermittent carriers and may represent a detection bias of unknown effect. Due to the enrichment step before plating of the nasal and throat swab cultures, quantification of *S. aureus* growth was irrelevant. Data on duration of HC use and thereby cumulative exogenous hormone exposure was unavailable. Thus, we were unable to test for these possible dose-response relationships.

We did not find the same association between HC use and *S. aureus* carriage based on throat samples as with nasal samples. This may represent a true difference in the host-microbe relationship between throat and nose. However, there are some methodological concerns relating to the throat swabs in our study. A possible source of bias can be the more complicated sampling method for throat swabs with lower compliance due to participant discomfort. Furthermore, we chose to include throat samples without any bacterial growth on the agar plates in our analysis. The enrichment broth promotes growth of staphylococci, while other members of upper respiratory microbiota are inhibited by the relatively high concentration of sodium chloride. Therefore, agar plates with no bacterial growth were included in the study as valid negative throat samples. The pilot study showed that nasal swab samples cultured within 9 days after sampling gave the same culturing results on both control agar and *S. aureus* agar plates as culturing on the day of sampling. We did not include throat swab samples in the pilot study. However, we assume that for throat swabs *S. aureus* culture has similar validity as nasal swabs, while growth on control agar has lower validity. All the potential sources of bias mentioned are sources of non-differential bias, as there is no obvious link to HC use.

In summary, we report novel evidence for an association between use of HC and risk of *S. aureus* nasal carriage in female adolescents. Furthermore we observe that progestin-estrogen combination users have higher risk while progestin-only users have lower risk of nasal carriage

compared to non-users. Our data support that exogenous estrogen is a major risk factor with potentially large impact on the *S. aureus* burden in the youth population. Our study has a biological foundation [33], demonstrates a dose response relationship, and the results are supported by data from another study including a different group of participants [19]. This study, together with existing knowledge, provides evidence for a causal association between exogenous estrogen exposure from hormonal contraceptives and nasal carriage of *S. aureus*. An experimental study design is needed to establish the role of exogenous sex steroids in *S. aureus* colonization in women.

Supporting information

S1 Table. Table of variables.
(DOCX)

S1 Fig. Figure of DAGitty models and corresponding AICs.
(PDF)

S1 Text. General questionnaire. Questionnaire from TFF2 in original language.
(PDF)

S2 Text. Interview. Interview from TFF2 in original language.
(DOC)

S3 Text. Metadata. Questionnaire and Interview for TFF2 in English.
(XLS)

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Paper I

Supplementary materials

Table of variables

Variable	Type
Sex	Categorical
Age	Continuous
Participation consent	Categorical
Attendance date	Date format
Healthy today	Categorical
Fever today	Categorical
Common cold today	Categorical
Infection today	Categorical
Infection today type	Categorical
Chronic disease	Categorical
Diagnosis chronic disease	Text
ICD10 Chronic disease(calculated)	Text
Age diagnosis chronic disease	Continuous
Diagnosis chronic disease 2	Text
ICD10 Chronic disease 2(calculated)	Text
Age diagnosis chronic disease2	Continuous
Diagnosis chronic disease3	Text
ICD10 Chronic disease3(calculated)	Text
Age diagnosis chronic disease3	Continuous
Diagnosis chronic disease4	Text
ICD10 Chronic disease4(calculated)	Text
Age diagnosis chronic disease4	Continuous
Diagnosis chronic disease5	Text
ICD10 Chronic disease5(calculated)	Text
Age diagnosis chronic disease5	Continuous
Chronic disease other	Text
Age chronic disease other	Continuous
Antibiotics the last 3 months	Categorical
Antibiotics last 3 months brand	Text
Antibiotics the last 3 months ATC-code	Text
Weeks since last taken antibiotic	Continuous
Antibiotics last 3 months brand2	Text
Antibiotics the last 3 months ATC-code2	Text
Weeks since last taken antibiotic2	Continuous
Antibiotics last 3 months brand3	Text
Antibiotics the last 3 months ATC-code3	Text
Weeks since last taken antibiotic3	Continuous
Antibiotics last 24 h	Categorical
Antibiotics last 24 h brand	Text
Antibiotics the last 24h ATC-code	Text
Antibiotics last 24 h brand2	Text
Antibiotics the last 24h ATC-code2	Text
Antibiotics last 24 h brand3	Text
Antibiotics the last 24h ATC-code3	Text
Menses	Categorical
Menses, regularity	Categorical
Menses, cycle length	Continuous
Last menses, certainty of start date	Categorical

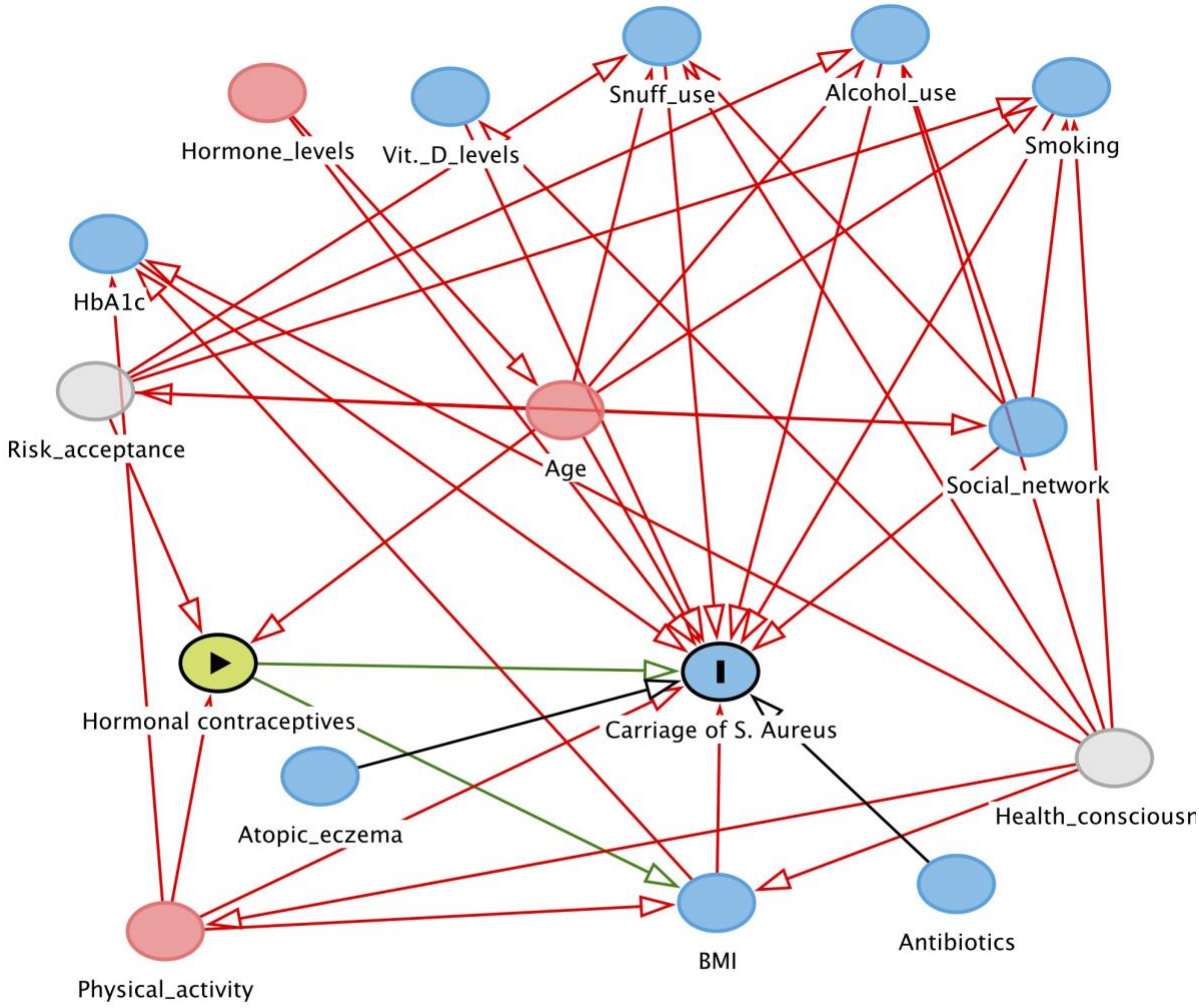
Date of last menstruation	Date format
Contraceptives	Categorical
Contraceptives type	Categorical
Oral contraceptives name	Text
Injected contraceptives name	Text
Subdermal contraceptives name	Text
Contraceptive skin patch name	Text
Vaginal contraceptives name	Text
IUD name	Text
Oral contraceptives ATC-code	Text
Injected contraceptives ATC-code	Text
Subdermal contraceptives ATC-code	Text
Contraceptive skin patch ATC-code	Text
Vaginal contraceptives ATC-code	Text
IUD ATC-code	Text
Chance of pregnancy	Categorical
Pregnancy test consent	Categorical
Pregnancy test result	Categorical
Eczema today	Categorical
Antibiotics local	Categorical
Antibiotics local brand	Categorical
Antibiotics local ATC	Categorical
Antibiotics local 2	Categorical
Antibiotics local brand 2	Categorical
Antibiotics local ATC 2	Categorical
UV-treatment last 14 days	Categorical
Height	Continuous
Weight	Continuous
Waist, 1. Measurement	Continuous
Hip, 1. Measurement	Continuous
Waist, 2. Measurement	Continuous
Hip, 2. Measurement	Continuous
Growth on control agar, nasal swab	Categorical
Growth on staphylococcal selective agar	Categorical
Coagulase test, nasal swab	Categorical
Staphylococcus aureus (coagulase positive), with bacterial growth	Categorical
Comments, lab staphylococcus aureus	Text
Staphylococcus culture date	Date format
BMI (calculated)	Categorical
Atopic eczema	Categorical
Smoking	Categorical
Smoking, start age	Categorical
Cigarettes per week	Categorical
Cigarettes per day	Categorical
Snuff	Categorical
Snuff, start age	Categorical
Snuff, portion per week	Categorical
Snuff portion per day	Categorical
Alcohol frequency	Categorical
Alcohol units	Categorical
Alcohol frequency of 6 units or more	Categorical
Physical activity	Categorical

Physical activity outside of school	Categorical
Physical activity, frequency	Categorical
Physical activity, intensity	Categorical
Acne, lifetime	Categorical
Acne, severity	Categorical
Acne, been seeing a doctor	Categorical
Acne, local treatment	Categorical
Acne, antibiotic therapy	Categorical
Acne, roaccutan treatment	Categorical
Dry skin	Categorical
Dry skin lotion	Categorical
Itchy rash	Categorical
Itchy rash duration	Categorical
Itchy rash head	Categorical
Itchy rash face	Categorical
Itchy rash ear	Categorical
Itchy rash neck	Categorical
Itchy rash wrist	Categorical
Itchy rash hands	Categorical
Itchy rash buttocks	Categorical
Itchy rash inner thigh	Categorical
Itchy rash truncus	Categorical
Itchy rash armpits	Categorical
Itchy rash groin	Categorical
Itchy rash outer extremities	Categorical
Itchy rash feet	Categorical
Itchy rash inner elbow/knee	Categorical
Itchy rash other	Categorical
Itchy rash debut age	Continuous
Itchy rash January	Categorical
Itchy rash February	Categorical
Itchy rash March	Categorical
Itchy rash April	Categorical
Itchy rash May	Categorical
Itchy rash June	Categorical
Itchy rash July	Categorical
Itchy rash August	Categorical
Itchy rash September	Categorical
Itchy rash October	Categorical
Itchy rash November	Categorical
Itchy rash December	Categorical
Itchy rash Cleared	Categorical
Itchy rash awake at night	Categorical
Itchy rash week	Categorical
Itchy rash week school	Categorical
Itchy rash week pain	Categorical
Itchy rash week sad	Categorical
Itchy rash week friends	Categorical
Itchy rash week clothes	Categorical
Itchy rash going out	Categorical
Itchy rash swimming	Categorical
Itchy rash week holiday	Categorical
Itchy rash week teasing	Categorical

Itchy rash week sleep	Categorical
Itchy rash week problems	Categorical
Eczema past year	Categorical
Eczema past year duration	Categorical
Eczema past year cortisone	Categorical
Eczema school/work	Categorical
Eczema leisure activities	Categorical
Eczema worries	Text
Hormonal Contraceptives (calculated)	Categorical
25-OH vitamin D	Continuous
HbA1c	Continuous
Staph. Eczema culture positive	Categorical
Staph skin culture positive	Categorical
Staph nasal culture positive	Categorical
Staph nasal all	Categorical
Staph throat all	Categorical

Model selection

Final model



Minimal sufficient adjustment sets for estimating the direct effect of HC on carriage of S. aureus:
Age, alcohol use, BMI, HbA1c, physical activity, smoking, snuff use, social network, vit. D levels

Directed Acyclic Graphs (www.dagitty.net) of final model

Likelihood Ratio Tests

Effect	Model Fitting Criteria: AIC of Reduced Model	Model Fitting Criteria: BIC of Reduced Model	Model Fitting Criteria: -2 Log Likelihood of Reduced Model	Likelihood Ratio Tests: Chi-Square	Likelihood Ratio Tests: df	Likelihood Ratio Tests: Sig.
Intercept	511,646	574,898	479,646[a]	,000	0	.
AGE_FF2	510,426	569,725	480,426	,780	1	,377
bmi_ff2	509,710	569,009	479,710	,064	1	,800
S_25_VITD_FF2	509,655	568,954	479,655	,009	1	,925
S_HBA1C_FF2	512,321	571,620	482,321	2,675	1	,102
SMOKE_GROUPS	514,214	573,513	484,214	4,568	1	,033
SNUFF_GROUPS	511,314	570,613	481,314	1,668	1	,197
ALCOHOL_GROUPS	514,670	566,062	488,670	9,024	3	,029
PHYS_ACT_GROUPS	512,482	567,828	484,482	4,836	2	,089
ANTIBIOTICS_GROUPS	509,826	569,125	479,826	,180	1	,671
ATOPIC_ECZEMA	517,317	576,616	487,317	7,671	1	,006
HORMONAL_CONTRACEPTIVES2	524,862	580,208	496,862	17,216	2	,000

The chi-square statistic is the difference in -2 log-likelihoods between the final model and a reduced model. The reduced model is formed by omitting an effect from the final model. The null hypothesis is that all parameters of that effect are 0.

a. This reduced model is equivalent to the final model because omitting the effect does not increase the degrees of freedom.

Akaike Information Criteria (AIC) of final model for nasal carriage

Likelihood Ratio Tests

Effect	Model Fitting Criteria: AIC of Reduced Model	Model Fitting Criteria: BIC of Reduced Model	Model Fitting Criteria: -2 Log Likelihood of Reduced Model	Likelihood Ratio Tests: Chi-Square	Likelihood Ratio Tests: df	Likelihood Ratio Tests: Sig.
Intercept	528,996	592,372	496,996[a]	,000	0	.
S_25_VITD_FF2	529,679	589,094	499,679	2,683	1	,101
S_HBA1C_FF2	531,088	590,503	501,088	4,092	1	,043
bmi_ff2	527,612	587,027	497,612	,616	1	,433
AGE_FF2	527,137	586,552	497,137	,141	1	,707
HORMONAL_CONTRACEPTIVE_GROUP2	527,022	582,476	499,022	2,026	2	,363
ATOPIC_ECZEMA	527,254	586,669	497,254	,257	1	,612
ANTIBIOTICS	531,893	591,308	501,893	4,896	1	,027
SMOKE_GROUPS	527,475	586,891	497,475	,479	1	,489
SNUFF_GROUPS	528,160	587,575	498,160	1,164	1	,281
ALCOHOL_GROUPS	532,211	583,705	506,211	9,215	3	,027
PHYS_ACT_GROUPS	528,773	584,227	500,773	3,777	2	,151

The chi-square statistic is the difference in -2 log-likelihoods between the final model and a reduced model. The reduced model is formed by omitting an effect from the final model. The null hypothesis is that all parameters of that effect are 0.

a. This reduced model is equivalent to the final model because omitting the effect does not increase the degrees of freedom.

Akaike Information Criteria (AIC) of final model for throat carriage

Paper II

Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general female population

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Abstract

Objective: Staphylococcus aureus is a major human pathogen, and nasal carriers have an increased risk for infection and disease. The exploration of host determinants for nasal carriage is relevant to decrease infection burden. Former studies demonstrate lower carriage prevalence in women and among users of progestin-only contraceptives. The aim of this study was to investigate the possible associations between circulating sex-steroid hormones and nasal carriage of *Staphylococcus aureus* in a general population.

Methods: In the population-based sixth Tromsø study (2007–2008) nurses collected nasal swab samples from 724 women aged 30–87 not using any exogenous hormones, and 700 of the women had a repeated nasal swab taken (median interval 28 days). We analysed a panel of serum sex-steroids by liquid chromatography tandem mass spectrometry, and collected information about lifestyle, health and anthropometric measures. Multivariable logistic regression was used to study the association between circulating sex-steroids and *Staphylococcus aureus* carriage (one swab) and persistent carriage (two swabs), while adjusting for potential confounding factors. Women in luteal phase were excluded in the analysis of androgens.

Results: Staphylococcus aureus persistent nasal carriage prevalence was 22%. One standard deviation increase in testosterone and bioavailable testosterone was associated with lower odds of persistent nasal carriage, (OR = 0.57; 95% CI = 0.35–0.92 and OR = 0.52, 95% CI = 0.30–0.92) respectively. Analysis stratified by menopause gave similar findings. Persistent carriers had lower average levels of androstenedione and DHEA, however, not statistically significant.

Conclusion: This large population-based study supports that women with lower levels of circulating testosterone may have increased probability of *Staphylococcus aureus* persistent carriage.

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Introduction

Staphylococcus aureus is the leading cause of skin and soft tissue infections, and can invade and infect any organ of the human body. *S. aureus* is the second most common cause of bloodstream infections (1), which continue to have high mortality among patients worldwide (2, 3). The burden of disease and mortality reflect *S. aureus'* extraordinary virulence factors, including highly evolved immune evasion strategies and resistance to antibiotic treatment (4).

S. aureus is frequent in the normal bacterial flora of healthy humans, and 20–50% of the population are nasal carriers (4, 5, 6, 7). Colonisation of the skin in the anterior nares is a major source of endogenous *S. aureus* infections and transmission (4). This motivates the search for lifestyle and environmental factors and associated biomarkers that may regulate immune responses to *S. aureus*. Among populations from different continents, male sex, younger age, atopic eczema, excess body weight, higher circulating glucose levels and diabetes, lower vitamin D levels, and work in health care services have been associated with higher probability of *S. aureus* nasal carriage, while smoking has been associated with both lower and higher probability of carriage (4, 5, 8, 9, 10, 11, 12, 13).

Sex-steroids are produced from cholesterol in testes or ovaries and the adrenal glands, and may be further converted to more potent sex-steroids in fat and other tissue. Sex-differences in immunological responses are largely caused by sex-steroid hormone actions, through binding to nuclear receptors and regulation of immune system genes expression in a variety of cells (14). In general, adult women have a more responsive immune system with faster clearance of pathogens and greater vaccine efficacy compared to men but are more prone to inflammatory and autoimmune diseases. Sex and age are the strongest risk factors for *S. aureus* nasal carriage (4, 8, 9). Thus, it has been hypothesised that sex-steroid hormones are important in the immune response to *S. aureus*. Smaller studies among women have found a positive association of nasal *S. aureus* carriage with biomarkers of endogenous oestrogen level (15), as well as of nasal *S. aureus* carriage with exogenous oestrogen and progesterone (i.e. hormonal contraceptive combination preparations) (16, 17). One small study among premenopausal women found higher prevalence of persistent throat carriage of *S. aureus* with increasing levels of free fraction testosterone (18). From the population-based sixth Tromsø study (2007–2008), we reported higher nasal carriage among men and sex-differences in the distribution of *S. aureus* spa types (genetic variance by *S. aureus* protein A (spa) typing) (9). Among 400 young women in the Tromsø study fit futures (2012–2013), where 50% used hormonal contraception, we found a positive dose-response relationship between oestrogen dose and *S. aureus* nasal carriage, and a negative association between exogenous progesterone alone and nasal carriage (17). To our knowledge, no epidemiological study has examined whether endogenous sex-hormone levels are associated with *S. aureus* nasal carriage among women. Thus, the aim of this study is to investigate the possible associations between circulating sex-steroid hormones and nasal carriage of *S. aureus* in a general female population.

Methods

Population and study design

The Tromsø study includes seven extensive health-screening surveys in the Tromsø municipality, North Norway, during 1974–2016 with invitation of total birth cohorts and large random samples of the population (5, 11). Each of these surveys consists of clinical examinations including anthropometric measures, non-fasting blood samples, interview covering menstrual history and hormonal medication, questionnaires on reproductive history, lifestyle, health and chronic disease (5, 11, 12). The present study uses data from the sixth Tromsø study (the sixth Tromsø study, participation proportion 66%).

Body height in centimetres (cm) and weight in kilograms (kg) were measured to the nearest 0.1 unit with participants wearing light clothing and no shoes. BMI was calculated as weight divided by height squared (kg/m^2). Non-fasting blood samples were drawn from an antecubital vein. Glycated haemoglobin (HbA1c) was determined in EDTA-blood by HPLC using an automated analyser (Variant II, Bio-Rad Laboratories Inc.). The total analytical coefficient of variation was <3.0%. Serum 25-hydroxyvitamin D (25(OH)D) was analysed by electrochemiluminescence immunoassay (ECLIA) using an automated clinical chemistry analyser (Modular E170, Roche Diagnostics). The total analytical coefficient of variation was 7.3%. There is a known overestimation of 25(OH)D levels in smokers when using the ECLIA (Roche) method (19). This necessitates stratification by smoking in the statistical analysis of 25(OH)D.

Nasal swabs for *S. aureus* culturing were collected from a random sample of 4026 participants attending the sixth Tromsø study screening centre during October 2007 to June 2008, 2285 of these were women. All women

without data on serum hormones, participants reporting antibiotic use or missing data on antibiotic use the last 24 h, ongoing pregnancy, use of hormonal contraceptives, use of hormonal replacement therapy, endocrine breast cancer therapy and IVF treatment were excluded from the analysis. The participants were invited to a second visit for repeated nasal swab sampling. Women with invalid or missing *S. aureus* culturing results were excluded from the analysis (Fig. 1).

Assessment of *S. aureus* carriage and serum sex-steroids

Nurses at the sixth Tromsø study screening centre collected nasal swab samples. Each nasal vestibule was sampled with the same NaCl (0.9%)-moistened sterile rayon-tipped swab which was rotated three times. The swabs were immediately placed in transport medium (Amies Copan, Brescia, Italy) and stored at 4°C for a maximum of 3 days. Trained personnel at the Department of Microbiology and Infection Control, University Hospital of North Norway, (UNN) Tromsø analysed the microbiological samples. The specimens were cultured on blood agar (Oxoid, UK) for growth control and chromID-plates (SAID) for *S. aureus* detection (bioMérieux, Marcy I'Etoile, France). The agar plates were incubated for 42–48 h at 37°C. To retain high specificity, colony morphology was examined, and the most dominating colony type on the SAID plate was confirmed as *S. aureus* by the Staphaurex plus agglutination test (Murex Diagnostic Ltd, Dartford, UK). Growth of any bacterial colonies on agar plates was registered as a valid culture.

The median interval between the first and the second nasal swab culture was 28 days.

S. aureus nasal carriage status was classified according to baseline sample status. *S. aureus* persistent carriage was defined as having two positive samples on the basis of van Belkum's definition of persistent carriage (20).

The Hormone laboratory at Haukeland University Hospital analysed a panel of serum sex-steroids by liquid chromatography tandem mass spectrometry (LCMS/MS, SCIEX API 5500 triple-quadrupole mass spectrometer, Applied Biosystems/MDS with an Agilent 1290 UPLC system), as part of the laboratory's work on establishing reference values within subgroups of the general population (i.e. by sex, age, and BMI categories) (21). LCMS/MS is the gold standard method for steroid profiling due to very high sensitivity and specificity (22). Serum concentrations of testosterone, androstenedione, dehydroandrostenedione (DHEAS), 17α-hydroxyprogesterone (17-OH progesterone), progesterone, luteinising hormone (LH), follicle-stimulating hormone (FSH), as well as the binding proteins sex-hormone binding globulin (SHBG) and albumin were measured using DPC immulite 200 XPi (Siemens Healthcare Diagnostics). For the estimation of bioavailable testosterone which includes free and albumin-bound testosterone we used the equation '(testosterone/SHBG) × 10' (23) and the equation derived by Morris *et al.* (24).

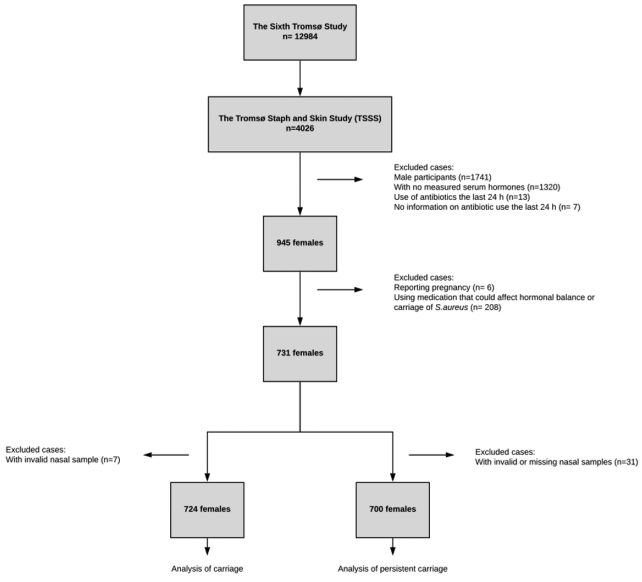

Figure 1
The study population. The sixth Tromsø study.

Classification of menopause and menstrual phase

To classify menopausal status, we used the following question from the interview: 'Do you still have natural menstruation?' (yes/no/irregular/unknown status). We also defined FSH-level over 21.7 IU as postmenopausal and FSH-level under 21.7 IU as premenopausal (25, 26). Female participants reporting no menses with FSH-levels under 21.7 IU were further classified by the question from the interview: 'If you do not have natural menstruation, why did it stop?' (stopped by itself/I had a hysterectomy/both ovaries were removed/other reasons (for instance radiation, cytotoxic mediation)). FSH-levels can be difficult to interpret because of the possible high levels in perimenopause, we therefore emphasised self-reported menstruation in classification of menopause. FSH-levels were used to confirm clinical history and to classify females with no data on self-reported menses. Among women with unknown status on menstruation from

the interview, age was used as an additional criterium in those with FSH-levels above 21.7 IU (age ≥ 51 years were classified as postmenopausal). One female participant reported no menses but had information about recent menstruation, we therefore concluded that this probably were due to misreporting on menstruation and classified the participant by FSH levels (Fig. 2).

Premenopausal women were further classified as follicular phase or luteal phase according to progesterone levels. Follicular phase were defined as progesterone levels

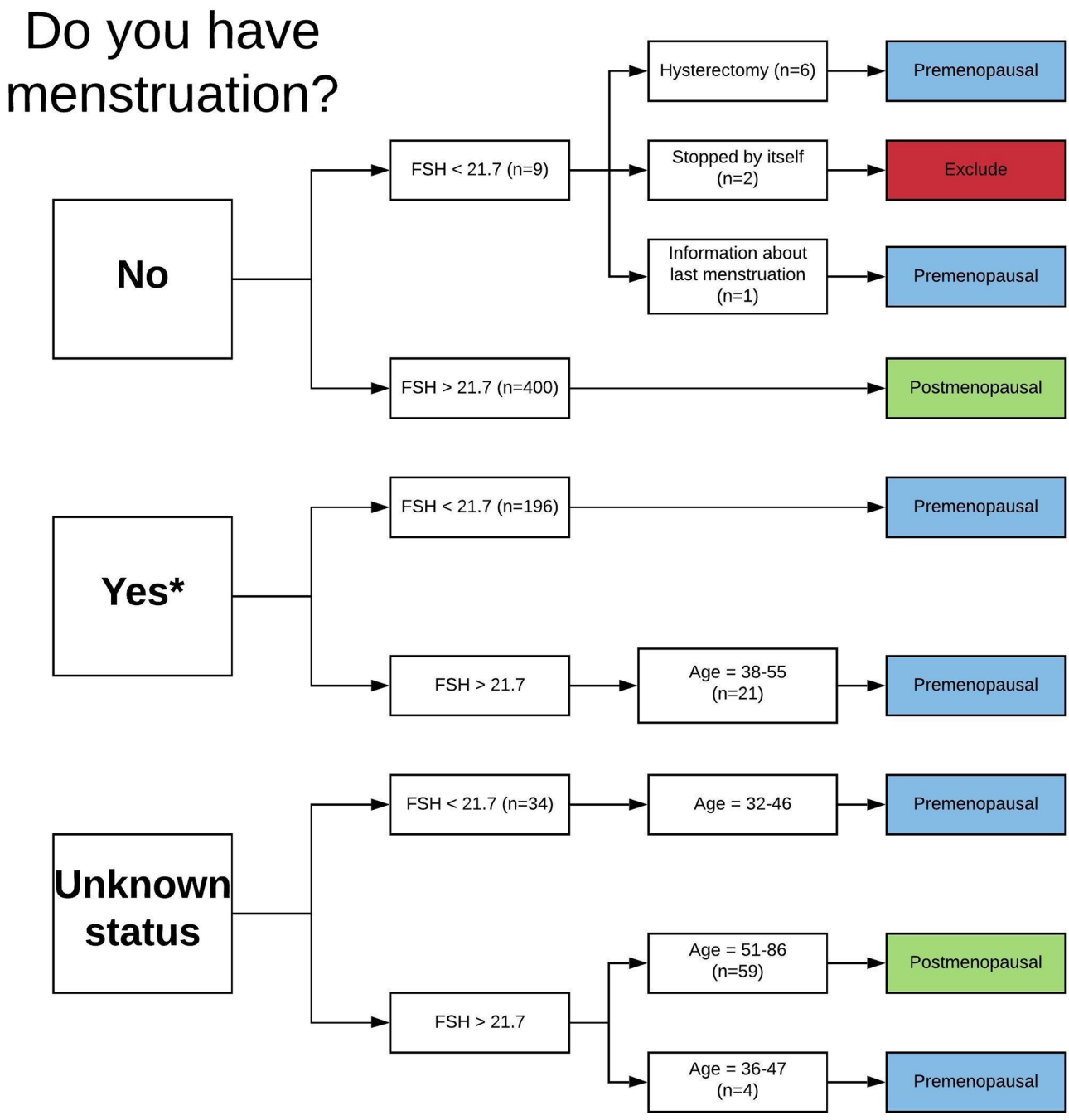

Figure 2

Classification of menstruation status, the sixth Tromsø study. 'Do you have menstruation?' corresponds with the interview question 'Do you still have natural menstruation?' *Participants reporting irregular menstruation (*n* = 2) are reclassified as 'yes'. FSH in IU, age in years. A full colour version of this figure is available at https://doi.org/10.1530/EJE-20-0877.

≤5.5 nmol/L and luteal phase as progesterone levels >5.5 nmol/L (27). In the analysis including premenopausal women, women in the luteal phase are excluded to increase accuracy of the testosterone measurement (28).

Statistical analysis

Statistical analysis was performed using Stata/MP 15.1 for Macintosh. Univariable associations were analysed in contingency tables by calculating means and s.d. using chi-square and t-test to quantify the potential role of chance. Independent samples t-test were used to test possible associations between sex-steroid hormones, carriage and persistent carriage. Multivariable logistic regression was fitted to estimate odds ratios (ORs) and 95% CIs to describe the associations between sex-steroid hormones and *S. aureus* nasal carriage and *S. aureus* persistent nasal carriage while adjusting for potential confounders. Missing values were excluded from the logistic regression analysis. DAGitty 3.0 was used for model selection (29). Testing for potential interaction between explanatory variables was done by including the multiplicative terms of two predictor variables in the model. *P*-values of ≤0.05 were considered statistically significant.

Ethics

Each participant in the sixth Tromsø study signed a declaration of consent. The study does not include data from participants with their declaration of consent withdrawn after participation. Tromsø 6 was approved by the Regional Committee for Medical and Health Research Ethics (REK) and the Norwegian Data Protection Authority. The present analysis was approved by REK (2018/1975/REK nord).

Results

In this population of female participants with age 30–87 years (mean = 53.5, s.d. = 13.6), the prevalence of *S. aureus* nasal carriage was 24% (180/724), and of *S. aureus* persistent nasal carriage 22% (151/700) (Table 1 and Supplementary Table 1, see section on supplementary materials given at the end of this article).

In postmenopausal women, mean level of testosterone were significantly lower in persistent nasal carriers compared to others (mean difference (MD) = 0.10 95% CI = 0.00–0.20) (Table 2). When postmenopausal women's carrier state was assessed from only one nasal swab, the mean level of bioavailable testosterone was 0.15 nmol/L for non-carriers and 0.13 nmol/L for carriers (MD = 0.02 95% CI = −0.00 to 0.04) (Supplementary Table 2). In postmenopausal women, serum levels of androstenedione, DHEAS, and 17α-hydroxyprogesterone were lower in nasal carriers than in others but not statistically significant (Table 2).

Using DAGitty (29), age and BMI were identified as sufficient covariates in the regression analysis when estimating the association between sex-steroids and *S. aureus* nasal carriage in women. In a multivariable logistic regression model adjusted for age and BMI, an increase of 1 s.d. (s.d. = 0.81 nmol/L) of testosterone lowered the odds of nasal carriage of *S. aureus* (OR = 0.60; 95% CI = 0.39–0.94) as well as persistent nasal carriage (OR = 0.57; 95% CI = 0.35–0.92; s.d. = 0.82 nmol/L) (Table 3).

When stratifying by menopausal status, this decrease in odds of persistent nasal carriage by higher testosterone was also found in postmenopausal women (OR = 0.74; 95% CI = 0.55–0.99; s.d. = 0.43 nmol/L). In premenopausal women, the association was similar but not statistically significant (OR = 0.35; 95% CI = 0.05–2.29; s.d. = 1.48 nmol/L). The odds of nasal carriage by higher

Table 1 Characteristics of the study population by *Staphylococcus aureus* nasal carrier state. The sixth Tromsø study. Data are presented as mean ± S.D. or as *n* (%).

	Carrier state, *n* = 724			**Persistent carrier state, *n* = 700**		
	Non-carrier	Carrier	*P*-value[a]	Others[b]	Persistent carrier	*P*-value[a]
n	544	180		549	151	
Age, years	55.9 ± 13.4	55.1 ± 13.7	0.485	56.1 ± 13.3	56.7 ± 13.3	0.628
BMI, kg/m^2	27.6 ± 5.2	28.2 ± 5.6	0.204	27.5 ± 5.1	28.4 ± 5.8	0.069
Menstruation phase						
Menopause	346 (63.6 %)	113 (62.8 %)	0.935	356 (64.9 %)	103 (68.2 %)	0.487
Luteal phase	87 (16.0 %)	28 (15.5 %)		87 (15.8 %)	18 (11.9 %)	
Follicular phase	111 (20.4 %)	39 (21.7 %)		106 (19.3 %)	30 (19.9 %)	

*Chi-square test for categorical and t-tests for continuous variables; [b]others; Intermittent carriers (one positive nasal samples of two samples in total) *n* = 49; non-carriers (two negative nasal samples of two samples in total) *n* = 500.

Table 2 Mean difference and CI in circulating sex-steroids, gonadotropins and binding protein levels among *Staphylococcus aureus* persistent nasal carriers compared to others. The sixth Tromsø study.

	Premenopausal, n = 237[a,b]		**Postmenopausal**, n = 445[a]	
	Mean difference[c]	95% CI[d]	Mean difference[c]	95% CI[d]
Testosterone, nmol/L	0.22	−0.38 to 0.83	0.10	0.00–0.20
Bioavailable testosterone[e], nmol/L	0.04	−0.11 to 0.18	0.18	−0.00 to 0.04
Androstenedione, nmol/L	0.17	−0.42 to 0.76	0.12	−0.13 to 0.36
Dehydroepiandrosterone, nmol/L	−0.04	−0.93 to 0.85	0.33	−0.04 to 0.69
17α-hydroxyprogesterone, nmol/L	0.88	−2.44 to 4.20	0.14	−0.18 to 0.46
Progesterone, nmol/L	1.57	−3.54 to 6.69	0.04	−0.04 to 0.11
Sex-hormone binding globulin, nmol/L	9.91	0.73–19.09	−0.89	−7.72 to 5.93
Albumin, nmol/L	0.05	−0.83 to 0.93	0.18	−0.42 to 0.78
Luteinising hormone, IU	−0.26	−2.99 to 2.47	0.80	−1.67 to 3.26
Follicle-stimulating hormone, IU	2.68	−1.65 to 7.01	1.39	−4.43 to 7.21

[a]Number may vary due to missing values; [b]women in luteal phase are excluded in the analysis of testosterone, bioavailable testosterone, androstenedione and DHEA; [c]mean difference = mean (others) – mean (persistent carriage); [d]independent sample t-test; [e]bioavailable testosterone calculated from the equation '(testosterone/SHBG) × 10'.

testosterone were lower but not statistically significant for premenopausal women (OR = 0.30; 95% CI = 0.06–1.56; S.D. = 1.41 nmol/L) and for postmenopausal women (OR = 0.78; 95% CI = 0.59–1.02; S.D. = 0.43 nmol/L).

Multivariable logistic regression analysis showed a lower odds of nasal carriage (OR = 0.53; 95% CI = 0.32–0.90) and persistent nasal carriage (OR = 0.52; 95% CI = 0.30–0.92) for an increase in bioavailable testosterone by one standard deviation (S.D. = 0.19 nmol/L) (Table 3).

As for testosterone a decrease in odds of persistent nasal carriage was also found for postmenopausal women (OR = 0.72; 95% CI = 0.52–0.99; S.D. = 0.09 nmol/L). In premenopausal women, there was a similar association (OR = 0.38; 95% CI = 0.06–2.45; S.D. = 0.34 nmol/L). The odds of nasal carriage were lower but not statistically significant for premenopausal women (OR = 0.28; 95% CI = 0.05–1.46; S.D. = 0.34 nmol/L) and for postmenopausal women (OR = 0.75; 95% CI = 0.56–1.01; S.D. = 0.09 nmol/L).

There was an inverse dose-response relationship between bioavailable testosterone and *S. aureus* carriage and persistent carriage, as well as for testosterone. In this female population, 98% had serum testosterone levels between 0 and 2 nmol/L, the estimates for values over 2 nmol/L are therefore more uncertain (Fig. 3).

Interaction analysis between all variables in the final model revealed no significant interactions. When the equation by Morris *et al*. was used to calculate bioavailable testosterone there was no change in the risk estimates for *S. aureus* carriage (Supplementary Table 5) (24). One female participant had an extreme value of testosterone (17.42 nmol/L, population-mean: 0.8 nmol/L). Estimates from the final model did not differ when including or excluding this observation, and the presented results therefore include this observation.

Table 3 Associations between testosterone, bioavailable testosterone and *Staphylococcus aureus* nasal carriage and persistent carriage. Analysis on menopausal women and premenopausal women in follicular phase. Odds ratios (ORs) and 95% CIs from logistic regression analysis. The sixth Tromsø study.

	Nasal carriage	**Persistent nasal carriage**
Testosterone		
n	573	560
per S.D.[a]	0.60 (0.39–0.94)	0.57 (0.35–0.92)
BMI, kg/m^2	1.03 (0.99–1.07)	1.03 (0.99–1.07)
Age, year	0.99 (0.98–1.01)	1.00 (0.98–1.02)
Bioavailable testosterone		
n	557	544
per S.D.[b,c]	0.53 (0.32–0.90)	0.52 (0.30–0.92)
BMI, kg/m^2	1.05 (1.01–1.09)	1.06 (1.01–1.10)
Age, year	0.99 (0.98–1.01)	1.00 (0.98–1.01)

[a]Serum testosterone (nmol/L) S.D.: analysis of nasal carriage, S.D. = 0.81; analysis of persistent nasal carriage, S.D. = 0.82; [b]bioavailable testosterone (nmol/L) S.D.: analysis of nasal carriage, S.D. = 0.19; analysis of persistent nasal carriage, S.D. = 0.19; [c]ioavailable testosterone calculated from the equation: (testosterone/SHBG) × 10.

Discussion

In this large population-based study of women not using any exogenous hormones, we report novel evidence of the association between circulating testosterone and bioavailable testosterone and *S. aureus* nasal carriage in women. This is to our knowledge the first study of circulating sex-steroids and *S. aureus* nasal carriage in a general female population. With increasing testosterone by one standard deviation, we see a 40% decrease in odds of *S. aureus* nasal carriage and a 43% decrease in *S. aureus*

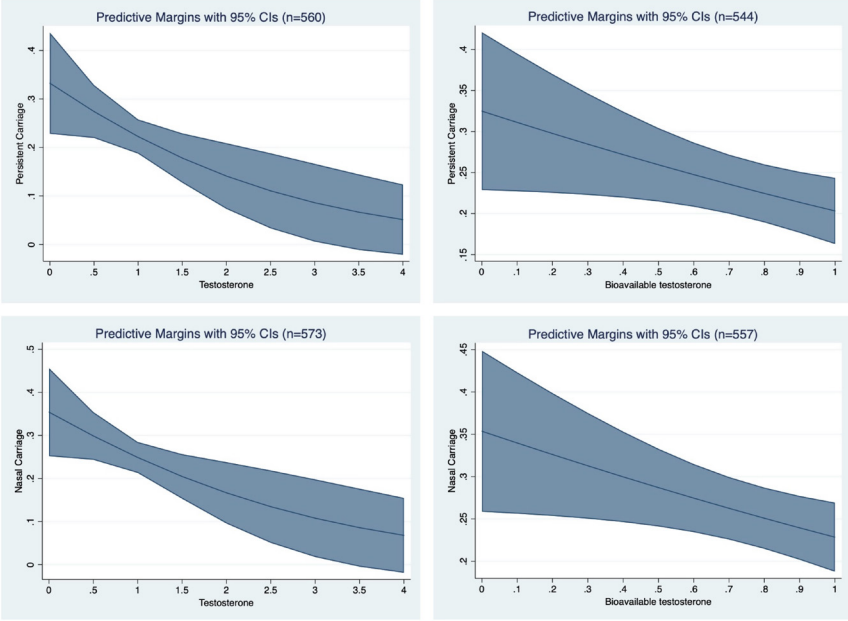

Figure 3
Probability of *S. aureus* nasal carriage (top) and persistent nasal carriage according to testosterone (left) and bioavailable testosterone (right). Analysis of postmenopausal women and premenopausal women in follicular phase. Testosterone and bioavailable testosterone per nmol/L. Bioavailable testosterone calculated from the equation '(testosterone/SHBG) × 10'. A full colour version of this figure is available at https://doi.org/10.1530/EJE-20-0877.

persistent nasal carriage compared to non-carriers and others (at least one of two nasal swab cultures negative for *S. aureus*), respectively. The same inverse associations are present in analysis stratified by menopausal status (range of ORs from 0.30 to 0.78), but statistically significant only for persistent nasal carriage in postmenopausal women.

We report a lowering in odds of *S. aureus* nasal carriage and *S. aureus* persistent nasal carriage of 47 and 48%, respectively, with increase of bioavailable testosterone by one standard deviation compared to non-carriers and others. In postmenopausal women, the association with *S. aureus* persistent carriage was weaker, but statistically significant. All other estimates from analysis stratified by menopausal status show the same inverse associations between bioavailable testosterone and both nasal carriage and persistent nasal carriage, range of ORs from 0.28 to 0.75, however, not statistically significant. Possibly, we lack the statistical power to identify a significant association because of the low number of participants in the subpopulations of premenopausal women (n = 135–148). Persistent nasal carriers also had lower average levels of androstenedione and DHEAS, however not statistically significant.

The knowledge of host response during *S. aureus* nasal carriage is limited. The presence of *S. aureus* nasal carriage induces both innate and adaptive immune responses, but the bacteria overcome host defence mechanisms to establish carrier status (6, 30). Innate immunity exerts the first response against microbes, and the association between delayed response of toll-like receptor 2 in mice and nasal carriage has been demonstrated (31). In animal models, hypogonadism is associated with an increase in immune response with castration of male mice increasing autoimmune encephalomyelitis severity (32) and the incidence and severity of thyroiditis and adjuvant arthritis in rats (33). A recent study demonstrated an immunomodulating role of dihydrotestosterone in both human and rat vaginal smooth muscle cells (34). In summary, studies in animals document a significant effect of sex-steroid hormones on the immune system, but the results from in vitro studies are conflicting (35). Testosterone has been characterised as immunosuppressive, but recent research suggests that testosterones immunomodulatory effects depend on the reproductive context and menopausal status. There are also inconsistency of results for testosterone in both postmenopausal and premenopausal women where testosterone has been characterised as both pro-inflammatory and anti-inflammatory (36).

Former studies have shown a positive association between combination-hormonal contraceptives and *S. aureus* nasal carriage, but lower odds of carriage with progestin-only contraceptives. Studies have demonstrated lower levels of bioavailable testosterone in women using combined oral contraceptives (37). This supports the finding of lower odds of *S. aureus* nasal carriage in women with higher bioavailable testosterone. Progestin is a synthetic progestogen and is classified from its chemical structure as a derivate of testosterone or progesterone (38). Therefore, progestin has both androgen and progestogen activity when used in contraceptives, and progestin-only

users may have a higher prevalence of androgenic side effects than combination-contraceptive users because of the lack of the oestrogen component (39).

High androgen and testosterone levels stimulate the sebaceous glands to increased sebum production, and plays an important role in the development of acne (40). In this study, 50–75% of women with polycystic ovary syndrome have high values of bioavailable testosterone and some of the diagnostic criteria are hyperandrogenistic features as acne (41). These hormonally related changes may also affect the microbiota of the skin and promote bacteria like *Cutibacterium acnes* that suppress the growth of other bacteria like *S. aureus* (42). This may be a plausible biological mechanism contributing to the lower probability of carriage of *S. aureus* with higher testosterone in our study.

Testosterone levels in women, as oestrogen levels, decline in the fourth decade of life and after menopause. Though the biological role of testosterone in women remains largely unclear, androgens are biological precursors of oestrogen production (43). High level of testosterone may therefore represent higher level of estrogen. However, oestrogen was not measured in our study. Estrogen has been theorised to have both pro-inflammatory and anti-inflammatory effects (44). One study found high testosterone and estradiol, and low SHBG levels associated with conditions that represent low-grade inflammation processes (45). The lower odds of *S. aureus* carriage with high levels of testosterone may be an indirect effect of possibly corresponding high levels of oestrogen or the unknown ratio between testosterone and oestrogen.

The major circulating steroids classified as androgens include DHEAS, androstenedione and testosterone (46). The lower levels of DHEAS and androstenedione in *S. aureus* persistent nasal carriers among postmenopausal women support our findings of lower odds of *S. aureus* carriage with higher levels of bioavailable testosterone and testosterone (Supplementary Fig. 2). This may represent an unknown effect of androgens on the immune system and increases the validity of the main finding.

We found no statistically significant association between androstenedione, LH, FSH, DHEAS, 17-OH progesterone, progesterone, SHBG, albumin and *S. aureus* nasal carriage and persistent carriage. This may be due to more complex relationships between sex-steroid hormones and the immune system. For premenopausal women, the female sex-steroid hormones have cyclic variations and data based on one measurement may not be representative for the participants' hormonal status.

It has been reported that the equation '(testosterone/SHBG × 10)' is not a reliable when SHBG concentration is low (47). Our analysis does not differ when using either this equation or the equation by Morris *et al.* (24) for the calculation of bioavailable testosterone. This may be due to our study population with mostly healthy women with normal BMI that has a low prevalence of very low measurements of SHBG (48, 49).

We used DAGitty for model selection, and the recommended confounders to adjust for in the analysis were BMI, HbA1c and age (Supplementary Fig. 1). Because of the minor insignificant effect on the main analysis of HbA1c, we decided to only adjust for BMI and age. We report a significant decrease of *S. aureus* with smoking, and a significant association with alcohol use. Because of the decision of not doing model selection by data driven statistical selection process, we did not include these variables in the final logistic regression model (Supplementary Tables 3 and 4). We include tables in Supplementary information for the reader to explore the odds ratio estimates including different possible covariates. The odds ratio estimates changed only slightly in these models. Although our data do not support any interactions among the explanatory variables, we cannot rule out the presence of smaller interaction effects that can only be detected by larger sample sizes.

Strengths of our study include a population-based design with high attendance, and consequently reduced risk of selection bias. Trained nurses collected nasal swab samples, and analyses of blood samples were performed with current gold standard assays for analysing sex-steroid hormones. Using both medical history and circulating FSH-levels to classify premenopausal and menopausal women reduces misclassification bias. One possible bias in the study is misclassification of *S. aureus* carrier status due to only two nasal swab samples, as van Belkum *et al.* recommend seven samples to correctly classify persistent carriers from intermittent carriers. Intermittent carriers has a lower risk of infections similar to non-carriers as well as similar elimination kinetics (20). Thus, we may have an unknown number of intermittent carriers misclassified as persistent in our study, which may have biased the risk estimates towards the null. However, large population-based data reduces the risk of selection bias and increases both the internal and external validity.

In conclusion, we report evidence of an association between circulating testosterone and bioavailable testosterone and *S. aureus* nasal carriage defined from both one and two swab samples in females. Higher levels of bioavailable testosterone are associated with lower

odds for *S. aureus* nasal carriage of 39–43%. The role of testosterone and bioavailable testosterone in *S. aureus* nasal carriage ought to be addressed in future prospective studies to identify risk groups for prevention of *S. aureus* carriage and disease.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EJE-20-0877.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general female population

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The authors of the above titled article published in the *European Journal of Endocrinology* (vol 184 Iss 2 pages 337–346) apologise for errors in the 'Methods' section. The authors state that the instruments for the analysis of testosterone, androstenedione, dehydroandrostenedione, 17α-hydroxyprogesteron and progesterone were incorrectly reported in the 'Methods' section: Assessment of *S. aureus* carriage and serum sex-steroids. The correct instruments for the analysis of the given hormones were LCMS/MS, SCIEX API 5500 triple-quadrupole mass spectrometer, Applied Biosystems/MDS with an Agilent 1290 UPLC system. Luteinising hormone, follicle-stimulating hormone, sex-hormone binding globulin and albumin were measured using DPC immulite 200 XPi and not as published.

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Paper II

Supplementary materials

Supplementary Table 1: Characteristics of the study population by *Staphylococcus aureus* nasal carrier state; additional covariates included. The 6th Tromsø Study

	Carrier state n=724[a]			Persistent carriage state n=700[a]		
	Non-carrier N (%) n= 544	**Carrier** N (%) n=180	**P-value** [b]	**Others**[c] N (%) n= 549	**Persistent carrier** N (%) n=151	**P-value** [b]
Age, years (mean, SD)	55.9 (13.4)	55.1 (13.7)	0.485	56.1 (13.3)	56.7 (13.3)	0.628
BMI, kg/m2 (mean, SD)	27.6 (5.2)	28.2 (5.6)	0.204	27.5 (5.1)	28.4 (5.8)	0.069
Menstruation phase						
Menopause	346 (63.6)	113 (62.8)	0.935	356 (64.9)	103 (68.2)	0.487
Luteal phase	87 (16.0)	28 (15.5)		87 (15.8)	18 (11.9)	
Follicular phase	111 (20.4)	39 (21.7)		106 (19.3)	30 (19.9)	
Smoking						
Yes	110 (20.9)	22 (12.5)	0.014	112 (21.0)	16 (10.9)	0.005
No	417 (79.1)	132 (87.5)		421 (79.0)	131 (89.1)	
Alcohol use						
More than 4 times a month	93 (17.5)	26 (14.9)	0.008	97 (18.0)	19 (13.0)	0.014
2-4 times a month	181 (33.9)	46 (26.3)		179 (33.3)	41 (28.1)	
Once a month or less	182 (34.1)	85 (48.5)		184 (34.2)	71 (48.6)	
Never	77 (14.5)	18 (10.3)		78 (14.5)	15 (10.3)	
HbA1c, % [d] (mean, SD)	5.6 (0.6)	5.6 (0.8)	0.555	5.6 (0.6)	5.7 (0.8)	0.168
Vitamin D nmol/L (non-smokers)[ef] (mean, SD)	55.4 (18.9)	53.2 (17.1)	0.220	55.1 (18.8)	54.2 (17.5)	0.620
Vitamin D nmol/L (smokers)[ef] (mean, SD)	77.6 (21.5)	78.87 (23.7)	0.801	77.5 (21.3)	80.1 (25.8)	0.663
Hospital admission last 12 months						
Yes	60 (11.3)	25 (14.4)	0.284	125 (20.9)	20 (24.7)	0.430
No	470 (88.7)	149 (85.6)		474 (79.1)	61 (75.3)	

[a]Number may vary due to missing values
[b]Chi-square test for categorical and t-tests for continuous variables
[c]Others; Intermittent carriers (one positive nasal samples of two samples in total) n=49; Non-carriers (two negative nasal samples of two samples in total) n=500
[d]HbA1c = EDTA-blood glycated hemoglobin (HbA1c)
[e]Vitamin D = serum 25-hydroxyvitamin D [25(OH)D]
[f]Vitamin D is stratified by smoking because of known overestimating of 25(OH)D levels in smokers by unknown mechanisms when using ECLIA

Supplementary Table 2: Mean difference and confidence intervals (CI) in circulating sex-steroids, gonadotropins and binding protein levels among *Staphylococcus aureus* nasal carriers compared to others. The 6th Tromsø Study

	Premenopausal n=261[ab]		**Postmenopausal** n=445[a]	
	Mean difference[c]	**95 % CI**[d]	**Mean difference**[c]	**95 % CI**[d]
Testosterone nmol/L	0.23	-0.29-0.76	0.09	-0.01-0.18
Bioavailable testosterone[e] nmol/L	0.04	-0.08-0.17	0.02	-0.00-0.04
Androstenedione nmol/L	0.29	-0.26-0.85	0.07	-0.16-0.31
Dehydroepiandrosterone nmol/L	0.06	-0.74-0.85	0.13	-0.23-0.48
17α-hydroxyprogesterone nmol/L	1.01	-1.76-3.78	0.16	-0.16-0.47
Progesterone nmol/L	0.74	-3.68-5.16	0.03	-0.04-0.11
Sex-hormone binding globulin nmol/L	5.73	-2.17-13.64	-1.16	-7.76-5.44
Albumin nmol/L	0.09	-0.66-0.85	0.23	-0.35-0.81
Luteinizing hormone IU	1.12	-1.68-3.91	0.96	-1.43-3.35
Follicle-stimulating hormone IU	2.95	-0.66-6.57	0.92	-4.72-6.56

[a]Number may vary due to missing values

[b]Women in luteal phase are excluded in the analysis of testosterone, bioavailable testosterone, androstenedione and dehydroepiandrosterone.

[c]Mean difference = mean (others) – mean (nasal carriage)

[d]Independent sample t-test

[e]Bioavailable testosterone calculated from the equation "(testosterone/SHBG) X 10"

Supplementary Table 3: Associations between testosterone and *Staphylococcus aureus* nasal carriage and persistent carriage. Odds ratios (OR) and 95% confidence intervals (95% CI) from logistic regression analysis. The 6th Tromsø Study

	Nasal carriage			Persistent nasal carriage		
	All women (n=567)[ab]	**Pre-menopausal** (n=147)[ab]	**Post-menopausal** (n=421)[a]	**All women** (n=554)[ab]	**Pre-menopausal** (n=134)[ab]	**Post-menopausal** (n=421)[a]
Testosterone[cd]	0.61 (0.38-0.95)	0.30 (0.06-1.55)	0.79 (0.60-1.04)	0.57 (0.36-0.94)	0.34 (0.05-2.25)	0.75 (0.57-1.00)
Testosterone[ce]	0.64 (0.41-1.00)	0.34 (0.06-1.86)	0.81 (0.62-1.06)	0.62 (0.38-1.00)	0.40 (0.06-2.80)	0.78 (0.59-1.04)
Testosterone[cf]	0.65 (0.41-1.02)	0.32 (0.06-1.75)	0.83 (0.64-1.08)	0.62 (0.38-1.00)	0.31 (0.04-2.32)	0.80 (0.60-1.06)
Testosterone[cg]	0.69 (0.44-1.08)	0.33 (0.06-1.86)	0.86 (0.66-1.11)	0.66 (0.41-1.07)	0.30 (0.04-2.30)	0.83 (0.63-1.09)

	Smokers		Non-Smokers	
	Carriage	**Persistent carriage**	**Carriage**	**Persistent carriage**
	All women[b] (n=98)	**All women**[b] (n=96)	**All women**[b] (n=428)	**All women**[b] (n=417)
Testosterone[ch]	0.53 (0.13-2.05)	0.56 (0.12-2.64)	0.69 (0.43-1.11)	0.64 (0.38-1.07)

[a]Number may vary due to missing values

[b]Women in luteal phase are excluded

[c]Testosterone divided by the standard deviation; Nasal carriage all women SD=0.81; Nasal carriage premenopausal SD=1.41.3387801; Nasal carriage postmenopausal SD=0.43; Persistent carriage all women SD=0.82; Persistent carriage premenopausal SD=1.47; Persistent carriage postmenopausal SD=0.43

[d]Adjusted for BMI, age and HbA1c

[e]Adjusted for BMI, age, HbA1c and smoking

[f]Adjusted for BMI, age, HbA1c, smoking and alcohol use

[g]Adjusted for BMI, age, HbA1c, smoking alcohol use and hospital admission

[h]Adjusted for BMI, age, HbA1c, alcohol use, hospital admission and vitamin D

Supplementary Table 4: Associations between bioavailable testosterone[a] and *Staphylococcus aureus* nasal carriage and persistent nasal carriage. Odds ratios (OR) and 95% confidence intervals (95% CI) from logistic regression analysis. The 6th Tromsø Study

	Nasal carriage			Persistent nasal carriage		
	All women (n=551)[bc]	**Pre-menopausal** (n=147)[bc]	**Post-menopausal** (n=405)[b]	**All women** (n=538)[bc]	**Pre-menopausal** (n=134)[bc]	**Post-menopausal** (n=405)[b]
Bioavailable testosterone[de]	0.53 (0.31-0.90)	0.27 (0.05-1.39)	0.76 (0.56-1.02)	0.52 (0.30-0.91)	0.36 (0.06-2.28)	0.72 (0.53-0.99)
Bioavailable testosterone[df]	0.54 (0.31-0.91)	0.25 (0.04-1.36)	0.77 (0.57-1.04)	0.53 (0.30-0.93)	0.32 (0.05-2.22)	0.73 (0.53-1.01)
Bioavailable testosterone[dg]	0.54 (0.32-0.91)	0.25 (0.05-1.33)	0.77 (0.57-1.04)	0.52 (0.29-0.92)	0.28 (0.04-1.91)	0.74 (0.53-1.02)
Bioavailable testosteronedh	0.56 (0.32-0.94)	0.30 (0.05-1.61)	0.78 (0.58-1.06)	0.54 (0.30-0.95)	0.31 (0.05-2.15)	0.75 (0.54-1.04)

	Smokers		Non-Smokers	
	Carriage	**Persistent carriage**	**Carriage**	**Persistent carriage**
	All women[c] (n=94)	**All women**[c] (n=92)	**All women**[c] (n=416)	**All women**[c] (n=405)
Bioavailable testosterone[di]	0.86 (0.40-1.81)	0.97 (0.64-1.47)	0.49 (0.27-0.87)	0.42 (0.22-0.80)

[a] Bioavailable testosterone calculated from the equation "(testosterone/SHBG) X 10"

[b] Number may vary due to missing values

[c] Women in luteal phase are excluded

[d] Bioavailable testosterone divided by the standard deviation; Nasal carriage all women SD=0.19; Nasal carriage premenopausal SD=0.34; Nasal carriage postmenopausal SD=0.09; Persistent carriage all women SD=0.19; Persistent carriage premenopausal SD=0.35; Persistent carriage postmenopausal SD=0.09

[e] Adjusted for BMI, age and HbA1c

[f] Adjusted for BMI, age, HbA1c and smoking

[g] Adjusted for BMI, age, HbA1c, smoking and alcohol use

[h] Adjusted for BMI, age, HbA1c, smoking alcohol use and hospital admission

[i] Adjusted for BMI, age, HbA1c, alcohol use, hospital admission and vitamin D

Supplementary Table 5: Associations between bioavailable testosterone and *Staphylococcus aureus* nasal carrier and persistent carrier state by Morris et al[a]. Odds ratios (ORs) and 95% confidence intervals (95% CIs) from logistic regression analysis. The Tromsø Study 6

	Nasal carrier			**Persistent nasal carrier**		
	All women (n=561)	**Pre-menopausal** (n=148)	**Post-menopausal** (n=414)	**All women** (n=548)	**Pre-menopausal** (n=135)	**Post-menopausal** (n=414)
Bioavailable testosterone, pr SD[a]	0.58 (0.37-0.92)	0.27 (0.05-1.43)	0.78 (0.59-1.02)	0.55 (0.33-0.91)	0.33 (0.05-2.20)	0.73 (0.54-0.98)
BMI, kg/m^2	1.03 (1.00-1.07)	1.09 (1.02-1.16)	1.01 (0.97-1.06)	1.04 (1.00-1.08)	1.10 (1.02-1.18)	1.01 (0.97-1.06)
Age, year	0.99 (0.98-1.01)	0.98 (0.90-1.06)	0.99 (0.97-1.02)	1.00 (0.98-1.01)	0.98 (0.90-1.08)	1.00 (0.97-1.03)

[a]ln BioT = -0.266 + (0.955 x lnTT) – (0.228 x lnSHBG)

[b]Bioavailable testosterone divided by the standard deviation; Nasal carriage all women SD=0.23; Nasal carriage premenopausal SD=0.40; Nasal carriage postmenopausal SD=0.12; Persistent carriage all women SD=0.23; Persistent carriage premenopausal SD=0.43; Persistent carriage postmenopausal SD=0.12

Paper III

Circulating sex-steroids and *Staphylococcus aureus* nasal carriage in a general male population

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Abstract

Background

Male sex is associated with higher risk of both colonization and infection with *Staphylococcus aureus* (*S. aureus*). However, the role of sex-steroids in colonization among men is largely unknown. Thus, the aim of this study was to investigate possible associations between circulating sex-steroids and nasal carriage of *S. aureus* in a general male population.

Methods

The population-based Tromsø6 study (2007-2008) included 752 males aged 31-87 years with serum sex-steroids measured by liquid chromatography tandem mass spectrometry and two nasal swab samples for the assessment of *S. aureus* carriage. Multivariable logistic regression models were used to study the association between sex-steroid concentrations and *S. aureus* persistent nasal carriage (two positive swabs versus others), while adjusting for potential confounding factors.

Results

S. aureus persistent nasal carriage prevalence was 32%. Among men aged 55 years and above (median age 65 years), there was an inverse dose-response relationship between serum concentration of testosterone and persistent nasal carriage, and carriers had significantly lower mean levels of testosterone (p=0.028). This association was attenuated when adjusting for body mass index and age (OR=0.96 per nmol/L change in testosterone; 95% CI=0.91-1.01). There was no association in the total population.

Conclusions

This large population-based study suggests that testosterone levels may be inversely related to *S. aureus* persistent nasal carriage in older men. Future studies addressing biological mechanisms underlying the male predisposition to *S. aureus* colonization and infection may foster preventive interventions that take sex-differences into account.

Keywords: Circulating sex-steroids; Staphylococcus aureus carriage; population-based study; testosterone

Background

Epidemiological research has shown that men are at increased risk of several different infectious diseases (*1, 2*). However, data addressing the underlying biological mechanisms are scarce. *Staphylococcus aureus* (*S. aureus*) is more frequent in men compared to women, both as a nasal colonizer and as a causative infectious agent (*3, 4*). Nasal colonization is a major risk factor for *S. aureus* infection (*5*). Thus, identification of biological pathways underlying sex differences in nasal colonization is important not only to enable a better understanding of host factors in colonization but also to enable the development of preventive interventions that take sex differences into account.

It is well known that immune functions differ by sex and age (*6-9*). Sex-steroids are key regulators of both the innate and adaptive immune system, and hormone levels and actions are context (i.e., sex and age) dependent. Recently, we showed for the first time that higher

levels of circulating testosterone in adult women (*10*) and use of progestin-only contraceptives (structurally related to testosterone) in younger women (*11*) are associated with lower prevalence of *S. aureus* nasal carriage. To our knowledge, no epidemiological study has examined whether endogenous sex-hormone levels are associated with *S. aureus* nasal carriage among men.

Thus, the aim of this study was to examine possible associations between endogenous sex-steroids and *S. aureus* nasal carriage in a large male population sample.

Methods

We used data from male participants in the population-based Tromsø6 study (2007-2008), North Norway, 66% attendance. The study included measurement of height and weight, blood samples, and interview and questionnaire on lifestyle and health. Nasal swab samples were collected from a sample of 1741 participants (Described – (*10*)) among whom serum concentrations of sex-steroids were measured in 888 individuals. After exclusion of 19 individuals taking antibiotics the last 24 hours and 117 individuals with only one nasal sample, 752 men were included in the present analysis.

We used direct culturing methods to identify *S. aureus* in two repeated nasal swabs to assess persistent carriage (*3*), and liquid chromatography tandem mass spectrometry (LCMS/MS) to measure serum concentrations of testosterone, androstenedione, 17α-hydroxyprogesterone (17-OH progesterone), and progesterone (*10*). Serum concentrations of gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH), binding proteins (sex-hormone binding globulin (SHBG) and albumin), dehydroepiandrostenedione sulfate (DHEAS) and 25-hydroxyvitamin D were assessed by immunoassay methods.

Statistical analyses were performed using Stata/MP 15.1 for Macintosh, with significance level set to $p < 0.05$. Univariable associations were assessed by chi-square test, Student's t-test, or Mann-Whitney U test. Multivariable logistic regression models were fitted to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for *S. aureus* persistent nasal carriage by change in sex-steroid concentrations, while adjusting for potential confounders. A sensitivity analysis on an age-stratified population (cut-off 55 years, median age) was performed, as both concentration of serum androgens and *S. aureus* persistent nasal carriage were inversely related to age. DAGitty 3.0 was used for model selection, and possible interactions were assessed for in the final model.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK) and the Norwegian Data Protection Authority. All methods were performed in accordance with relevant guidelines and regulations.

Results

Among the 752 males, age 31-87 years, the prevalence of *S. aureus* persistent nasal carriage was 32%. Persistent nasal carriers were younger, had lower vitamin D levels and lower prevalence of current smoking than others (intermittent or non-carriers; results not shown).

We found no association between any circulating sex-steroid and *S. aureus* nasal carriage in the total population when adjusting for age and body mass index (BMI) in a multivariable logistic regression model (Table 1). There was a statistically significant interaction between BMI and age, but the interaction term was not included in the model as this did not alter the main results.

Table 1. Associations between hormonal status and *S. aureus* persistent nasal carriage in men.	
	Persistent nasal carriage[a] OR[b] (95% CI)
Testosterone, nmol/L	0.98 (0.95-1.01)
Bioavailable testosterone, nmol/L	0.96 (0.83-1.12)
Androstenedione, nmol/L	1.03 (0.91-1.17)
Dehydroepiandrostenedione, nmol/L	0.96 (0.89-1.03)
17α-hydroxyprogesterone, nmol/L	0.97 (0.87-1.08)
Progesterone, nmol/L	1.11 (0.62-1.98)
Sex-hormone binding globulin, nmol/L	0.99 (0.98-1.00)
Albumin, nmol/L	0.96 (0.88-1.04)
Luteinizing hormone, IU	0.99 (0.94-1.03)
Follicle-stimulating hormone, IU	1.00 (0.98-1.01)
Adjusted odds ratios (OR) and 95% confidence intervals (95% CI) of carriage by one unit increase in serum hormone biomarkers The Tromsø6 study, n=752 [a]Persistent nasal carriage: two *S. aureus* culture positive nasal swab samples [b]Adjusted for age and body mass index (BMI) in multivariable logistic regression analysis	

Among men aged 55 and above, persistent nasal carriers had lower mean serum concentration of both testosterone and SHBG compared to others (p=0.028 and 0.052, respectively, Table 2). When adjusting for BMI, the OR for persistent nasal carriage was 0.96 (95% CI=0.91-1.01) per nmol/L increase in testosterone in the oldest age group (result not shown).

Table 2. Serum concentrations of sex-steroids, gonadotropins, and binding proteins by *S. aureus* nasal carrier state.

	<55 years n=387[a]			**≥55 years** n=365[a]		
	Persistent carriage n= 141	**Others[b]** n= 246	**P-value[c]**	**Persistent carriage** n=96	**Others[b]** n= 269	**P-value[c]**
Testosterone nmol/L	14.73 (5.97)	14.72 (5.63)	0.728	13.22 (4.51)	14.89 (5.90)	0.028
Bioavailable testosterone[d] nmol/L	4.19 (1.21)	4.18 (1.32)	0.944	2.97 (0.94)	2.94 (0.92)	0.845
Androstenedione nmol/L	3.02 (1.70)	2.89 (1.10)	0.353	2.43 (0.94)	2.50 (1.09)	0.777
Dehydroepiandrostenedione nmol/L	5.51 (2.19)	5.51 (2.62)	0.601	3.29 (2.32)	3.45 (2.10)	0.259
17α-hydroxyprogesterone nmol/L	2.56 (1.21)	2.62 (1.59)	0.778	2.49 (1.45)	2.61 (1.79)	0.497
Progesterone nmol/L	0.23 (0.23)	0.22 (0.24)	0.608	0.23 (0.27)	0.23 (0.31)	0.889
Sex-hormone binding globulin nmol/L	37.11 (16.59)	38.22 (16.50)	0.377	47.81 (17.92)	53.25 (21.49)	0.052
Albumin nmol/L	47.69 (2.00)	47.64 (2.42)	0.949	46.17 (2.51)	46.54 (2.36)	0.198
Luteinizing hormone IU	4.25 (2.13)	4.87 (3.69)	0.137	7.06 (5.30)	7.18 (5.50)	0.952
Follicle-stimulating hormone IU	5.63 (3.12)	7.05 (10.52)	0.242	13.02 (14.27)	12.59 (13.45)	0.564

Age group (median split) in men. Data are presented as mean (SD). The Tromsø6 study.
SD = standard deviation
[a]Number may vary due to missing values
[b]Others; Intermittent carriers (one positive nasal samples of two samples in total) or non-carriers (two negative nasal samples of two samples in total)
[c]Mann-Witney U test
[d]Calculated by the equation "(testosterone/SHBG) X 10"

There was an inverse dose-response relationship between serum testosterone concentration and *S. aureus* persistent carriage. The dose-response relationship was most evident among men aged 55 and above (Figure 1).

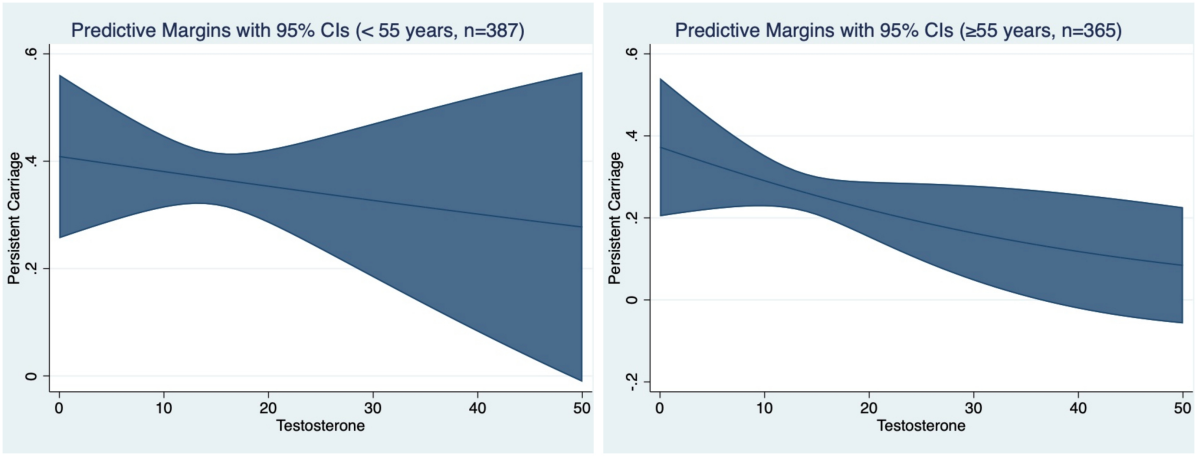

Figure 1 Probability of S. aureus persistent nasal carriage according to serum testosterone concentration *(nmol/L), range 0.4-44.3). The Tromsø6 study, male participants.*

Discussion

In a recent study among women in the Tromsø6 study, we showed that higher levels of testosterone and bioavailable testosterone were associated with lower prevalence of *S. aureus* nasal carriage (*10*). In the present study of the male population, we found no statistically significant associations of sex-steroids, gonadotropins, and binding-proteins with the prevalence of *S. aureus* carriage when adjusting for BMI and age. In the age-stratified sensitivity analysis, we found an inverse association for testosterone among the oldest group (≥55 years).

In our population-based data, there was a strong inverse association between age and serum testosterone (results not presented), that is consistent with the described progressive decline in testosterone levels in healthy men between 25 and 75 years (*12-14*). The decline in prevalence of *S. aureus* nasal carriage across adulthood is well known (*15*). Both age-related changes in testosterone and bacterial flora may be adaptations to ageing, but the

contribution of ageing *per se* versus lifestyle/nutrition and comorbidities (i.e. confounding factors) to these changes is not clear. Importantly, when adjusting for both age and BMI in our analysis, we found no statistically significant associations between sex-steroid concentrations and *S. aureus* nasal carriage. Thus, we cannot conclude that testosterone is a predictor for *S. aureus* nasal carriage in men.

In this study we collected only one venous blood sample for analysis of sex-steroid hormones. Male sex-steroid hormones are diurnal, but less so compared to women and this may result in a more representative value with only one measurement. Testosterone in men has a circadian rhythm with optimal sampling from 8 to 10 am. In our study, the blood samples were taken from 8 am to 8 pm, thus an unknown proportion of the samples are not optimal. Studies have shown that the circadian rhythm is lost in elder men (*16*), and we believe that the stratified model of men over 55 years of age could be more representative.

Conclusions

We are not able to conclude from our data that circulating sex-steroid concentrations are related to *S. aureus* nasal carriage in men. This is in contrast to our recent findings in women (*10*), and may represent, among others, imprecision in measurements, a too broad age range, or a different relationship between sex-steroids and immunity in men and women. The role of endogenous sex-steroids in *S. aureus* colonization should be addressed in future prospective studies.

List of abbreviations

BMI – Body Mass Index

CI - confidence interval

DHEAS - dehydroepiandrostenedione sulfate

FSH - follicle-stimulating hormone

LCMS/MS - liquid chromatography tandem mass spectrometry

LH – luteinizing hormone

OR - odds ratio

SHBG - sex-hormone binding globulin

Declarations

Ethics approval

Tromsø6 was approved by the Regional Committee for Medical and Health Research Ethics (REK) and the Norwegian Data Protection Authority. The present analysis including all methods was approved by the Regional Committee for Medical and Health Research Ethics (2018/1975/REK nord). All methods were performed in accordance with relevant guidelines and regulations.

Consent to participate

Participants in Tromsø6 were informed to read the information folder before the survey and signed the informed consent form when they attended the study sight. The study does not include data from participants with their declaration of consent withdrawn after participation. Informed consent was obtained from all subjects included in the study.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from The Tromsø Study but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon request and with permission of The Tromsø Study. Proposals for data should be directed to tromsous@uit.no. Statistical analysis and consent form will be available on request. Proposals should be directed to dina.b.stensen@uit.no.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ASF, CSN, GSS and GG contributed with the conception and design of the work. BH performed biochemical analysis of sex-steroids and binding proteins. JUES performed microbiological analysis of nasal samples. ASF, DBS, KO and LSA interpreted the data. DBS

performed the statistical analysis and wrote the first draft. All authors read and approved the final manuscript.

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Paper IV

Appendix A

Questionnaire from the sixth Tromsø study

English translation

https://uit.no/Content/401052/Questionnaire_T6_1.pdf
https://uit.no/Content/531228/cache=20172908084211/Questionnaire_T6_2.pdf

Appendix B

Questionnaire and Interview from the Fit Futures 1 study

English translation

Fit Futures 1

Questionnaire

Selected questions from questionnaire about lifestyle and health

How often do you drink alcohol?

☐ Never

☐ Once per month or less

☐ 2-4 times per month

☐ 2-3 times per week

☐ 4 or more times per week

☐ Daily, or almost daily

Do you use snuff?

☐ No, never

☐ Sometimes

☐ Daily

Do you smoke?

☐ No, never

☐ Sometimes

☐ Daily

Which description of your exercise and exertion in leisure time fits best? If your activity varies much, for example between summer and winter, then give an average. The question refers only to the last twelve months.

☐ Reading, watching TV, or other sedentary activity.

☐ Walking, cycling or other forms of exercise at least 4 hours a week

☐ Participation in recreational sport, heavy outdoor activities, snow clearing etc.

☐ Participation in hard training or sports competitions, regularly several times a week.

Fit Futures 1

Interview

Selected questions from the interview on hormonal contraceptives. Female participants only.

Menstruation;

Have you started menstruating? ☐ Yes | ☐ No

If you have started menstruating;

Do you use any kind of contraceptives? ☐ Yes | ☐ No

If you use any kind of contraceptives: What type?

☐ Tablets

☐ Injections

☐ Implants

☐ Condom

☐ Transdermal contraceptive patch

☐ Vaginal contraceptive ring

☐ Intrauterine device (IUD)

☐ Other

If you use any oral contraceptive pill, what is the name of the medicine?

_____

If you use any injected contraceptive, what is the name of the medicine?

_____

If you use any hormonal contraceptive subdermal implant, what is the name of the medicine?

_____

If you use any hormonal contraceptive skin patch, what is the name of the medicine?

_____

If you use any intrauterine device, what is the name of the medicine?

_____

Fit Futures 1

Interview

Selected questions from interview about social network.

Which students at first level of high school have you had most contact with the last week? Name up to 5 students at your own school or other schools in Tromsø and Balsfjord.

1_____
2_____
3_____
4_____
5_____

For each nominated friend, please answer the following questions.

Did you have physical contact?

☐ Yes | ☐ No

Have you been together at school?

☐ Yes | ☐ No

Have you been together at sports?

☐ Yes | ☐ No

Have you been together at home?

☐ Yes | ☐ No

Have you been together at other places?

☐ Yes | ☐ No

To what degree does this table of friends give an overview of your social network? Please indicate on a scale from 0 (small degree) to 10 (high degree)

☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 | ☐ 6 | ☐ 7 | ☐ 8 | ☐ 9 | ☐ 10

Fit Futures 1
fitfutures@uit.no
+47 922 12 891

https://uit.no/research/fitfutures

Appendix C

Questionnaire and Interview from the Fit Futures 2 study

English translation

Fit Futures 2

Questionnaire

Selected questions from questionnaire about lifestyle and health

How often do you drink alcohol?

☐ Never

☐ Once per month or less

☐ 2-4 times per month

☐ 2-3 times per week

☐ 4 or more times per week

☐ Daily, or almost daily

Do you use snuff?

☐ No, never

☐ Sometimes

☐ Daily

Do you smoke?

☐ No, never

☐ Sometimes

☐ Daily

Which description of your exercise and exertion in leisure time fits best? If your activity varies much, for example between summer and winter, then give an average. The question refers only to the last twelve months.

☐ Reading, watching TV, or other sedentary activity.

☐ Walking, cycling or other forms of exercise at least 4 hours a week

☐ Participation in recreational sport, heavy outdoor activities, snow clearing etc.

☐ Participation in hard training or sports competitions, regularly several times a week.

Fit Futures 2

Interview

Selected questions from the interview on hormonal contraceptives. Female participants only. Additional question about antibiotic use for all participants.

Menstruation;

Have you started menstruating? ☐ Yes | ☐ No

If you have started menstruating;

Do you use any kind of contraceptives? ☐ Yes | ☐ No

If you use any kind of contraceptives: What type?

☐ Tablets ☐ Injections

☐ Implants ☐ Condom

☐ Transdermal contraceptive patch

☐ Vaginal contraceptive ring

☐ Intrauterine device (IUD)

☐ Other

If you use any oral contraceptive pill, what is the name of the medicine?

_____

If you use any injected contraceptive, what is the name of the medicine?

_____

If you use any hormonal contraceptive subdermal implant, what is the name of the medicine?

_____

If you use any hormonal contraceptive skin patch, what is the name of the medicine?

_____

If you use any intrauterine device, what is the name of the medicine?

_____

Have you taken any antibiotics (tablets or oral suspensions, nasal ointment eye drops or eye ointment applicated in the nose/eye for the last 3 months?

☐ Yes | ☐ No

Fit Futures 2

Questionnaire

Selected questions from questionnaire about atopic eczema.

In the past 12 months, have you used cortisone on your skin?

☐ No

☐ Yes, less than 1 month

☐ Yes, 1 to 6 months

☐ Yes, more than 6 months

Have you had an itchy rash at any time in the past 12 months?

☐ Yes | ☐ No

Has this itchy rash at any time affected the skin on the face?

☐ Yes | ☐ No

Has this itchy rash at any time affected the skin on the neck?

☐ Yes | ☐ No

Has this itchy rash at any time affected the skin in areas around the joints of the hands or feet?

☐ Yes | ☐ No

Has this itchy rash at any time affected the skin folds on the elbow or behind the knees?

☐ Yes | ☐ No

In the past 12 months, have you had eczema?

☐ Yes | ☐ No

Have you had dry skin in the past 12 months?

☐ Yes | ☐ No

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Questionnaire continued

Selected questions from questionnaire about atopic eczema.

For how long have you had eczema the past 12 months?

☐ Less than 1 month

☐ 1 to 3 months

☐ 4 to 6 months

☐ More than 6 months

Have a doctor ever said that you have children's eczema or atopic eczema?

☐ Yes

☐ No

☐ Don't know

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