Doctoral theses at NTNU, 2022:360

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Epidemiological studies of bloodstream infections, sepsis and COVID-19

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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Department of Circulation and Medical Imaging

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Trondheim, November 2022

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To my husband Torstein and my children Sindre and Mirja.

To my parents Anne Lise and Åge and my sister Trude.

I could not have done this without you!

"Mankind is endowed with very effective mechanisms of natural resistance that are responsible for our survival as species during countless millenia in the past".

- John J. Bullen

Norsk sammendrag

EN STUDIE AV JERN OG KJØNN SOM RISIKOFAKTORER FOR BLODFORGIFTNING, SEPSIS OG COVID-19

Alvorlige infeksjonssykdommer har alltid vært en trussel mot liv og helse. På verdensbasis rammer sepsis nesten 50 millioner mennesker hver år, og 11 millioner mennesker dør. Opp gjennom menneskehetens historie har pandemier og epidemier satt sitt preg på menneskeheten, og når vi ser på menneskets genom har infeksjonssykdommer, forårsaka av både tidligere og nåværenede patogener, satt tydelige spor i våre gener. De siste 2 årene har COVID-19, satt sitt preg på hverdagen over hele jordkloden. Mange har blitt smitta, mange har hatt behov for medisinsk behandling, også intensivmedisinsk behandling, og dessverre har mange mennesker mista livet sitt under denne pandemien. På mange måter har COVID-19 vist for verden hva sepsis er, og hvor resurskrevende det er å få en sepsispasient levende gjennom et intensivforløp.

Mi forskning starta i møtet med sepsispasienter. Sepsis er når et patogen, f.eks bakterier eller virus, påvirker immunforsvaret vårt slik at en dysregulert immunrespons oppstår. Hos ca 30% av pasienter med sepsis kan man påvise bakterier i blodet, dette kalles en blodbaneinfeksjon (BSI). Forekomsten av BSI er rapportert til mellom 174 og 215 per 100000 personår med en dødelighet på 23.5 til 37.8 per 100000 personår avhengig av populasjonen som er studert. Forskningsgruppa jeg er en del av har sett på ulike risikofaktorer for BSI, og denne avhandlinga handler om hvordan jernstatus og kjønn påvirker framtidig risiko for BSI. For jernstatus har jeg også sett på risiko for sepsis og COVID-19. I studiene som er grunnlaget for avhandlinga har vi brukt ulike epidemiologiske metoder for studere sammenhengene mellom jerrnstatus og risiko for alvorlig infeksjoner, og sammenhengen mellom kjønn og risiko for BSI.

Jern er det vanligste metallet på jorda og 5% av jordas overflate er jern. Jern er relatert til opprinnelsen av liv og er et viktig sporstoff i mange cellulære prosesser, også i immunforsvaret vårt. Jernmetabolismen i kroppen vår er regulert for å sikre nok tilgang til vev som trenger, mesteparten brukes til å danne røde blodceller. Samtidig har kroppen mekanismer for å sikre at nivået av fritt jern er lavt, både fordi fritt jern er svært reaktivt, men også for at patogener (som også er avhengige av jern for sin virulens) ikke skal ha lett tilgang til jern i kroppen vår. Kontroll av jern er en del av immunforsvaret vårt, og jernstatus endrer seg betraktelig i forbindelse med en infeksjon eller som følge av inflammasjon.

Jernmangel er den vanligste mangelsykdommen i verden, mest vanlig blant kvinner og barn i utviklingsland, men jernmangel er også vanlig i vestlige land med en forekomst på 10-15% av befolkninga. I den andre enden finner vi også ulike tilstander med for høyt jern, enten hemokromatose som er en arvelig genetisk akkumulering av jern, eller som følge av jernsupplement eller hyppige blodtransfusjoner.

Utgangspunktet for studie 1 var forskningsspørsmålene om jernstatus påvirker framtidig risiko for BSI og risikoen for å dø av en BSI. I studie 1, undersøkte vi jernstatus (serumjern, transferrin metning (TSAT) og total

jernbindingskapasitet (TIBC)) målt i Helseundersøkelsen i Nord-Trøndelag (HUNT2) og sammenhengen med framtidig risiko for BSI og død innen 30 dager etter en BSI-episode i en prospektiv cohort studie. Studiepopulasjonen var stor med ~61000 deltakere og oppfølgingstida var nesten 17 år. Vi brukte overlevelsesanalyser hvor vi justerte analysene for en rekke potensielle confoundere. Lav jernstatus var forbundet med økt risiko for framtidig BSI, og en tendens til økt risiko for død etter BSI. I tillegg så vi på kumulativ insidens av BSI hvor vi fant økt forekomst av BSI hos personer med lav jernstatus, men også tendens til høyere forekomst hos personer med høy jernstatus.

Det vil alltid være en mulighet for gjenværende confounding og/eller revers kausalitet i observasjonsstudier og vi ønska å undersøke videre sammenhengen mellom jernstatus og risiko for alvorlige infeksjoner. Mendelsk randomisering (MR) er en metode som bruker genetiske varianter som instrument for eskponering og utfallet som studeres. Siden våre gener er tilfeldig fordelt ved unfangelsen, vil de ikke være gjenstand for confounding og revers kausalitet. I studie 3, undersøkte vi sammenhengen mellom jernstatus og risiko for sepsis og alvorlig COVID-19 i en MR studie. Vi brukte genetiske instrument som er assosiert med jernstatus i en stor genom-vid assosiasjonsstudie (GWAS) som utganspunkt for å vurdere om en persons genetisk-predikerte jernstatus også viser seg å gi økt genetisk-predikert risiko for sepsis og alvorlig COVID-19. Vi fant i denne studien at personer med høyere genetisk-predikert jernstatus har høyere risiko for sepsis, og en tendens til at høyere serum jern er relatert til å bli innlagt på sykehus med COVID-19. I denne studien utnytta vi muligheten for å se etter kjønnsforskjeller fordi vi hadde tilgang til kjønnseparate data fra GWAS for jernstatus og GWAS for COVID-19. Vi så at kvinner med genetisk-predikert høyere serum jern har høyere risiko for å bli innlagt på sykehus med COVID-19 sammenligna med kvinner med normalt serum jern. Vi hadde ikke funn forenlig med å konkludere med en kjønnsforskjell.

Det å være kvinne eller mann er en fundamental biologisk variabel som utgjør store forskjeller i kvinners og menns sykdomspanorama. Kjønnsforskjeller i epidemiologi, patogenese, symptombilde og diagnostikk er vist for flere sykdommer blant annet; hjerte-/karsykdommer, kreft, kronisk obstruktiv lungesykdom, altzheimer demens, diabetes, nyresvikt, leversykdom, depresjon og autoimmune sykdommer. Når det gjelder infeksjonssykdommer er det velkjent at mange patogener har ulik forekomst og intensitet mellom kjønnene. Dette har vært tilegna både biologiske forskjeller som kjønnshormoner, kjønnskromosomer og anatomi, men også forskjeller i adferd og tilgang til helsetjenester. Likevel er anbefalt behandling av BSI og sepsis lik for kvinner og menn. Over lang tid har medisinsk forskning, både eksperimentell og klinisk, vært gjort på mannlige celler, hanndyr og på menn. På denne måten er mye av vår kunnskap om sykdommer og om behandling basert på mannlig fysiologi.

Tidligere studier har vist at det er kjønnsforskjeller i risiko for å få sepsis og å dø av sepsis. Studiene har ofte vært utført i små pasientkohorter, gjerne intensivkohorter på enkeltsykehus, og resultatene er sprikende med noen studier hvor kvinner har økt risiko, mens andre viser at menn har økt risiko. På befolkningsnivå er kjønnsforskjellen ulik når man studerer sepsisforekomst i utviklingsland eller industrialiserte land. Det er få

studier som har studert kjønnsforskjeller ved BSI, men befolkningsstudier fra Canada, Finland og USA har vist økt risiko for BSI blant menn.

Dette var utgangspunkt for studie 2 hvor forskningsspørsmålene var om det er kjønnsforskjell i forekomst og dødelighet av BSI? Om menn og kvinner får BSI som følge av ulike bakterier? Og om kjønnsforskjellen kan tilskrives ulikhet i helseadferd, utdanningsnivå eller kroniske sykdommer? Studie 2 er en prospektiv cohort studie som følger HUNT2 deltakere i nesten 17 år. Vi finner at menn har høyere risiko for å få BSI, menn har en to-doblet risiko for å bli ramma av BSI som følge av *Staphylococcus (S.) aureus*, og menn har høyere risiko for å dø som følge av en BSI. Vi undersøkte om ulike faktorer målt ved inklusjon i HUNT2 påvirker sammenhengen mellom kjønn og risiko for BSI. I denne analysen finner vi at helseadferd (røyking og alkoholforbruk), utdanningsnivå, risikofaktorer for hjertekarsykdom (systolisk blodtrykk, non-HDL kolesterol, body mass index (BMI), og komorbiditeter (hjertesykdom, kronisk lungesykdom, diabetes, nyresvikt) utgjør omtrengt 30% av den økte risikoen for BSI som vi ser hos menn.

Oppsummert har studiene som er inkludert i denne avhandlinga funnet sammenheng mellom jernstatus og risiko for BSI og sepsis og en tendens til økt risiko for å bli innlagt på sykehus med COVID-19. De to studiene som så på jernstatus hadde sprikende resultater, hvor observasjonsstudien viste at lavt jern var en risikofaktor for BSI, mens MR studien viser at høyere genetisk-predikert jernstatus gir økt risiko for sepsis. Vi viser også at menn har høyere risiko for BSI gjennom overlevelsesanalyser og medieringsanalyser, og kun ca 30% av menns økte risiko for BSI skyldes helseadferd og komorbiditeter. Men har en betydelig høyere risiko for BSI forårsaka av *S. aureus,* og høyere risiko for å dø av BSI.

"It became clear that the innate immune system itself is what makes microbes poisonous, for its attemt to combat infection, the host may harm its own tissues and undermine its own survival"

- Bruce Beutler

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Table of Contents

Sepsis is a word of Greek deviation mening “biological decay” and described in ancient Greek and Roman litteratur. Physicians from Hippocrates and Galen via Semmelweis, Lister and Armauer Hansen, to health care workers of to day around the world, have sougth for ways to prevent and cure this malady[1]

List of Papers

This thesis contributes with increased knowledge about risk factors for BSI, sepsis and Covid-19. The studies performed in this thesis uses different epidemiological methods to assess and explore the associations studied. **Paper 1** present the association of iron status with the risk of BSI and **paper 2** explored sex differences in incidence and mortality of BSI in HUNT2. **Paper 3** uses a Mendelian randomization approach to further assess iron status and risk of sepsis, using UK Biobank, and risk of Covid-19 using HGI. The three studies studies resulted in the following papers:

Paper 1

Association of iron status with the risk of bloodstream infections: results from the prospective population-based HUNT Study in Norway

Randi Marie Mohus, Julie Paulsen, Lise Gustad, Åsa Askim, Arne Mehl, Andrew T. DeWan, Jan Egil Afset, Bjørn Olav Åsvold, Erik Solligård, Jan Kristian Damås

Intensive Care Medicine. 2018; 44:1276-1283.

Paper 2

Explaining sex differences in risk of bloodstream infections: mediation analysis in the population-based HUNT Study in Norway

Randi Marie Mohus, Lise T. Gustad, Anne-Sofie Furberg, Martine Kjølberg Moen, Kristin Vardheim Liyanarachi, Åsa Askim, Signe E. Åsberg, Andrew T. DeWan, Tormod Rogne, Gunnar Skov Simonsen, Tom Ivar Lund Nilsen, Bjørn Olav Åsvold, Jan Kristian Damås, Erik Solligård

Scientific Reports. 2022; 12:8436

Paper 3

Iron status and the risk of sepsis and severe COVID-19: A two-sample Mendelian randomization study

Randi Marie Mohus, Helene Flatby, Kristin V. Liyanarachi, Andrew T. DeWan, Erik Solligård, Jan Kristian Damås, Bjørn Olav Åsvold, Lise T. Gustad, Tormod Rogne

Submitted

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The work for this thesis was carried out during a part-time employment as a PhD student at the Department of Circulation and Medical Imaging in Gemini Center for Sepsis Research. Financial support was granted from The Liaison Committee for Education, Research and Innovation in Central Norway. I want to express my gratitude to Nord-Trøndelag Hospital Trust, the HUNT Research Center and to the many participants that have contributed with health data to this research.

I started my PhD together with Åsa Askim and Julie Paulsen looking into risk factors for BSI. We used data from the Norwegian HUNT Study linked with hospital-confirmed clinically relevant BSI registered in The Nord-Trøndelag Hospital Trust (HNT HF) Sepsis Registry to assess risk factors for BSI and BSI-related death. I also worked with Arne Mehl who founded the HNT HF Sepsis Registry as a quality register, and with Lise T. Gustad who has contributed as a post doc in our group with substantial knowledge in epidemiology and academic writing. Kristin Vardheim Liyanarachi and Marianne Stenbekk Thorkildsen joined our group focusing on risk factors and the trajectories of BSI and sepsis. I have also worked in projects on risk factors for sepsis and BSI together with Signe Åsberg and Martine Kjølberg Moen.

During the fall of 2020, in the midst of the COVID-19 pandemic, I brought my family for a research stay at Yale School of Public Health. Supervised by Professor Andy DeWan, I worked with genetic epidemiology and audited his lectures in “Concepts in Genetic Epidemiology”. For the genetical part of my work I have used summary-level data from UK Biobank and the COVID-19 Host Genetics Initiative (HGI) and worked closely with Andy DeWan, Tormod Rogne and Helene Flatby.

I have also had invaluable help from my supervisors; Professor Erik Solligård, Professor Jan Kristian Damås and Professor Bjørn Olav Åsvold. I have received supervision and advice on methodological considerations, help with statistical programs (Stata, SPSS, R and PLINK), and academic writing from Lise T. Gustad, Professor Tom Ivar Lund Nilsen, Signe Åsberg, Professor Andrew T. DeWan, Associate professor Tormod Rogne and my fellow PhD students Julie Paulsen, Åsa Askim, Helene Flatby, Kristin Vardheim Liyanarachi and Marianne Stenbekk Thorkildsen.

During these years, I have started projects that would not lead to any publications, like reading through patient records of approximately 400 cases of *E. coli* BSI to the HNT HF registry, and performing an X chromosome wide association study on risk of severe COVID-19. For the *E. coli* project, I had to realize that the work was too time consuming to finish in a PhD schedule. For the X-chromosome wide association study I met hurdles on my way, although this work was inspiring with lessons learned and valuable knowledge to put into future projects.

I started my residency in anesthesiology at Telemark Hospital in Skien in 2008, guided and inspired by all my colleagues, especially Kristin Hauss, Harry Achterberg and Torkjell Nøstdahl. My residency continued at St. Olavs hospital in 2012. I started the work with this thesis in 2015 and most of the time I have worked part-time as a clinician. I would like to thank all my colleagues at Clinic of Anesthesia and Intensive care and Clinic of

Cardiothoracic Surgery at St. Olavs hospital, my superiors Sigurd Fasting, Guri Greiff and Øystein Karlsen, and a special thank you to my collegial friends, Stein Dragsund, Hilde Flesseberg, Signe Palludan, Guro M. Krüger and Annamaria Forsmark for support and positive attitude, but also for reminding me that the best stories come from inside the hospital where excellent patient management takes place.

"Every day is a beautiful day to save people's lives"

- Grey's Anatomy

Finally, my deepest gratitude to my family; my parents Åge and Anne Lise and my sister Trude, for always believing in me, for safe upbringing and education, for making me wonder and search for answers, for support, faith and encorage to persue my goals. To my in-law, Rannveig, for advises, collaboration and fruitfull discussions, and my father in-law, Roar, for invaluable days and nights caring for our children, for countless professional discussions and advises, and for outdoor adventures. To my lovely husband, Torstein, who have been supportive and loving throughout these years, encoraging me to persue a PhD. The countless hours of invaluable help with computers, networks, wi-fi, VPN, (all computer problems where I have been in need of an orakel), rehersals of lectures and academic writing. To my beloved children, Sindre and Mirja, who admist the pandemic came with Torstein and me on an adventure to the US and New Haven, making new friends in a new school despite hours of remote schooling, and who always remind me what is the most important in life.

"Men are from Mars, women are from Venus. Ironically, both planets contain more iron than Earth".

- Torstein Sakshaug

Abreviations

ARDS: Acute respiratory distress syndrome

APC: Antigen presenting cell

B cells: B lymphocytes

BMI: Body mass index

BSI: Bloodstream infection

CI: Confidence intervals

COVID-19 HGI: COVID-19 Host Genetics Initiative

CVD: Cardiovascular disease

DAG: Directed acyclic graph

eGFR: Estimated glomerular filtration rate

GRASP: Genome-Wide Repository of Associations Between SNPs and Phenotypes

GWAS: Genome wide association study

HDL: High density lipoprotein

HNT HF Sepsis Registry: The Nord-Trøndelag Hospital Trust Sepsis Registry

HR: Hazard ratio

HUNT: The Trøndelag Health Surveys

ICD: International classification of disease

ICU: Intensive care unit

IOW: Inverse odds weighting

IVW: Inverse variance weighted

LD: Linkage disequilibrium

MR: Mendelian randomization

qSOFA: quick Sequential Organ Failure Assesment

REK-midt: Regional Committee for Medical and Health Ethics of Central Norway

SIRS: systemic inflammatory response syndrome

SNP: Single nucleotide polymorphism

SOFA: Sequential Organ Failure Assesment

T cells: T lymphocytes

TIBC: Total iron binding capacity

TSAT: Transferrin saturation percentage

X-CHR: X chromosome

Y-CHR: Y chromosome

Introduction

The many faces of sepsis have intrigued me since I began working as a medical doctor, but I also found them a challenge and cause for concern. Sepsis is the dysregulated host response to infections leading to organ failure and premature death. Even now—in the era of antibiotics and high-tech intensive care units (ICUs)—sepsis remains a life-threatening disease that can kill a healthy person like me in matter of hours. Bloodstream infections (BSIs) are a major global burden and may lead to sepsis. An estimated 48.9 million incident cases of sepsis, and 11 million sepsis-related deaths were recorded worldwide in 2017[2]. In response to an urgent need to impose better preventive measures and treatment for severe infections, the last 20 years have seen an increased effort to identify risk factors. This thesis uses a large, population-based cohort study (the HUNT2) linked with prospectively recorded information on blood-culture positive BSIs to assess risk factors including, iron status and sex, for first-time BSI and BSI mortality. The thesis further uses different epidemiological methods to explore confounding and mediating factors of the associations and uses of genetic epidemiology concepts to examine the causal relationship between iron status and risk of sepsis and COVID-19.

This thesis contributes to increased knowledge about risk factors for severe infections, in particular BSIs, sepsis and COVID-19. Additionally, the thesis shows the importance of population-based longitudinal health surveys, including well maintained health registries, and the possibility to link data to investigate potential risk factors using an array of epidemiological methods. In this thesis we present epidemiological studies investigating the association between iron status and risk of severe infections. We explore this association using survival analyses, cumulative incidence and Mendelian randomization (MR). Finally, we investigate sex differences in risk of BSI using survival analyses, cumulative incidence and mediation analyses.

The period in which I worked on this thesis coincided with the COVID-19 pandemic, which fortunately all my loved ones survived. In the beginning, however, we were afraid—of both the knowns and unknowns. This fear was compounded by my knowledge about severe sepsis, and the urgency and intensive care resources these patients often need to survive. COVID-19 has shown the world what an infection without effective therapy might look like. With the development of vaccines effective against a severe course of COVID-19, restrictions were lifted; nevertheless, more than two years after the pandemic began, for individuals who develop severe COVID-19, we are left with organ supportive intensive care treatment. For some patient groups, steroids[3] and tocilizumab[4] have shown promising results, although the studies are small and the cohorts studied may not always be directly comparable with the patients we encounter in our ICU. These are questions and challenges faced by every health care worker: namely, how best to translate the results of research articles and case reports into quality patient care.

MY SEPSIS STORY

Sepsis is a condition in which one's body overreacts to an invading pathogen, leading to organ failure and death. The infection causing sepsis could arise from bacteria, virus, fungi, or parasites. BSIs are infectious diseases

defined by the presence of viable bacteria or other pathogens in the bloodstream that lead to an inflammatory response with alterations of clinical, hemodynamic, and laboratory parameters. Sepsis and BSI are overlapping phenomena since sepsis is an infectious syndrome triggered by an infection, and a BSI can proceed to sepsis, following or concomitant with a localized infectious disease (e.g., pneumonia, urinary tract infection, or endocarditis)[5].

COVID-19 gave sepsis a face

In early 2020, reports about a new virus, SARS-CoV2, began spreading around the world. The public was suddenly faced with news about overwhelmed hospitals and ICUs with severely ill patients, including a worryingly high death toll. Images of ICUs, with their respirators, pumps, and monitors, became pervasive as country after country shut down to reduce the spread of the virus. In the hospital, we prepared for numerous patients in need of intensive care treatment, and COVID-19 reminded us that severe infections are a daily threat to our existence. At the time of this writing, the pandemic has been raging around the globe for over two years, with more than 500 million people infected and 6.3 million deaths (worldometer May 2022); the suffering, despair around treatment and prevention, and hope and efforts to develop treatments and vaccines have been part our everyday life.

Stories of sepsis, showing diversity in age and sex

I can reference many sepsis-related examples: a woman in her 30s, feeling sick while preparing Christmas dinner for her husband and children, and hours later fighting for her life in our ICU with fulminant septic shock. A young man who was in a car accident but survived his multiple injuries and the numerous operations, about to convalescence, but dying from multi-organ failure caused by sepsis several weeks after his accident. An old woman treated for her medical disease in the hospital, then reporting with fever and disorientation, ultimately diagnosed with sepsis caused by indwelling bacteria from her intravenous catheter. A newborn who felt hot, breathed rapidly, and barely roused by his mother's stimuli. With the arrival of COVID-19, I can add the image of a middle-aged man with chronic medical illnesses, now barely breathing on his own, and finally transferred to the ICU to start mechanical ventilation. We who work with ICU patients know that his life is threatened, but that his loved ones will not be able to visit during these times, to give him one last moment of love and hope.

It was in these encounters that my research started. The stories of sepsis are diverse, indicating that this is not one disease, but multiple immune responses leading to the body's self-destruction, which characterizes sepsis. Compared to other medical disorders, like cardiovascular diseases and cancers where new therapeutic strategies have evolved and tremendously changed outcomes, there is no magic bullet for sepsis. Despite numerous efforts to develop new treatments, trials fail to show an effect on sepsis outcomes. In the latest Surviving Sepsis Campaign (from October 2021) the guidelines include early recognition, obtaining blood cultures, and administering broad-spectrum antibiotics within one hour for septic shock and within three hours for sepsis without evidence of shock. Further, they recommend that hospitals apply a performance improvement program for sepsis, including sepsis screening for high-risk patients[6].

For sepsis patients, we are thus left with **identification** of patients at risk, **prevention**, **early recognition**, and **detection**. Clinicians, patients, and their relatives can only hope that highly specialized treatment proves effective and that we continue improving management of septic patients in the future.

Origin of life—iron, sex, and infections

In my PhD work, I have explored iron status related to the risk of BSI, sepsis, and COVID-19. Iron is the second-most abundant metal on earth, comprising about 5% of the earth's crust. To understand the importance of iron as a trace element, we must look back 4,000 million years, when biological precursor molecules began to form as iron-sulfur clusters in alkaline hydrothermal vents on the seafloor[7-10]. The high iron availability on the very early Earth was likely a key component in the synthesis of biological precursor molecules[7]. Iron and sulfur are part of both enzymes and co-enzymes in metabolic cycles like the Krebs cycle, Calvin cycle, and electron transport system. The energy released from redox reactions of iron sulfides became available for the synthesis of organic molecules, leading to iron dependency in (almost) all living organisms[7,11].

2,000 million years later, sexual reproduction first appeared in early eucaryotic life[12]; multi-cellular life dates back 1,500 million years, and *homo sapiens* 300,000 years (Figure 1). Organisms need to efficiently and reliably replicate their genetic material—the need to repair genetic damage is one of the leading theories explaining the origin of sexual reproduction. However, sexual reproduction does not come without cost[12] and evolution has left us with important differences between the sexes.

Infections have always been a daily threat to human existence, and patterns in our immune system have evolved under this threat. We share common features in our immune functions with invertebrates that date back 650 million years[13]. The risk of acquiring a BSI and sepsis depends on bacterial virulence, host characteristics, and biological factors[14-20]. The global epidemiology of BSIs and sepsis is difficult to assess, since studies have been conducted with different methodologies and among different populations. Large, population-based studies are lacking, and geographical differences are substantial in both incidence and outcomes of BSI and sepsis[2].

FIGURE 1: Life timeline

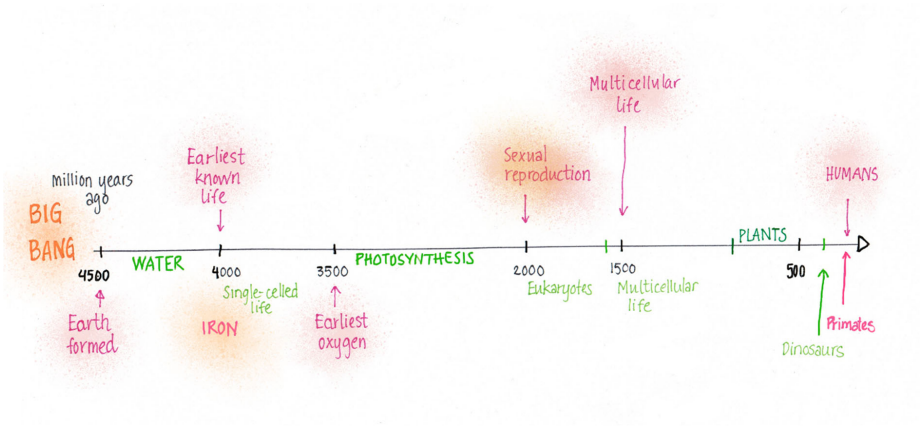


Figure1:
Timeline of life on earth, beginning 4,500 million years ago. The iron-sulfur vents evolved 4,000 million years ago with the earliest form of life. Oxygen in the atmosphere is dated back 3,500 million years. Sexual reproduction evolved 2,000 million years ago.

BURDEN OF BSI AND SEPSIS

A BSI occurs when a pathogen invades the bloodstream. These infections may be classified as primary when no focus of infection is evident or may arise secondary to a specific focus of infection. BSIs are often classified according to the site of acquisition—hospital-acquired, health-care-associated, or community-acquired—the epidemiology of which differs[21]. Even in the era of antibiotics and modern ICUs, a diagnosis of a BSI is a serious condition with overall case fatality rates of 15 to 20%[22-25]. Although BSIs and sepsis are often linked, the two terms refer to different concepts. A BSI, like any other infection, can lead to the dysregulated host response seen in sepsis, but sepsis is not the inevitable result of a BSI. Moreover, not all cases of sepsis are caused by a BSI, as reflected by the number of positive blood cultures from septic patients ranging from 15 to 30%[26-29].

The Global Burden of Disease Study estimated that sepsis affects up to 50 million people every year and at least 11 million die from it, meaning that 20% of all deaths worldwide are related to sepsis[2]. Sepsis is the most common cause of hospital-related death (in high-income countries), and the health care costs are immense[30]. Sepsis remains a major cause of health loss globally, and the recent COVID-19 pandemic is not included in these numbers. COVID-19 has shown the world what is at stake when we cannot control the invading pathogens

and the dysregulated host response to an infection: an immense global death toll, and untold survivors suffering from long-term COVID-19-related health issues.

The definition of sepsis has changed many times over the past three decades. In 1991, sepsis was defined as a systemic response to proven or suspected infection, defined by the presence of two or more systemic inflammatory response syndrome (SIRS) criteria[31]. The SIRS criteria were revised in 2001, to include several variables relating to the inflammatory, hemodynamic, general clinical, and organ response to the infection. *Severe sepsis* was defined as sepsis-induced organ dysfunction, and *septic shock* was defined as hypotension despite adequate fluid resuscitation[32]. In 2016 the Third International Consensus Definitions for Sepsis and Septic Shock was established (Sepsis-3) and sepsis was defined as "a life-threatening organ dysfunction caused by a dysregulated host response to infection"[33]. Organ dysfunction was defined as a score of two or higher on the Sequential Organ Failure Assessment (SOFA). This scoring system assesses the response to infection in several organ systems, including the respiratory, hematology, cardiovascular, central nervous system, and renal systems[34]. For Sepsis-3 quickSOFA (qSOFA) was introduced as a screening tool (two or more organ failure symptoms of either hypotension, altered mental status and/or tachypnea). In the Sepsis-3 classification, the term "severe sepsis" was removed, and septic shock was defined as a need for vasopressors to maintain a mean arterial blood pressure of >65 mmHg and serum lactate levels of >2 mmol/L despite adequate fluid resuscitation[33]. A recent update on the management of sepsis and septic shock was published in 2021, the classification follows that of Sepsis-3, but they recommend against using qSOFA as a single screening tool[6]. The poor performance of qSOFA was also the conclusion in a study of emergency department patients at St. Olavs Hospital from our study group[35].

The burden of BSIs is quite difficult to assess due to the heterogeneity of the populations studied. Many studies reply on hospital data, despite the knowledge that population-based studies are optimal for defining the epidemiology of infectious diseases, including BSIs[36]. A population-based design ensures that all cases of disease occurring in residents of a defined geographical area are identified, minimizing risk of selection bias. Population-based studies provide data about all residents within a specified geographical area, and when the population at risk is known, incidence rates can be determined and further compared to other regions. In their review, Goto et al. found a BSI incidence rate of 174 to 204/100.000 (person-years) and a mortality rate of 23.5 to 27.5/100.000 in North America; the corresponding incidence rate in Europe was 166 to 189/100.000 and the mortality rate 21.6 to 37.8/100.000[37]. The same year, Laupland et al. reported in a review of population-based studies of BSIs, an overall average rate of 140-160/100.000 in high income countries[38]. In a recent review of community-onset BSI in Canada, Laupland et al. found an overall annual incidence rate of 147.1/100.000[20], Kontula et al. found and average annual BSI incidence of 216/100.000 in Finland[25], and Buetti et al. reported an incidence rate of 240/100.000 in Switzerland in 2014[39]. In the Finnish study they reported 1 month fatality rate of 13%, and the case-fatality rate was higher among men in all age groups[25]. Importantly, BSI risk varies by age and sex, with the youngest and oldest most at risk, and a progressively increasing risk after the age of 70—the older age group also shows a predominance among males[22,25,40].

In Norway, few studies have investigated the epidemiology of BSI and sepsis. Mehl et al. used The Nord-Trøndelag Hospital Trust Sepsis Registry (HNT HF) data from 2002 to 2013 at Levanger Hospital, and found an overall BSI incidence of 215/100.000, and an exponential increase with age—particularly in men. BSI mortality was 32/100.000. They also showed that during the study period BSI incidence increased and the case fatality rate decreased[22]. A recent study by Knoop et al. aimed to investigate sepsis epidemiology in Norway. They performed a retrospective study using data from the Norwegian Patient Registry and Statistics Norway and showed an annual incidence of hospitalized sepsis of 140/100.000. Hospital mortality for sepsis admissions was 19.4%, and sepsis-related deaths constituted 12.9% of all hospital fatalities[41].

RISK FACTORS FOR BSI, SEPSIS, AND COVID-19

Knowledge about risk factors for severe infections is important for identifying at-risk groups and guiding preventive measures. Chronic medical disorders such as chronic lung disease, chronic kidney disease, myocardial infarction, coronary artery disease, peripheral artery disease, stroke, deep vein thrombosis, hypertension, atrial fibrillation, cancer (including cancer treatment), dyslipidemia, diabetes, and BMI have been identified as risk factors for BSI, sepsis, and/or COVID-19[15,18-20,42,43]. Psychiatric disorders such as anxiety and depression, health behaviors (e.g., smoking, alcohol use, and level of physical activity) and thyroid function have been identified as risk factors for BSI[15-17]. Social demographics (e.g., poverty, education level, and income) have also been linked to risk of severe infections[19]. Other important factors are the ability to mount an adequate immune response to invading pathogens[14], and factors accompanying the invading pathogen that contribute to vulnerability and severity; the latter includes the virulence and antimicrobial susceptibility of infecting microbes[44].

In this landscape of risk factors, I have looked into *iron status* and *sex*. As iron is essential for both immune function and microbial growth, alterations in iron status could influence the risk of infections. Iron is tightly bound to transferrin to control the balance between the host's need for iron in cellular metabolism and restricting invading bacteria from obtaining iron[45]. Disturbances in iron homeostasis might influence the risk of BSI and sepsis. Iron deficiency is the most common nutritional disorder affecting more than a quarter of the world's population and a leading risk factor for disability and death worldwide, with children and women most at risk[46]. Iron deficiency is more prevalent in low-income countries, but this nutritional deficiency is also prevalent in industrialized countries, affecting about 10 to 20% of the population[47-49]. On the other hand, iron overload—as seen in hemochromatosis and in patients in need of repetitive blood transfusions—also poses infection risk[50]. The modifiable properties and wide variation in iron status in both the healthy population and in individuals with pathologies makes iron status a research priority[51].

There are indications of sex differences in the incidence and mortality rates of severe infections, but previous studies have reported conflicting results[2,52-55]. Many studies tend to adjust for sex in their analyses without focusing on mechanisms between sex and infection risk[56]. Knowledge about these differences and mechanisms is important for clinicians when treating patients, for policy makers and public health leaders when they impose

prevention strategies, and for researchers analyzing results and planning future research trials. Large, population-based studies are needed to investigate sex differences in the epidemiology of severe infections, and further to elucidate how sex influences important immune functions.

Host genetic variation has considerable influence on an individual's susceptibility. Infectious pathogens are arguably among the strongest selective forces acting on human populations[57]. In the history of human existence, pathogens that diminish reproductive potential drive selection for genetic variants that affect resistance. This natural selection varies in timing, strength, and the direction and shape of patterns within the human genome[58]. An important feature of most human infections is that only a proportion of exposed individuals develop clinical disease. This is a challenge when performing epidemiological research in infectious diseases. Even if an individual holds the risk factor(s) or the genetic variant(s) investigated, they would have to be exposed to and infected by the pathogen before they could develop disease.

A BRIEF OVERVIEW OF THE IMMUNE SYSTEM

Our immune system provides protection from a wide range of pathogens and is a collection of different cells, molecules, and tissues[59]. It is designed to carry out rapid, specific, and protective responses against harmful pathogens or their biologic products. In other words, the aim of our immune functions is to resolve infections, and to recognize and reject tumors. On the other hand, immune functions may lead to autoimmunity and allergies, and tolerance or rejection of transplanted tissues or organs. The immune system must be capable of doing three things: 1) recognizing a diverse array of pathogens, 2) killing those pathogens once recognized, and 3) sparing host tissues[60]. When encountering a pathogen, the immune system responds to and eliminates the pathogen via pro-inflammatory mechanisms, before returning to homeostasis through anti-inflammatory mechanisms. Immune responses are both amplified and suppressed by different mediators and within this process some infections become more severe, leading to sepsis. We often subdivide the overall system into the innate and the adaptive immune systems, recognizing that there is an interplay between these[60].

Innate immune system

The innate immune system is the evolutionary, ancient part of our immune system and is shared across species[61]. It is constantly active and is costly, even in healthy individuals[62]. It launches an immediate and rapid response against invading pathogens. Here, the discrimination between self and non-self is of utmost importance and constitutes the basis of the innate immune system. Another hallmark of the innate immune response is swelling, the recruitment and activation of leucocytes, and a deconstruction and then remodeling of affected tissues. Chemotactic mediators are released along with cytokines and other small molecules. These cause vasodilation and vascular adhesion, enabling phagocytes to kill pathogens and infected cells. The complement system is often referred within the innate immune system but serves merely as a bridge between the innate and adaptive responses and can be activated in different pathways, including tissue injuries, microbes, toxins, and when one's blood is exposed to foreign materials[63].

Adaptive immune system

The adaptive immune system consists mainly of B lymphocytes (B cells), T lymphocytes (T cells), antigen-precenting cells (APCs), and the antibodies and cytokines they produce[60]. B and T cells dictate the specificity and orchestrate the responses of the adaptive immune system[59]. An important advantage of the adaptive immune system is that it expands quickly in response to infections, and there is a low maintenance cost in its resting state. While the innate immune response is instantaneous, the adaptive immune response takes days or even weeks to develop maximum efficacy[64].

THE IMMUNE RESPONSE IN SEPSIS

When encountering a pathogen, the immune system responds and eliminates the pathogen via pro-inflammatory mechanisms, before returning to homeostasis through anti-inflammatory mechanisms. Immune responses are both amplified and suppressed with different mediators, and within this process some infections become more severe either because the pathogen is not cleared, or a dysregulated host-response may develop, leading to sepsis. In sepsis, there is an enduring and simultaneous pro-inflammatory and anti-inflammatory state driven by a dysfunctional, innate, and suppressed adaptive immune response. We face an overwhelming immune response with the activation of the innate immune system, the release of inflammatory mediators, and further activation of the complement system, coagulation system, and vascular endothelium; this leads to a disruption of the endothelial barrier, referred to as a “cytokine storm”. The result of what should have been a normal response to infection can result in tissue injury, microvascular thrombosis, disruption of endothelial barrier function, organ dysfunction, and ultimately death[65].

The cytokine storm seen in sepsis arises from elevated levels of circulating cytokines and immune-cell hyperactivation. Infections are the most common cause of the cytokine storm, but it can also be triggered by certain therapies, cancers, autoimmune conditions, graft-versus-host disease following stem-cell transplantation, and monogenic disorders. No single definition of the cytokine storm is widely accepted, and to distinguish between elevated levels of cytokines with or without pathogens is difficult, as certain cytokines can be helpful when controlling an infection, but harmful to the host when there is no infection. Fever is a clinical hallmark of the cytokine storm, however[66]. The development of septic shock with organ failure is a consequence of disseminated infections with excessive inflammation, hypoxia, cell death, coagulopathies, and eventually multi-organ failure. The patients’ condition may rapidly progress with disseminated intravascular coagulation, hypoxemia, vasodilatory shock, and death. Many patients have respiratory symptoms, need ventilatory support, and eventually develop acute respiratory distress syndrome (ARDS). Other organs may be affected by renal failure, liver failure, bone marrow suppression, and cardiomyopathy.

Nevertheless, the exact mechanisms behind sepsis remain unanswered. A complex, interconnected network of cell types, signaling pathways, and cytokines are involved. The specific immune cells and which cytokines they

secrete are not fully understood. Treatment directed at specific cytokines and hemofiltration to remove cytokines from circulation have failed in clinical trials[67,68].

In addition to factors within the hosts' immune system, virulence factors associated with the invading pathogen are also at play when an infection turn into sepsis. Within the immune system, virulence factors differ from pathogen to pathogen, activating different cascades. These variations in microbial characteristics necessitate different responses from the immune system. Certain bacteria (e.g., *Staphylococcus (S.) aureus*) can produce superantigens, which leads to the hyperactivation of T-cells[69]. Shiga toxins associated with some *Escherichia (E.) coli* strains are responsible for a cytokine-derived hemolytic-uremic syndrome[70]. Moreover, Some viruses can trigger the cytokine storm, such as SARS-CoV2, influenza viruses, and herpes viruses[66].

Following the pro-inflammatory response in sepsis, we encounter a compensatory anti-inflammatory response. Adding to this, sepsis patients often experience an immune suppression characterized by lymphocyte exhaustion and a reprogramming of APC, and lymphocytes and dendritic cells also die by apoptosis in septic patients. This phase leaves sepsis patients immunocompromised and at increased risk of secondary infections. Patients who survive the acute phases of sepsis but have prolonged ICU treatment often die from secondary infections.

IRON METABOLISM AND IRON IN THE IMMUNE SYSTEM

Iron is essential for life

Iron is one of the most abundant elements in the earth's crust, but the oxidized Fe^{3+} (ferric) form is insoluble and therefore poorly absorbed by plants and animals. Iron metabolism in vertebrates is mainly based on recycling and conserving iron[71]. Iron is essential in almost every biochemical process dating back to the origins of life—including cellular respiration (electron transfer reaction in the mitochondria), synthesis of oxygen-carrying molecules, gene regulation (nucleic acid synthesis and repair), regulation of cell growth, and differentiation—and works as a co-factor in many enzymatic reactions. It is also important in red blood cell formation, immune function, fetal development, and physical and mental well-being[72]. Iron homeostasis is tightly regulated on both cellular and systemic levels.

The main physiological processes involved in iron metabolism are iron sensing and storage, absorption from the gut, iron recycling, erythropoiesis, bleeding/menstruation, and inflammation. Iron metabolism is orchestrated by the liver hormone hepcidin, which shifts iron into storage when it encounters increased levels and reduces iron absorption. This results in iron restriction. Reduced levels of hepcidin will normalize iron absorption and iron release from storage. As inflammation is a strong signal to increase hepcidin levels, inflammation leads to iron restriction and is thought to reduce the iron available for invading pathogens. This iron restriction will eventually also affect erythropoiesis and lead to "anemia of inflammation"[73]. Iron-regulating mechanisms have been demonstrated to protect us from infection by denying iron to invading pathogens because most microbes depend on iron for their pathogenicity, leaving iron central in a constant battle between the host and the invaders[45].

Iron metabolism

Adequate iron levels must meet the needs of different tissues, but excess iron causes cellular damage; the maintenance of iron homeostasis is therefore essential. A simplistic overview of iron metabolism is presented in Figure 2. Iron metabolism is a closed system because there is no regulated mechanism for iron excretion through the liver or kidneys. Iron losses occur through bleeding and sloughing of mucosa and skin cells[74]. The iron content of the body is maintained mainly by recycling iron from senescent erythrocytes and by regulating the absorption of iron via the enterocytes in the duodenum[75]. The adult human body contains approximately 3 to 5 g of iron and more than 70% of body iron is present as heme. Within cells, iron is bound to *ferritin*, the major iron storage protein. Ferritin possesses a large cavity that can accumulate great amounts of iron, necessary to protect the cells from Fenton reactions (the process of iron oxidation and mineralization). Ferritin is characterized as having a structure with a compact and mineral iron core in which the iron is protected, maintained in solution, and biologically available[76].

In healthy humans, the plasma concentration of iron is stable, and iron is stored in hepatocytes and in splenic and hepatic macrophages at constant levels. In plasma, iron is bound to transferrin (normally 20 to 40% saturated), and serves in a buffering capacity to avoid free iron. Plasma contains only 0.1% of the iron content in the body. Erythropoiesis happens in bone marrow erythroblasts and requires approximately 20 to 30 mg of iron per day. During erythroid cell differentiation in the formation of hemoglobin, the iron demand increases; this process is sensitive to decreasing plasma iron concentrations[77].

Four iron biomarkers are used for the clinical assessment of iron status: **serum iron**, **total iron binding capacity (TIBC)**, **transferrin saturation (TSAT)**, and **ferritin**. Serum iron is tightly bound to transferrin (normal range in adults is 9–34 µmol/L). TIBC is a measure of how many of the transferrin binding sites are bound to iron, meaning that high TIBC is a sign of iron depletion whereas low TIBC is a sign of iron overload (normal range is 49–83 µmol/L). TSAT is derived from [serum iron]/[TIBC]%, measures the availability of iron for erythropoiesis, and is low (<20%) in iron deficiency and high during iron overload (>50%). Ferritin correlates well with body iron stores but is heavily affected by inflammation (normal range in adult men is 20–300 µg/L, and in women 15–200 µg/L)[46,78].

Figure 2

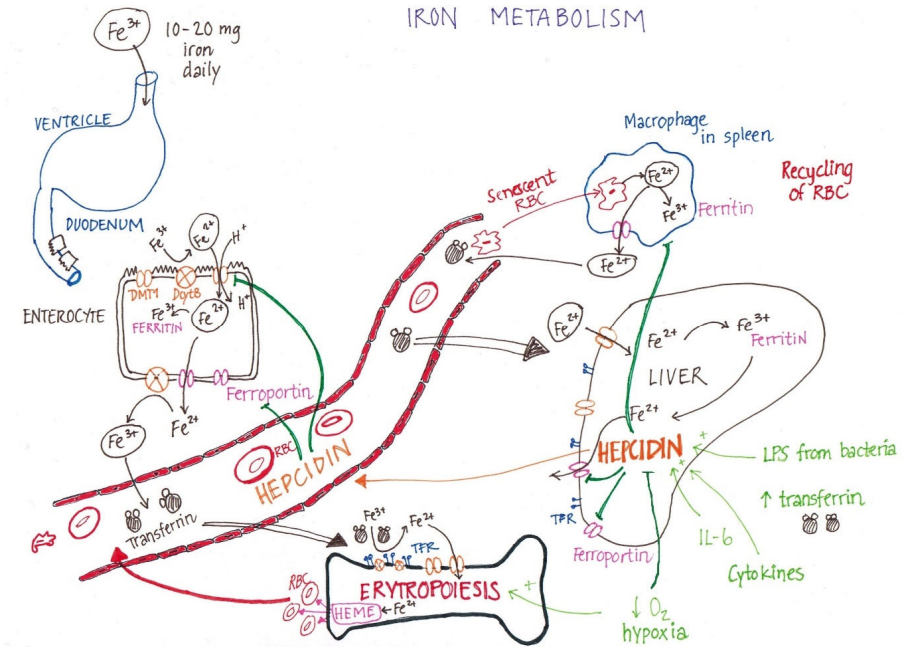


Figure 2: A simplistic overview of iron metabolism.

From the food, the daily intake of iron is approximately 10 to 20 mg (mostly Fe^{3+}). At the apical membrane of enterocytes, the divalent metal transporter 1 (DMT1) takes up iron from duodenum after reduction from Fe^{3+} to Fe^{2+} by duodenal cytochrome B (DcytB). In the enterocyte, small amounts of iron can be stored in ferritin, but most will be transported through ferroportin at the basolateral membrane and oxidized before being taken up by transferrin. Most iron in plasma is bound to transferrin, which delivers iron to all cells, binding it to transferrin receptors (TFRs). The TFRs then undergo endocytosis. Iron is bound within heme proteins in red blood cells (RBCs) as Fe^{2+}. RBC iron is recycled by macrophages via engulfing of senescent RBCs and the release of iron through ferroportin or storage in ferritin.

Hepatocytes sense transferrin saturation and iron stores, and release hepcidin accordingly. Other stimuli for hepcidin release are, cytokines, and lipopolysaccarides (LPS) from the cell membrane in some bacteria (indicated with light green arrows). Hepcidin reduces the uptake of iron in enterocytes and transportation through ferroportin, and induces storage of iron in intracellular ferritin molecules (indicated with dark green lines). Hypoxia suppresses hepcidin via erythroferrone and facilitates erythropoiesis.

Illustration drawn and digitalized by R. M. Mohus in Adobe Photoshop and Illustrator.

Iron status pathologies

Iron deficiency, defined as the decrease of total iron content in the body, is the most common nutritional deficiency worldwide, affecting four to six billion people—with women and children in low-income countries most at risk[46,79]. Iron deficiency is the main cause of anemia, which affects 1.25 billion people, and is 1 of the 5 leading causes of global disease burden[46,49]. Reducing the prevalence of iron deficiency anemia is one of the WHO's six

priorities[80]. The etiology of iron deficiency includes inadequate dietary intake, impaired absorption, increased losses, and increased requirements during growth and pregnancy. Iron deficiency may lead to anemia, but is also related to reduced immune functions, altered thermoregulation, and alteration in energy metabolism and exercise and work performance[72]. Iron deficiency is attributed to cognitive dysfunction[81], which has been shown to improve by iron supplementation[82]. In children, iron deficiency may cause delayed mental and physical development, and during the perinatal period, anemic women and their infants are at greater risk of dying[46,78]. Anemia of inflammation is a functional anemia associated with diseases that involve immune activation and the release of pro-inflammatory cytokines and is considered to be the second most common reason for anemia in the world after iron deficiency anemia[73].

Iron overload is a result of dietary or therapeutic intake, genetic causes, or a combination of the two. A genetic hepcidin deficiency leads to hemochromatosis, a group of iron-overload disorders, in which different mutations have been identified. Hemochromatosis is characterized by low and even undetectable hepcidin levels, iron overload, and iron deposition in the liver, heart, and endocrine organs[83]. Some anemias are associated with iron overload that develops even in the absence of a blood transfusion (e.g., thalassemia)[84]. Iron overload is also seen in end-stage kidney failure as a result of unbalanced iron supplementation and blood transfusions, although iron deficiency due to anemia of inflammation, reduced iron intake, or iron loss (from occult bleeding), remains an issue in these patients[85].

Iron status and the immune response

As a critical part of cellular activity, iron plays an important role in the fight for survival between hosts and pathogens. Both display a vast array of mechanisms to control iron acquisition and utilization[86]. Hosts have developed mechanisms to make iron less available for their invaders, leading to the characteristic hypoferremia of infection which develops within hours of systemic infection[86]. It has been assumed that the iron deficiency seen during infection has a role in the host defense against extracellular microorganisms, and that this response provides an increased capacity to bind iron that is released during tissue destruction at sites of infection[45].

Iron is a central regulator of immune cell proliferation and function. Studies have shown that iron deficiency affects numerous immune cells and processes, including 1) neutrophils with reduced function and decreased myeloperoxidase activity, 2) decreased and defective T cells and reduced lymphoproliferation, 3) impaired IL-2 production by lymphocytes, 4) decreased cytokine production and natural killer-cell activity, and 5) reduced production of macrophages' migration inhibition factor[87-91]. Iron deficiency may increase the risk of infection since iron is required for normal immune function, including in the bactericidal activity of macrophages where iron is a critical component of peroxide- and nitrous oxide-generating cellular enzymes called the "oxidative burst"[72]. This latter is the production of reactive oxygen species to damage invading pathogens within macrophages and neutrophils.

On the other hand, patients with iron overload, as seen in hemochromatosis, show increased susceptibility to some infections[50,92,93]. In hemochromatosis, the high levels of TSAT compromise bacteriostatic properties and

pathogens can much more easily procure iron from transferrin. Iron overload compromises the bactericidal capacity of phagocytic cells and decreases chemotactic responses[94]. Iron supplementation has also been associated with acute exacerbations of infections—in particular, malaria: Sazawal et al. had to discontinue their intervention study in Tanzania before completion because of a substantial increase in mortality and hospitalization among the children who were given iron and folic acid supplement[95]. This has led to the recommendation that iron supplementation in malaria-endemic regions should only be performed with strict malaria control measures[87,96,97]. Iron supplementation and risk of infection has been long noted, already in 1872, Trousseau reported that patients recovering from active tuberculosis had a relapse when given iron-rich supplements and he warned against their use[98]. In 1977, Barry and Reeve reported increased incidence of *E. coli* sepsis in newborn given intramuscular iron dextran[99].

This U-shaped risk profile, where both low iron status and high iron status is linked to increased infection risk is illustrated in Figure 3 and have been elucidated in previous work[100-102]. The optimum level of host iron status might be different from pathogen to pathogen[100], and depending on other medical conditions[73]. As iron deficiency is common and efforts to increase iron status in populations is a high priority, timing and control of the correction is of utmost importance.

Figure 3

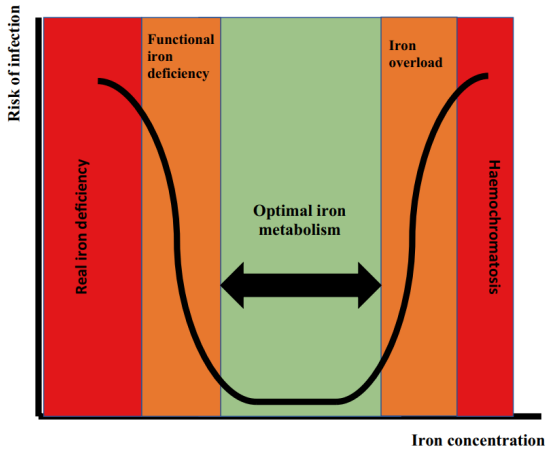


Figure 3:
The U-shaped risk between iron status (iron concentration) and risk of infection. The red color represent high susceptilblitity, orange represent moderate, and green represent low susceptibility to infections related to iron status. Functional iron deficiency represent iron restriction due to inflammation. Iron overload includes; red blood cell transfusion and iron supplementation.

Illustration used with permission from Springer Nature; Swenson, E.R., Porcher, R. & Piagnerelli, M. Iron deficiency and infection: another pathway to explore in critically ill patients?. *Intensive Care Med* **44,** 2260–2262 (2018).

Low iron status is found in up to 40% of critically ill patients[103]. Another study found that ferritin and hepcidin levels were elevated in intensive care patients, and especially in septic patients, serum iron and transferrin levels

were decreased. Interestingly, the same study found that higher levels of serum iron were associated with mortality[104]. This is an expected physiological reaction when one experiences inflammation and/or infection that reflects a functional iron deficiency. While it may be protective in the short term by restricting iron to invading pathogens, it can become harmful for ICU patients in the long term, as low iron status has been linked to critical illness and cognitive, neuromuscular, and cardiopulmonary dysfunctions[102]. For survivors of critical illness, anemia is common and linked to a functional iron deficiency due to high levels of hepcidin[105]. It is associated with poor quality of life[106], and often challenging to diagnose (i.e., commonly used iron biomarkers will be confounded by inflammation), and due to the high hepcidin concentrations patients are unlikely to absorb oral iron supplements[105]. The reduced iron availability during and in the convalescent period of critical illness is not only linked to anemia, but many challenges faced by ICU survivors (e.g., cognitive, neuromuscular, cardiopulmonary dysfunction and recurrent infections)[107].

Concerning COVID-19, there are indications of an association between the virus and iron status[108]. Severe COVID-19 has been related to hyperferritinemic syndrome, which is associated with hyperinflammation[109]. Studies have linked excess serum iron, TSAT, and lower TIBC (i.e., an indication of iron overload) and hyperferritinemia to be associated with a more severe course of COVID-19[110,111]. Another study showed lower-serum iron and TSAT levels in patients with COVID-19 compared to non-COVID-19 patients, independent of severity, while COVID-19 patients defined as severe and critical had substantially higher ferritin levels[112]. On the other hand, an observational study found evidence of a more severe course in patients with iron deficiency measured at hospitalization[113], also seen in a study from Austria where low iron status was associated with need of in-hospital management[114] and low serum iron was linked to severe hypoxemia in a small study from the United Kingdom[115]; moreover, a study using genetic variants to assess nutritional status in European populations linked low iron status to higher mortality from COVID-19[116].

SEX DIFFERENCES IN THE EPIDEMIOLOGY OF SEVERE INFECTIONS AND IN IMMUNE FUNCTIONS

Sex differences between men and women have been studied in a variety of fields. Here, two important terms need to be defined: *sex* and *gender*. *Sex* refers to one's biological sex as determined by sex chromosomes, where males have one X chromosome (X-CHR) and one Y chromosome (Y-CHR), while females have two X-CHRs. *Gender* refers to the socially constructed norms that affect and determine roles, relationships, and positional power for all people across their lifetime. Gender and sex interact, but lately gender has evolved from being a binary term to including intersex identities[117].

Sex affects susceptibility to and the severity and mortality of infections, and varies depending on pathogen, geographical location, and socioeconomic factors[118]. During our work on risk factors, we observed a substantial sex difference and had to adjust our analyses to account for these differences. This is often the case in the biological sciences: we compare exposed versus unexposed, treated versus untreated, and susceptible versus

resistant, but often neglect or forget the most fundamental difference in biology—*male versus female*. Historically, most knowledge about diseases originated from studies done on male cells, male mice, and men[119]. This has led to medical research and care that is largely centered on male physiology. Is this problematic? If we look at infectious diseases, in most vertebrates both the prevalence and the severity of infection is typically higher in males[120]. Sex-specific infection and mortality rates have been reviewed by Giefing-Kröll et al.[118].

Epidemiological studies indicate a male predominance in sepsis, but studies on sex differences in the incidence and mortality of sepsis have given conflicting results[2,52,54,55,121-123]. Most studies on sex differences in BSI and sepsis have been conducted among small, intensive care cohorts, and there are limited population-based studies. Few studies have investigated the influence of sex in the incidence of BSIs[20,25,40,124]. Nevertheless, all of these studies report higher burden of BSI in men[20,25,40,124], higher risk of BSI caused by gram-positive bacteria in men and higher risk of *E. coli* BSI in women[25,124], and higher BSI-related mortality in men[25]. Uslan et al. identified no overall difference in mortality by sex[124].

Sex differences in the immune system

Men and women differ in the intensity, prevalence, and pathogenesis of infections caused by viruses, bacteria, parasites, and fungi as well as the strength of immune responses[125]. Esper et al., looking at sex and race disparities in sepsis in the United States, found that sex differences in sepsis varied according to the source of infection, where gram-positive bacteria were more common in men, as were respiratory sources of infection. They also found that men had a 25% higher risk of sepsis than women[52]. Nevertheless, the Global Burden of Disease study found higher sepsis incidence in women, with pregnancy-related infections contributing to this sex bias. In the same study, the authors found that men were more likely to die from sepsis[2].

In this work, I have focused on sex differences in BSIs. The underlying reasons for the observed sex differences in infectious disease incidence and severity have not been fully addressed, but are likely due to sex hormones, genetic make-up, anatomy, behavioral and lifestyle differences, as well as socioeconomic differences. The sex hormones (testosterone, estrogen, and progesterone) have different levels in men and women, especially during the years between puberty and reproductive senescence. Immune responses are costly in terms of energy, nutrients, and immunopathologies. The investment in immunity is considered a trade-off between other important functions and activities[126,127]. Another theory is that the benefits and costs of immunity depend not only on the host genotype, but also on the presence and phenotype of the pathogens we encounter frequently. Pathogens have been shown to play a significant role in the evolvement and selection of immune defenses[126,128].

Several differences exist between men and women in both innate and adaptive immune responses. These include variations in cellular number and activity, where women have higher phagocytic activity in macrophages and neutrophils and more efficient APCs, while men have higher natural killer-cell activity and higher levels of pro-inflammatory cytokines[129]. In adaptive immune responses, women are predominantly driven by B cells and CD4+ T-helper 2 cells, in contrast to men whose adaptive immune responses are driven by CD8+ T cells and CD4+ T-helper 1 cells[130].

Anatomical differences between the sexes obviously also contribute to differences in susceptibility. Anatomy affects the exposure and transmission of pathogens: for example, the anatomy of the female genital tract influences the transmission of certain infections more efficiently in females. Characteristics of the genital mucosa are also different, and altered by sex hormones[131]. Behavioral differences between the sexes also influence the types of pathogens to which we are exposed, and the time of exposure. In addition, behavioral differences vary substantially across different geographical areas and cultures. In many countries, more women than men have been exposed to the SARS-CoV2, as more women work as front-line health care providers[132,133]; men, by contrast, have had more opportunities to work from home[134]. Women also tend to care for sick family members, increasing their exposure to pathogens, while men who work outside the house typically bring the pathogens from outside and infect their families. Behavioral differences also include prioritizing health needs, accessing health care, and participating in health care promotion programs—all areas where boys and men surpass girls and women around the world[132-135]. Food intake and food composition also affect immune function, and in many areas of the world, inadequate nutrition is more common in women[136,137]. Poverty is a major gender bias in access to health care in any society and should be considered an indirect female immunomodulatory factor; moreover, in developing countries, women are confronted by a myriad of socio-cultural factors which negatively influence physical well-being and access to appropriate health care services[138,139]. As such, behavioral differences likely affect susceptibility to and outcome of infectious diseases differently between the sexes, depending on the population being studied.

Sex hormones

At the turn of the 19th century, Calzoari proposed a link between sex hormones and the immune system when he recognized changes in the thymus after castration[140]. Sex hormones include androgens, estrogens, and progestogens, of which the most important human derivates are testosterone, estradiol, and progesterone, respectively. Testosterone is the most important androgen and is considered the “male sex hormone”. Estradiol and progesterone are considered “female sex hormones”, although all sex hormones are present in both sexes (at different levels). Sex hormones exert powerful modulatory effects at all levels of the innate and adaptive immune systems. Testosterone and progesterone are generally considered to be immunosuppressive while estrogen tends to be immunoenhancing. Sex hormones influence the functioning of host immune cells by binding to specific receptors that are expressed in most immune cells, including lymphocytes, macrophages, and dendritic cells. The sex hormones influence signaling pathways associated with the production of cytokines and chemokines[120].

Testosterone has a dampening effect on immune responsiveness and many innate immune cells. It also enhances cell-mediated immunity via CD8+ T cell activation and induces important pro-inflammatory cytokines[141]. The overall effect of estrogen depends on the concentration (physiological vs. supraphysiological (i.e., pregnancy) associated levels) cell type, and the relevant receptor signaling pathways[142]. Further complexity arises across the estrous cycle, with serum estrogen peaking during the ovulatory phase and serum

progesterone peaking during the luteal phase. Physiological levels of estradiol enhance pro-inflammatory capacity, whereas supraphysiological estradiol levels during pregnancy suppress the pro-inflammatory effects[143]. Estrogen has stimulating effects on humoral immunity, and dampening effects on cell-mediated immunity[144]. Progesterone is known to downregulate many immune functions, mainly to ensure maternal tolerance of the fetus[145]. Altogether, female sex hormones are associated with enhanced adaptive immune functions, with an upregulation of B cells, activated macrophages, dendritic cells, eosinophils, and basophils[143]. The hypothesized sex-dependent effects on immune functions are illustrated in Figure 4, as a U-shaped relationship between the intensity of the immune response and the host damage.

Figure 4

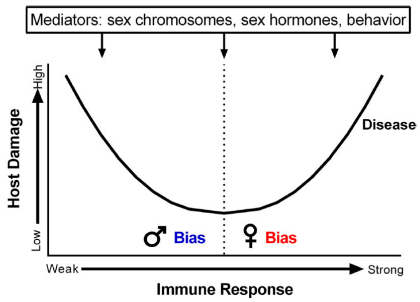


Figure 4:
The hypothesized U-shaped relationship between the host damage and the immune response. When an immune response is weak and not sufficient to clear the pathogen, the damage caused by the pathogen is high. If the immune response is strong, it could cause tissue damage. Several factors related to sex may contribute to male and female bias: for example, sex hormones, sex chromosomes, and behavior might contribute to bias in the outcome of infections.

Sex chromosomes

Sex chromosomes have intrigued researchers for a long time. Their special mode of inheritance and atypical patterns of evolution have shaped their present structure and are at the root of differences between the sexes. Many genes on the X-CHR regulate immune functions and could explain partly why women have heightened immune responses compared to men. In females one X-CHR is subject to inactivation at random in the individual cells a process called X-inactivation to control the quantity of gene product. Females are thus composed of a mosaic of cells, with genes from either the maternal or paternal X-CHR, providing greater diversity also regarding immune functions[146]. About 10% of X-CHR genes escape X-CHR inactivation and heterogeneity in X-CHR inactivation patters are likely to contribute to differences in immune responses between women and men at both cellular and molecular levels[147]. Little is known about the immune modulating properties of genes on the Y-CHR,

but recent discoveries have found some evidence of immune and inflammatory responses in men are related to Y-CHR genes[148].

GENETIC CONTRIBUTION TO THE RISK OF INFECTIOUS DISEASES

As noted earlier, infectious pathogens are among the strongest selective forces acting on human populations[57]. As humans migrated throughout the world, populations encountered distinct pathogens and natural selection increased the prevalence of alleles that were advantageous in the new ecosystems, in both host and pathogens. Host genetics thus strongly influence an individual's vulnerability to infectious disease. Pathogens that diminish reproductive success, either through death or poor health, drive selection for genetic variants that affect *resistance*. *Natural selection* leaves distinctive signatures in the genome. Genetic variants that improve reproduction and survival will increase in frequency (positive selection), while detrimental genetic variants eventually vanish (negative selection)[58]. Balancing selection favors diversity. Population events that alter genetic diversity include *bottlenecks*, *expansions*, *splits*, and *admixture*. This natural selection influences human infectious disease susceptibility and human microbiome homeostasis, but at the same time contributes to common diseases that show geographical disparities (e.g., autoimmune and metabolic diseases). A classic example is the effect of genetic red-blood-cell disorders on malaria susceptibility[149].

When we consider evolutionary mechanisms that infer risk of infectious diseases, we must keep in mind that our pathogens also adapt to natural selection. The outcome of exposure to an infectious agent reflects the interaction between both human and pathogen genotypes. This might drive the co-evolution of the host and pathogen, and adaptation of pathogens to host polymorphisms in specific human populations may partly explain the geographical distribution of pathogen strains[150]. A study of genetic variation in 50 populations around the world revealed that pathogens are primary drivers of local adaptations[58]. The Great pandemics of history have inflicted to a large extent mortality and are of particular interest to evolutionary genetics[57], as illustrated in Figure 5. A characteristic feature of many human infections is that only a proportion of exposed individuals develop a clinical disease, which renders genetic studies very challenging. Another challenge of genetic research is the need for large population samples, so that studies have enough power to detect significant differences, and that systematic ancestry differences—*population stratification*—will potentially lead to biased results if not accounted for[151,152].

Figure 5

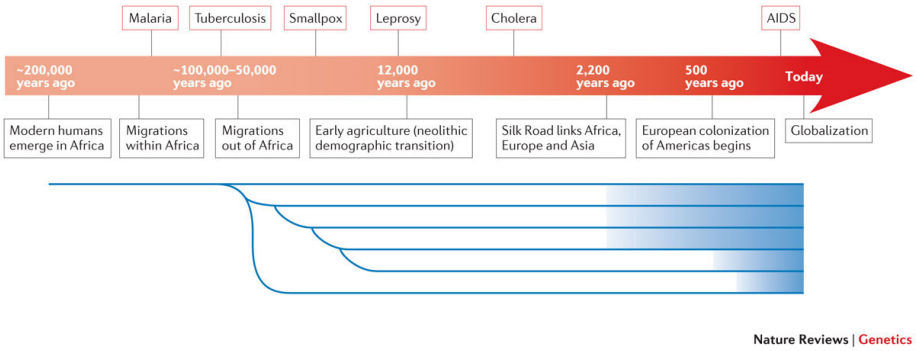


Figure 5:
Key events in recent human evolution (boxes outlined in black) are juxtaposed with the estimated ages of infectious disease emergence (boxes outlined in red). The fragmentation of the human lineage into genetically and geographically distinct populations (blue lines) accelerates with migration out of Africa. Later, these populations started mixing more (blue shaded regions between the populations) along trade routes (such as the Silk Road), through colonization and through high rates of global travel nowadays.

Genome-wide-association study

Genome-wide-association study (GWAS) aim to identify susceptibility to different diseases or traits. The method takes advantage of regions in the human genome where differences are common. GWAS identify many genetic variants in correlation with the phenotype of interest, and the genetic polymorphisms (e.g., single nucleotide polymorphisms (SNPs)) identified often have small correlations with the phenotype. The GWAS method relies on the phenomenon that variation across the genome is clustered because we inherit segments of chromosomes together—this results in polymorphisms being identified in one location if highly correlated with nearby genetic variation[153]. For GWAS population stratification might introduce confounding in different settings; 1) if the genetic risk of disease depend on the ethnic background of the individual, 2) if the genetic variants under investigation are differently frequent depending on ethnic subgroups, and 3) if cases and controls in the study are heterogeneous with regard to ancestry[151]. To adjust for potential population stratification we use GWASs from same ancestry (for our study European ancestry) and the GWAS have been adjusted for principal components which is the standard method for estimating and addressing population structure and sample ancestry in genetic datasets[151].

When GWAS reveal locations susceptible to novel infectious diseases, considerable work is needed to identify the causative alleles, their functional consequences, and the biological mechanisms by which they influence

disease pathogenesis[154]. A major challenge is to develop strategies for translating insights from the genetic basis of an infectious disease into improved patient outcomes. The use of genetic information to predict infectious disease risk in individuals is unlikely to alter clinical practice in the near future. Clinical translation is more likely to result from characterizing molecular pathways involved in disease, and identifying novel targets for immunomodulatory drugs or vaccines[155]. However, as the numbers of GWAS have increased—and, importantly, their results are provided as publicly available, summary-level data—the use of GWAS data to assess causal relationships has evolved substantially.

Mendelian randomization study

This is an increasingly important tool for studying causal relations in observational epidemiology. Using GWAS with robust associations between genetic variants and exposure of interest, the use of MR studies has evolved[156]. MR exploits the principle that genotypes are not generally susceptible to reverse causation and confounding bias, as they are inherited randomly at conception. This method is subject to important assumptions: 1) that the genotype is associated with the exposure, 2) that the genotype is associated with the outcome through the studied exposure only, and 3) that the genotype is independent of other factors which affect the outcome. When performing MR, it is important to control for potential threats to the validity of these assumptions, such as population stratification, linkage disequilibrium (i.e., the non-random association of alleles at different loci in a given population)[153], pleiotropic effects (i.e., the genotype's effects on multiple biological pathways), or the genotype's association with confounders[157]. It is important to assess the association between the genotype(s) used and the exposure of interest to avoid weak instrument bias[158]. To support causal inferences from MR studies, thorough analytical methods are needed, as these can assess potential violations of the assumptions. Many MR studies include multiple genetic variants, which can be used in sensitivity analyses[159].

As the aim of most observational studies is to inform preventive measures and/or interventions to improve public health, reliable research is essential. In some situations, it is difficult, unethical, and impossible to perform randomized control studies, and MR approaches have been developed with this context in mind. MR uses genetic instruments that are fixed across the lifespan to estimate lifetime effects of the exposure[160]. The rapid evolvement of analytical methods and the increase in GWAS have made it more complicated to interpret MR results. The assumptions required may be impossible to validate[161], the relationships studied may not fit into the statistical methods at hand (i.e., non-linear relationships)[162], or the outcomes studies may be vulnerable to "survivor bias" (i.e., survival to study inclusion)[163].

With the paucity of observational studies and inconsistency regarding iron status' role in severe infections, we wanted to explore the relationship between genetically predicted iron status and risk of sepsis and severe COVID-19 using a two-sample MR method. A novel GWAS study of the iron-status biomarkers among 246,139 participants of European descent was used to identify the genetic instruments[164]. For the outcomes of interest, for sepsis, we used summary-level data obtained from UK Biobank, and for COVID-19, we obtained summary-

level data from the COVID-19 host genetics initiative (HGI). As we also wanted to include sex-disaggregated analyses to explore potential sex differences, we used sex-stratified data on COVID-19 from UK Biobank.

Aim of the studies

OVERALL AIM OF THE PROJECT

The overall aim of this project was to identify potential modifiable risk factors for BSI, sepsis, and COVID-19, and to characterize sex differences in risk and outcome of BSI.

SPECIFIC AIMS OF THE STUDIES

PAPER 1: To assess the association of iron status with risk of first-time BSI and BSI mortality.

PAPER 2: To identify sex differences in incidence and mortality of BSI and examine sex differences in BSI incidence due to the most common causal bacteria. Further, to investigate whether the observed sex differences in incidence of BSI are mediated by known BSI risk factors.

PAPER 3: In the framework of a Mendelian randomization study, to investigate the causal relationship between iron status and risk of sepsis and COVID-19.

Methods

STUDY DESIGN

Table 1: Summary of the three different studies included in this thesis

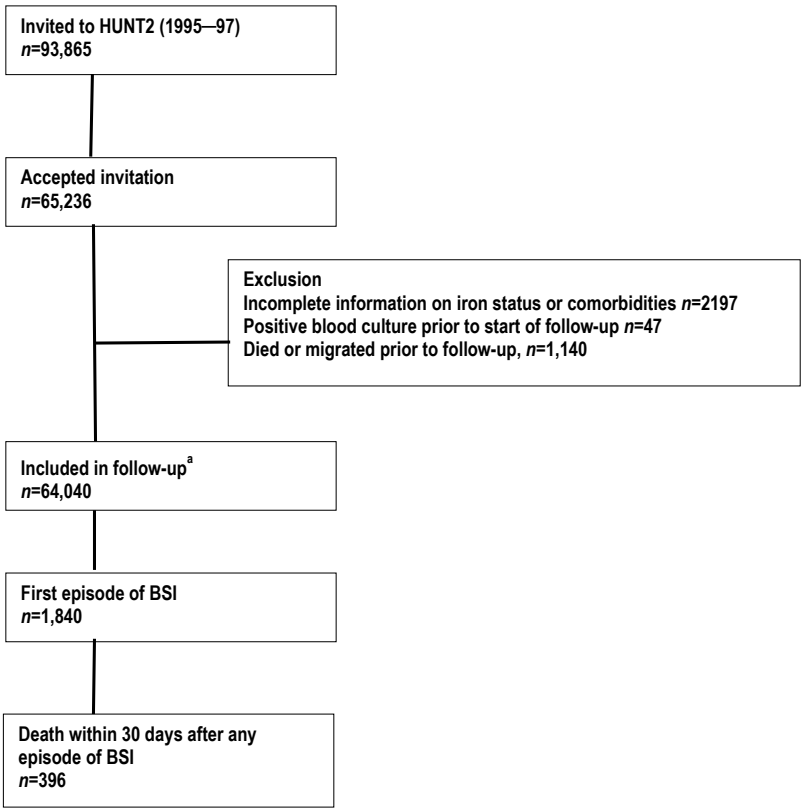
Study	Study design	Study question(s)	Baseline information/ Exposure information	Outcome information
1	Prospective observational study	Is there an association between iron status and risk of first-time BSI and BSI-related mortality?	HUNT2 Cancer Registry of Norway	HNT HF Sepsis Registry
2	Prospective observational study	Are there sex differences in the risk of first-time BSI and BSI-related mortality? Are there sex differences in the causative bacteria? Do known risk factors for BSI mediate the association?	HUNT2	HNT HF Sepsis Registry
3	Two-sample Mendelian randomization	Is there a causal relationship between iron status and risk of sepsis, bacterial infections, viral infections, and Covid-19?	GWAS on iron status	UK Biobank and COVID-19 Host Genetics Initiative

HUNT2: The second survey of the Trøndelag Health Study. HNT HF Sepsis Registry: The Nord-Trøndelag Hospital Trust Sepsis Registry

The studies published in Papers 1 and 2 are prospective population-based cohort studies using participants in the HUNT2 survey, with a follow-up of BSI incidence of almost 17 years. Participants were followed for a first-time BSI identified in the HNT HF Sepsis Registry. BSI-related mortality was defined as all-cause mortality occurring within 30 days after detection of a BSI. For Paper 2, we also included analyses of the three most prevalent bacteria. Paper 1 assessed the association between iron status and risk of first-time BSI and BSI mortality. Paper 2 assessed sex differences in the incidence and mortality of BSI, sex differences in causative bacteria and potential mediators of the association between sex and risk of first-time BSI. Paper 3 reports the results from an MR study investigating the causal relationship between genetically predicted iron status with genetically predicted risk of sepsis and severe COVID-19. The study uses publicly available summary-level data from large GWASs on iron status, sepsis, and COVID-19.

Paper 1: We identified 65,236 HUNT2 participants. Censoring before follow-up was performed for 47 (0.07%) participants that had a positive blood culture, 1,140 (1.8%) migrated or died prior to follow-up and 2,197 (3.4%) had incomplete information on iron status or comorbidities. A total of 61,852 participants were eligible for analyses (Figure 6).

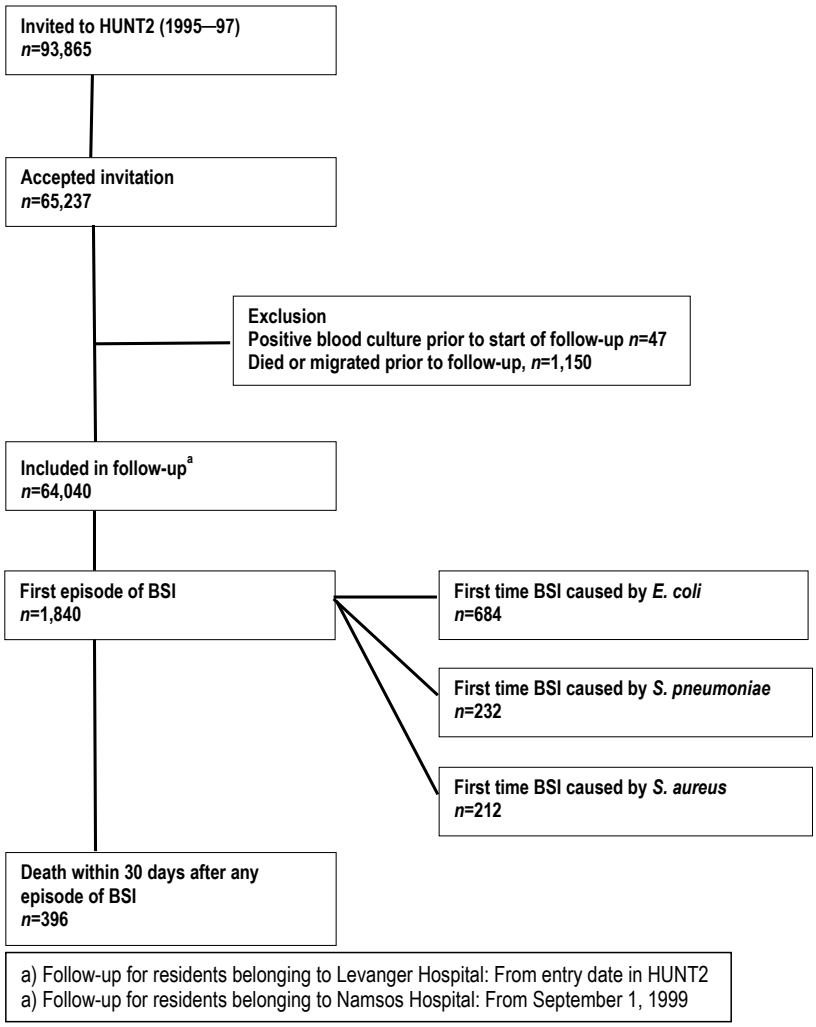
Figure 6: Flow-chart of study recruitment and follow-up Paper 1



a) Follow-up for residents belonging to Levanger Hospital: From participation date in HUNT2
a) Follow-up for residents belonging to Namsos Hospital: From September 1, 1999

Paper 2: We identified 65,237 HUNT2 participants. Censoring before follow-up was performed for 47 (0.07%) participants that had a positive blood culture and 1,150 (1.8%) migrated or died prior to follow-up. A total of 64,040 participants were eligible for analyses (Figure 7).

Figure 7: Flow-chart of study recruitment and follow-up Paper 2



Paper 3: We examined the causal relationship between iron status and risk of severe infections using Mendelian randomization. The genetic instruments for the systemic iron status biomarkers were collected from a recent GWAS of 246,139 participants from Iceland, Denmark, and the United Kingdom. The genetic associations with the outcome of interest were collected from the IEU OpenGWAS with summary-level data of GWAS for sepsis obtained from UK Biobank which included 10,154 sepsis cases and 452,764 controls (https://gwas.mrcieu.ac.uk/). Sepsis was defined as explicit sepsis using the International Classification of Disease (ICD)-9 and ICD-10 codes and the Global Burden of Disease description. Summary genetic associations estimates for hospitalization and risk of severe COVID-19 were obtained from release 5 (18 Jan 2021) from the COVID-19 HGI. This included *hospitalized COVID-19 versus non-hospitalized COVID-19* (4,829 cases and 11,816 controls) and *hospitalized COVID-19 versus population* (9,986 cases and 1,877,672 controls). All participants included in the GWAS were of European descent (Figure 8).

Figure 8: Flow-chart for the two-sample MR study

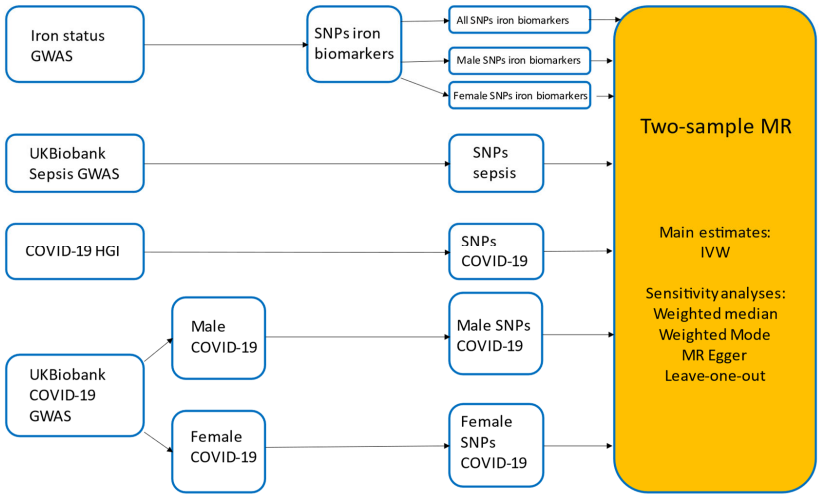


Figure 8:
Left: The included GWASs for Iron status, sepsis and COVID-19. For the COVID-19 outcomes we included two separate COVID-19 GWASs in order to conduct sex-separate MR analyses.
Right: The applied MR methods with sensitivity analyses

GWAS; genome-wide-association study, SNP; single nucleotide polymorphism, HGI; host genetics initiative, IVW; inverse variance weighted

Illustrated by R.M. Mohus in Power Point.

STUDY SETTING

The studies described in this thesis were conducted as part of the Gemini Centre for Sepsis Research, a group consisting of clinicians and researchers from several clinical disciplines, with interest in sepsis epidemiology, pathophysiology, and improved diagnosis and management. The initiative was started by Dr. Arne Mehl (1948–2021) at Levanger Hospital with the registration of clinical data on all patients with BSI as part of a quality improvement effort launched in 1994. Over the years, Dr. Mehl recruited interested colleagues and increased the awareness of sepsis and improved follow-up of patients at Levanger hospital. Namsos Hospital was also included in this work from 1999. The work has resulted in improved prognosis for sepsis patients locally[165] and publications on the epidemiology of BSI and antibiotic resistance[22,166,167]. Linking information from the HNT HF Sepsis Registry to the HUNT studies has revealed insights into risk factors for BSI and sepsis[15-17,168-170], as well as identifications on genetic variants associated with susceptibility[42,171-173].

DESCRIPTION OF THE INCLUDED DATA SOURCES

This thesis is based on data from the Trøndelag Health Study (HUNT), HNT HF Sepsis Registry, the Cancer Registry of Norway, and summary-level GWASs data on iron status, on sepsis from UK Biobank, and on COVID-19 from the COVID-19 HGI. The cohorts in Papers 1 and 2 were recruited from (former) Nord-Trøndelag county where, as in all of Norway, the entire population is provided with healthcare that is free at point of delivery. For Paper 3, we used summary-level data from large GWASs with relevant exposure and outcome data.

The HUNT Study

The HUNT Study is a population-based health study conducted in the Trøndelag region in Norway and consists of four consecutive surveys distributed among the total adult population approximately every 10th year. The second Trøndelag Health Survey (HUNT2 1995–1997) invited all inhabitants ≥20 years old (*n*=93,865) in the Nord-Trøndelag region to participate in a clinical examination that included non-fasting blood sampling, and to complete questionnaires covering a range of health-related topics. Of these, 65,237 (69%) chose to participate. The self-report questionnaires were extensive and distributed in a two-step procedure. Questionnaire 1 (Q1) was delivered by post together with the invitation to participate; participants were to answer Q1 at home and bring it with them to the clinical examination. At the clinical examination, the participants received Questionnaire 2 (Q2) to complete at home and return via a prepaid envelope. Participants signed an informed consent form and accepted linkage of their data to other medical registries. The HUNT Study database is regularly updated with information on date of out-migration and death from the National Registry[174]. The Nord-Trøndelag region in Norway has a population of 130,000, of which approximately 70% is served by Levanger Hospital and 30% is served by Namsos Hospital. The tertiary referral center is St. Olavs Hospital in Trondheim. The population is ethnically homogeneous (97% Caucasian) and stable, with a net out-migration of 0.3% per year[175].

The Nord-Trøndelag Hospital Trust Sepsis Registry

The HNT HF Sepsis Registry has prospectively recorded information on all clinically relevant BSI events at Levanger Hospital from January 1, 1995, and Namsos Hospital was included in the registry from September 1, 1999. The microbiology laboratory at Levanger Hospital exclusively provided all microbiology services in the Nord-Trøndelag region. In addition, all HUNT2 participants with a positive blood culture recorded at St. Olavs Hospital were included in the registry from January 1, 1995 to assure completeness of the study cohort.

The Cancer Registry of Norway

The Cancer Registry of Norway was established in 1951 and has information on all cancer diagnoses on inhabitants in Norway. All medical doctors in the country are instructed by law to notify the registry of new cancer cases. The registry may be linked to other medical registries using Norwegian residents' unique personal identification number (www.kreftregisteret.no).

UK Biobank

UK Biobank is a large-scale biomedical database containing genetic and health information from approximately 500,000 UK participants aged 40 to 69 years old and living in the United Kingdom, out of 9.2 million individuals who were invited to the baseline assessment, indicating a participation rate of 5.5%. Participants were recruited between 2006 and 2010. The database is regularly augmented with additional data and is globally accessible to approved researchers. The participants regularly provide blood, urine, and saliva samples, and information about their lifestyle. This information is linked to their health records (www.ukbiobank.ac.uk)[176]. A non-participation study has been conducted and showed a higher participation rate among women, in older age groups, and in less socioeconomically deprived areas. Participants were also less likely to be obese or smoke, and had lower prevalence of self-reported health conditions. In a follow-up of UK Biobank participants, they show lower all-cause mortality and lower cancer incidence compared to the general population[177].

The COVID-19 Host Genetics Initiative

Early during the COVID-19 pandemic, this international initiative was established to bring together the human genetics community to better generate, share, and analyze genetic data. The aim is to discover and learn the genetic determinants of COVID-19 susceptibility, severity, and outcomes, and with increasing knowledge contribute to improved understanding, prevention, and treatment of SARS-CoV2. Summary-level data are free to download, and as of May 2022, there have been seven consecutive releases of data. For Paper 3, the fifth release (comprised of only those of European descent) was used.

STUDY VARIABLES

Exposure and outcome variables in Papers 1 and 2

The exposure of interest in Paper 1 was iron status, assessed by serum iron, TSAT, and TIBC measured at inclusion in HUNT2. Sex as registered in the National Registry was the exposure of interest in Paper 2. Data on the HUNT2 participants were linked to the HNT HF Sepsis Registry. All HUNT participants consented to linkage between their HUNT information and information from other medical registries. The two main outcomes were first-time BSI and BSI mortality as registered in the HNT HF Sepsis Registry. BSI mortality was defined as all-cause mortality within 30 days after a BSI episode. As secondary outcomes for Paper 2, we assessed first-time BSI caused by the most common bacteria: *E. coli, S. aureus*, and *Streptococcus (S.) pneumonia*. All BSI were clinically relevant and confirmed at the microbiology laboratories in Levanger or at St. Olavs Hospital. Blood cultures solely containing microorganisms associated with skin contamination such as coagulase negative *Staphylococcus* species, *Corynebacterium* species, and *Cutibacterium* (former *Propionibacterium)* species were not considered as BSI[178].

Covariates for Papers 1 and 2

Age as registered in HUNT2 was included as the underlying time-scale for the Cox regressions models in Papers 1 and 2[179], and as the mean of the study population for the cumulative incidence plots performed in Papers 1 and 2. Sex as registered in the National Registry was the exposure of interest in Paper 2 and a covariate in Paper 1, where we stratified the analyses by sex. The HUNT database is regularly updated with information on place of residence, date of death or migration out of the county from the National Registry.

For HUNT2, trained nurses performed the clinical examinations at baseline after a standardized protocol. Weight was measured with light clothing to the nearest 0.5 kg, height measured without shoes to the nearest 1.0 cm. Body mass index (BMI) was calculated as weight (kg) divided by the squared value of height (m^2) and categorized as recommended by the World Health Organization (<18.5, 18.5–24.8, 25–29.9, 30–34.9, 35–39.9, and ≥40 kg/m^2). Systolic blood pressure was measured three times after the participant had been seated for at least two minutes with the cuff placed and cuff size adjusted to the arm circumference. All blood pressure measurements were performed with the Dinamap 845XT (Criticon) based on oscillometry. The average of the second and third measurements was used in the analyses.

Fresh, non-fasting serum samples were analyzed at the Central Laboratory at Levanger Hospital. Iron status was assessed in HUNT2 as a population screening for hereditary hemochromatosis where they estimated a prevalence for hereditary hemochromatosis of 0.75%[180]. Serum iron was measured after a reduction of transferrin with ascorbic acid, complexed with bathophenanthroline and quantitated colorimetrically (Boehringer, Germany). TIBC was calculated from serum transferrin analyzed via an immunoturbidimetric method from DAKO A/S, Denmark. Transferrin saturation percentage (TSAT) was calculated as 100 x (serum iron/2 x TIBC)%. Serum creatinine was analyzed using the Jaffé method (Roche Diagnostics, Germany). Estimated glomerular

filtration rate (eGFR) was estimated from recalibrated creatinine values using the Modification of Diet in Renal Disease formula[181]. Non-HDL cholesterol was calculated as the difference between total and HDL cholesterol. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol were analyzed using enzymatic colorimetric methods (Boehringer Mannheim, Germany).

Comorbidities were based on self-report in questionnaires in HUNT2 and included in Papers 1 and 2; cardiovascular disease (history of myocardial infarction, stroke, and/or angina), lung disease (asthma or chronic obstructive pulmonary disease,) and diabetes. Chronic kidney disease was defined as an eGFR of <60 ml/min/1.73 m^2. In Paper 2, cancer was defined as answering "Yes" to "Have you ever been diagnosed with cancer?".

In Paper 1, we retrieved information on cancer diagnoses, including dates of the cancer diagnosis, from the Cancer Registry of Norway for all patients with a registered BSI event (from January 1, 1953 to January 1, 2014). This included 368 BSI cases. We also included information on rheumatic illnesses (rheumatoid arthritis and ankylosing spondylitis) and inflammatory bowel disease (ulcerative colitis and Crohn's disease) from a review of the records of patients with BSI at Levanger Hospital. We identified a total of 111 BSI cases with a rheumatic illness or inflammatory bowel disease out of 1,145 BSI cases at Levanger Hospital during follow-up.

In Paper 2 we included information on education level, smoking habits and alcohol consumptions as covariates. Education attainment was categorized into three groups: ≤9 years, 10–12 years, >12 years of schooling. Smoking habits were self-reported and categorized as current, previous, or never smoking. Smoking was categorized based on several questions targeting past and current smoking habits: "current smoking" (smoking tobacco daily), "prior smoking" (any prior daily tobacco smoking), or "never smoked". Alcohol was categorized from reported alcohol use: "never drink alcohol", "1–7 units of alcohol in two weeks", "8–12 units alcohol in two weeks", or "more than 15 units in two weeks".

Papers 1 and 2 Microbiology laboratory diagnostic

The microbiology laboratory at Levanger Hospital is accredited (NS-EN ISO/IEC 15189) and handles all blood cultures from both hospitals in the HNT HF and in the hospital's catchment area. The blood culture media was obtained in BACTEC 9240 Vacutainer Culture Bottles (Becton Dickinson Diagnostic Instrument System, sparks, MD). From 2010, it was replaced with BACTEC FX. A blood culture set consisted of one aerobic and one anaerobic BACTEC bottle obtained from a single draw. For cases where antibiotic treatment was indicated immediately, a second draw comprising one aerobic bottle was taken at the same time from another venipuncture site. In other cases, a second blood culture set with one aerobic and one anaerobic bottle was taken after two to three hours.

Paper 3 Genetic instruments for iron status

The exposure was iron status assessed by four iron biomarkers (serum iron, TSAT, TIBC, and ferritin). None of the iron biomarkers reflect iron status perfectly and iron status in populations is challenging to assess[46,78]. The

genetic instruments for the iron-status biomarkers were collected from a GWAS of 246,139 participants of European descent. They identified SNPs associated with one or more of the four iron biomarkers[164]. To limit bias from weak instrumental variables, we calculated F statistics and F statistic above 10 was required for sufficient strength[158]. We adjusted for correlation between SNPs using the linkage disequilibrium (LD) reference panel of European populations (10,000 kb and R^2<0.01) in the TwoSampleMR package (version 0.5.6)[182] and MendelianRandomization package (version 0.6.0)[183]. Sex-disaggregated genetic associations were reported for all iron biomarkers in the iron-status GWAS, which enabled us to conduct sex-stratified MR analyses for the COVID-19 outcomes[164].

Paper 3 Genetic instruments for sepsis and COVID-19

The outcomes studied were sepsis and COVID-19. Sepsis was defined as explicit sepsis from International Classification of Disease (ICD) -9 and ICD-10 codes and as described in the Global Burden of Disease[2]. The genetic associations with the outcome of interest were collected from the IEU OpenGWAS, with summary-level data of GWAS for sepsis obtained from UK Biobank, which included 10,154 sepsis cases and 454,764 controls[182,184]. For COVID-19 we evaluated hospitalization due to COVID-19, which included two different comparisons: hospitalization with COVID-19 compared to COVID-19 cases who were not hospitalized (**hospitalized vs. non-hospitalized COVID-19**) and hospitalization with COVID-19 compared to the general population (**hospitalization with COVID-19 vs. population**)[185]. To allow for sex-stratified analyses, we used the sex-disaggregated summary-level data on the two COVID-19 outcomes from UK Biobank using the National Heart, Lung, and Blood Institute's Genome-Wide Repository of Associations Between SNPs and Phenotypes (GRASP) catalogue (release date 06.18.21)[186]. To avoid possible bias related to population stratification, both exposure and outcome cohorts included individuals of European descent.

STATISTICAL ANALYSES

Directed Acyclic Graphs

Before I explain the statistical analyses used in my work, I would like to describe the associations investigated more thoroughly. For the three papers, the associations are illustrated using directed acyclic graphs (DAGs). DAGs are used to illustrate potential bias in epidemiological research[187] and to help determine whether the effect of interest can be identified from the available data[188]. Consideration of variables beyond the exposure and outcome of interest is important when performing such research. DAGs have provided insight into diagnosing sources of bias and helped researchers to select a set of covariates that allow the estimation of causal effects from observed data. The DAGs in this thesis have been used to encode the projects' a priori assumptions about the associations and relationships studied. Importantly, the assumptions are presented visually, making it easier to identify which variables require control to minimize bias and which could introduce bias if controlled in the analyses.

An example of a DAG is presented in Figure 9. A DAG is a graph with arrows that show the direction of hypothesized causal effects from exposure to outcome, and a path in the DAG is connected by arrows. There are two types of paths, directed or nondirected, where the directed paths follow the direction from exposure to outcome and all other paths are considered nondirected. The path Exposure → Mediator → Outcome represents a direct path, which partly explains the effect of the relationship between exposure and outcome. Mediators are part of the directed path and controlling for them in the analyses could remove part of the effect of exposure. The path Exposure ← Confounder → Outcome illustrates a confounder, often called a backdoor path, and could introduce bias to the associations if not controlled. The path Exposure → Collider ← Outcome illustrates a collider and would potentially introduce bias if adjusted for. Both confounders and colliders are considered nondirected paths[189].

Figure 8:

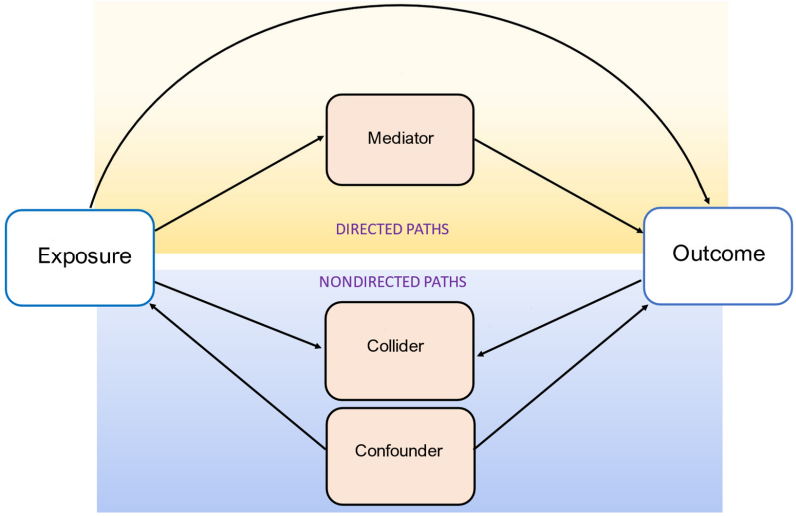


Figure 8:
The directed paths illustrate the effect of the exposure on the outcome either directly or via the mediator. Bias to the directed paths can be reduced by controlling for confounder(s) to close this nondirected path. If we control for a collider, we open a nondirected path and this may introduce bias.

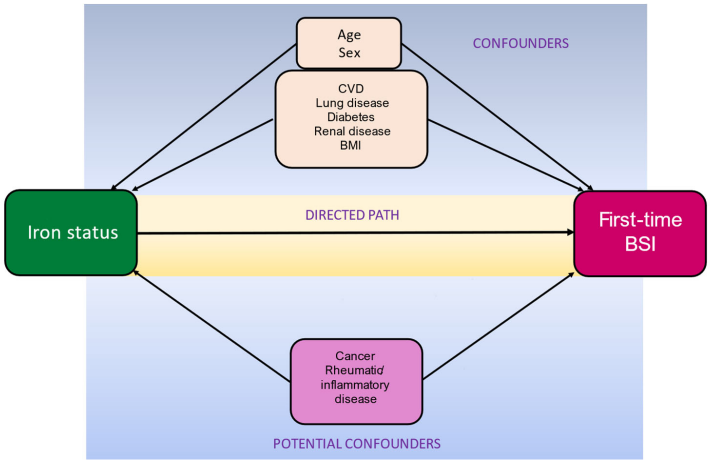
Illustrated by R.M. Mohus in PowerPoint.

Paper 1

We wanted to assess the relationship between iron status and future risk of a BSI. The DAGs prepared for this study are presented as Figure 9A and B. Potential confounders were identified and adjusted for in the analyses. A DAG will always be a simplified illustration of the true associations, leaving the possibility of unknown or unmeasured confounders, and also the possibility of reverse causation[188]. In our work for Paper 1, we identified two covariates, cancer and rheumatic/inflammatory diseases, with limited information in HUNT2 (Figure 9A). We therefore decided to acquire supplemental information on these diagnoses in BSI cases. This gave us the opportunity to identify BSI cases with a cancer diagnosis within five years prior to BSI or two years after, and in a sensitivity analysis we omitted cancer-related BSI. For rheumatic illnesses (e.g., rheumatoid arthritis, ankylosing spondylitis) and inflammatory bowel disease (e.g., ulcerative colitis, Crohn's disease), we retrieved information from medical records in BSI patients, and in a sensitivity analysis we omitted these BSI cases from the outcome definition (Figure 9B).

Figure 9:

A



B

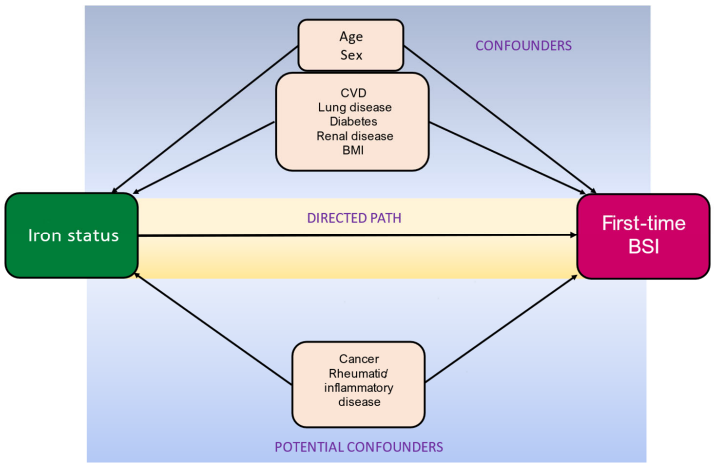


Figure 9: Simplified DAGs presenting associations in Paper 1:
A) DAG describing the association between iron status (exposure = green box) and risk of first-time BSI (outcome = dark pink box) with potential confounders (yellow boxes) including cancer and rheumatic/inflammatory disorders (pink box) that we realized needed precautions.
B) DAG describing the same association with potential confounders (yellow boxes) that have been adjusted for in the analyses.
Arrows indicate the directions of the relationships. Blue boxes indicate nondirected paths, and the yellow box in the middle indicates the direct path of the association.

Illustration prepared in PowerPoint by R.M.Mohus

Paper 2

We examined the association between sex and risk of BSI. Interestingly, as biological sex is fixed at conception, there are no confounding factors on the associations between sex and risk of BSI. Nevertheless, there could be mediating factors that partly or completely explain the observed sex differences. Mediators are variables that are causally located between exposure and outcome variables. Pearl has proposed three assumptions to identify natural direct and indirect effects from observational data; these assumptions require that there is no unmeasured confounding of the 1) exposure on the mediator, 2) mediator on the outcome, or 3) exposure on the outcome. There must also be no confounding variables of the mediator–outcome relationship affected by the exposure[190].

In epidemiology, we often want to disentangle different pathways that may explain the associations between exposure and outcome. Mediation analyses are important for understanding causal relationships. When we want to assess whether or not the effect of the exposure is explained by a given set of mediators, mediation analyses may be applied. In the association between sex and risk of BSI, we wanted to examine the total effect, but also assess how much of the total effect on the outcome was explained by the mediators (the *natural indirect effect*) and how much of the total effect was not explained by the mediators (the *natural direct effect*) (Figure 10).

Figure 10:

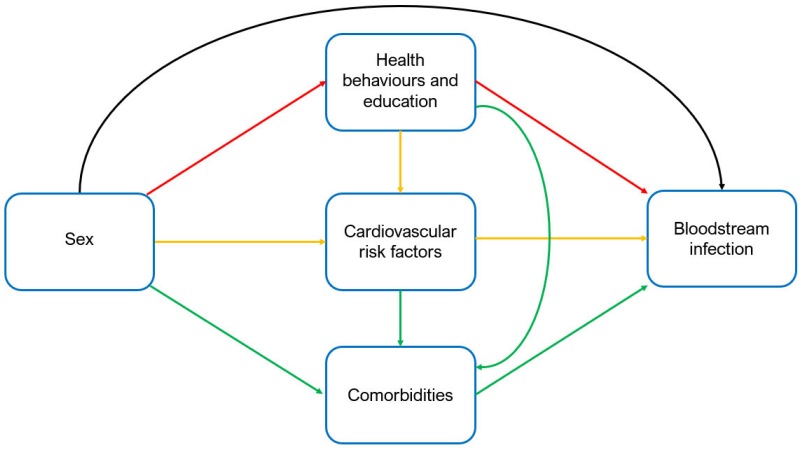


Figure 10: Mediation analysis in Paper 2:
Diagram of the direct and indirect (i.e., mediated) effects of **sex** on **bloodstream infection**. The black arrow represents the natural direct effect of the association. Red arrows represent Model 1, proportion mediated by health behaviours and education attainment. Yellow arrows represent Model 2, proportion mediated jointly by health behaviours, education, and cardiovascular risk factors. Green arrows represent Model 3, proportion mediated jointly by health behaviours, education, cardiovascular risk factors, and comorbidities. The total effect reflects the sum of both the natural direct effect and the natural indirect effects that work through the mediators.

Model 1) Smoking, alcohol use, and educational attainment
Model 2) Systolic blood pressure, non-high-density lipoprotein cholesterol, and body mass index
Model 3) Cardiovascular disease, chronic kidney disease, diabetes, history of cancer, and chronic lung disease

Illustration prepared in PowerPoint and Adobe Illustrator by R.M.Mohus

Paper 3

We wanted to explore the causal associations of iron status and risk of sepsis and COVID-19. MR methods use genetic variants as instrumental variables for exposures of interest to overcome problems of confounding and reverse causality. Since alleles follow random distribution during conception this minimizes confounding, and as genetic alleles are always assigned prior to disease onset they are not influenced by reverse causation[159].

The use of genetic variations in MR studies serves as a natural experiment to investigate causal relations between potentially modifiable risk factors and health outcomes, but this depends on specific assumptions: 1) the *relevance assumption*—the genetic variance associate with the risk factor of interest; 2) the *independence assumption*—there are no unmeasured confounders of the associations between the genetic variants and outcome; and 3) the *exclusion restriction*—the genetic variants affect the outcome only through their effect on the risk factor studied[159]. Other important considerations are the strength of the variant–exposure association, and if any variants exert effects depending on ancestry, sex, or age[191].

Figure 11

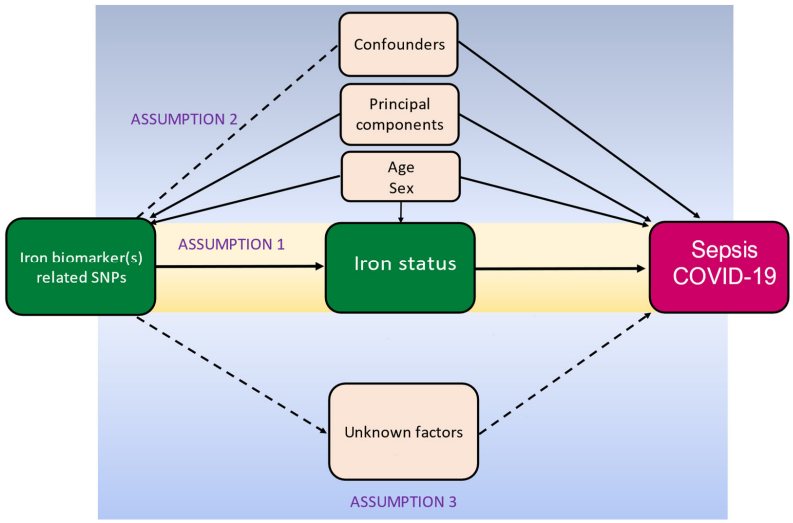


Figure 11 showing a DAG of the MR studies performed in Paper 3:
The *relevance assumption* 1 was assessed using SNPs associated with iron-status biomarkers in a large GWAS. The GWAS was adjusted for principal components, sex, and age. The *independence assumption* 2 was assessed using sensitivity analyses. The *exclusion restriction* (assumption 3) was assessed using the PhenoScanner to search for other potential pathways between the genetic variants and the outcome studied.

Illustrated in PowerPoint by R.M. Mohus

Performing an MR study enabled us to explore the causal path between iron status and risk of sepsis and COVID-19 using another framework than that used in the prospective cohort study in Paper 1. In this scenario, we used iron-related SNPs identified in a GWAS on iron status as instruments[164]. In the DAG presented in Figure 11, potential confounders include sex, age, and principal components, which were adjusted for in both the exposure and outcome GWASs included[164,184,185]. To assess potential horizontal pleiotropy—namely, whether the iron-related SNPs could affect the outcome through other paths than iron status—we conducted sensitivity analyses; (e.g, MR Egger and PhenoScanner search).

Paper 1: Incidence Rates, Survival Analyses, and Cumulative Incidence

Incidence rates

The incidence rates of first-time BSI and BSI mortality were computed as the number of events divided by the total person-years at risk for each outcome. Survival analyses are typical statistical methods that analyze the associations, which include information on time. The survival function is a key term in survival analyses and is defined as the probability of the outcome event not occurring up to a specific point in time[192]. To examine the associations of iron status and risk of first-time BSI and BSI mortality, we performed Cox proportional hazards regression by categories of the iron indices, using the middle quintile as the reference. Levels of serum iron, TSAT, and TIBC were categorized into values (≤2.5th percentile (low) or ≥97.5th percentile (high)) and the values in between were categorized into quintiles. The HUNT Study population is representative of the Norwegian adult population, and we therefore based iron values on the distribution of the entire study cohort (Figure 12 A–C).

Figure 12: Histograms of the iron biomarkers' normal distribution in HUNT2

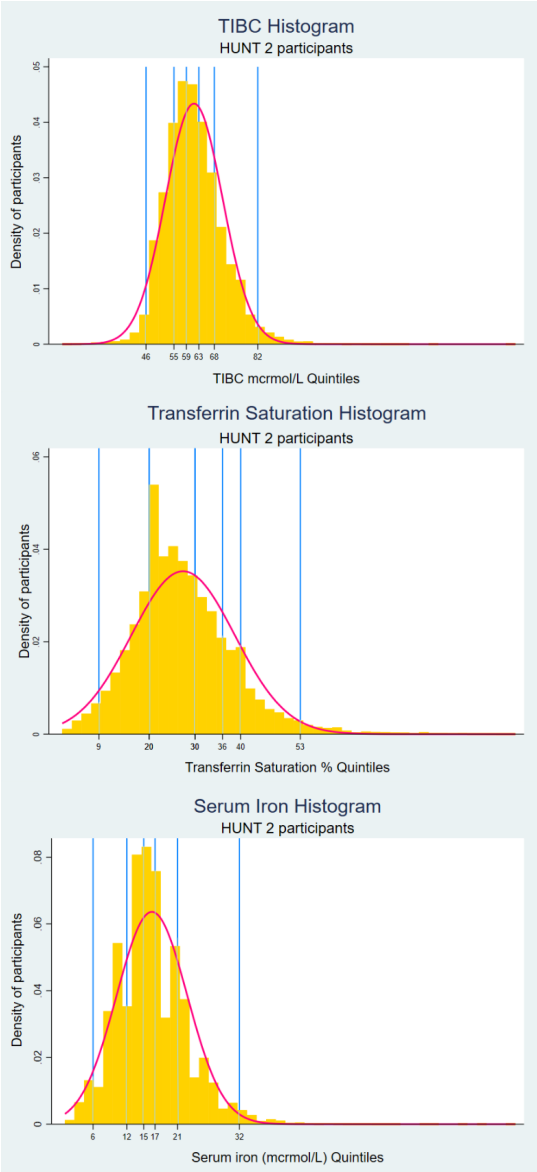


Figure 12:
Histograms with normal distribution of serum iron, TSAT, and TIBC measurements in HUNT2 participants. Blue lines mark the range of the quintiles used.

Illustrated using Graph Editor in Stata version 17 by R.M. Mohus.

Survival analyses

Cox proportional hazards regression is a survival analysis that allows for time-to-event associations, where the hazard of the event of interest is compared between the exposed and the unexposed participants. The purpose is to simultaneously evaluate the effect of several factors on survival. It allows investigators to evaluate how specified factors influence the rate of a particular event happening at a particular point in time but can also harbor censoring when data about the outcome are unknown from a time during the study follow-up period (in our study, migration out of the capture area or death from other causes).

Cox regression is based on two assumptions: first, the survival function is an exponential function; and second, the hazard ratio (HR) for the two compared groups is constant throughout the study period[193]. The proportional hazards assumption was examined by visual inspection of log–log plots of the survival by time of study for each exposure variable and for sex. In Paper 1, the curves for sex were not proportional, and because of this, we adjusted for sex by stratification. For both Paper 1 and 2, we also tested this assumption using Schoenfeld residuals. Attained age was used as the time scale because this has been shown to give a better adjustment for age in cohort studies[179].

Start of follow-up was defined by the availability of data in the sepsis registry. For patients referred to St. Olavs Hospital, the tertiary referral center, BSI information was included depending on their primary hospital. Participants contributed person-years from inclusion date in HUNT2 except for those having Namsos as their primary hospital; the latter contributed from September 1, 1999. In the analyses of BSI risk, the participants were followed until their first BSI, migration out of the region, death, or end of follow-up on December 31, 2011, whichever occurred first. For BSI mortality, participants were followed until migration out of the region, death, or end of follow-up. The first model was adjusted for age and stratified by sex. The second model was additionally adjusted for BMI and chronic illnesses, as these conditions would be considered confounders to the association between iron status and risk of first-time BSI and BSI mortality. Increasing age, sex, BMI, and chronic medical conditions (cardiovascular disease, diabetes, chronic lung disease, and chronic kidney disease) may increase the risk of BSI[18,194] and cause “anemia of inflammation” and altered iron status[73].

Sensitivity analyses

First, we explored the impact of rheumatic illnesses and inflammatory bowel disease by omitting BSI potentially related to this patient group in 1,145 cases of BSI at Levanger Hospital. Cox regression analyses were performed solely in participants having Levanger Hospital as their primary hospital (*n*=43,280). Second, because cancer and cancer treatment may confound iron status and BSI risk, we omitted cancer-related BSI (*n*=368) (defined by a cancer diagnosis within five years prior to or two years after a BSI) from the outcome. Third, to reduce potential confounding by prevalent but unknown disease at time of serum measurements, we excluded the first two years of follow-up after HUNT2 participation.

Cumulative incidence

To account for competing risks, we performed cumulative incidence from start of follow-up to first-time BSI and BSI mortality; in particular, death from other causes than BSI would appear during this long follow-up and count as a competing risk for this outcome. For each category of serum iron, TSAT, and TIBC, we estimated the age-adjusted cumulative incidence of first-time BSI. The analyses were adjusted for the mean age of the study population. The Stata command *stcompadj* was used to estimate the cumulative incidence. The Stata Graph Editor was used to make the cumulative incidence curves.

All statistical analyses for Paper 1 were performed using Stata version 13.

Paper 2: Survival Analyses and Cumulative Incidence

We examined the association between sex and risk of first-time BSI, risk of first-time BSI caused by *E. coli*, *S. aureus*, and *S. pneumoniae*, and BSI mortality with a follow-up of 16.8 years. Cox proportional hazards regression analysis was performed in the same manner as described for Paper 1, with attained age used as the time scale in all models. Start of follow-up was defined by the availability of data in the HNT HF Sepsis Registry as in Paper 1, as was follow-up time and censoring.

We investigated cumulative incidence, as this analysis better accommodates competing risks such as death by other causes than BSI. We assessed cumulative incidence for first-time BSI, for first-time BSI caused by the specific bacteria, and cumulative mortality after a BSI event. All analyses were adjusted for the mean age of the population (49.9 years) and performed using the Stata command *stcompadj*.

To address whether menopause affects women's risk of BSI, and to assess sex differences in BSI risk with advancing age, we conducted age-stratified analyses on risk of first-time BSI: <50, 50 to <65, 65 to 79, and ≥80 years.

Paper 2: Mediation Analyses

As sex is determined at fertilization of the ovum, there are no confounding factors to sex and risk of BSI. The goal of the mediation analyses was to investigate alternative causal pathways by examining intermediate variables that lie in the causal paths between exposure (*sex*) and outcome (*first-time BSI*). Mediators are variables that are causally located between exposure and outcome variables, and that partly explain the effect of exposure on outcome. Mediation analysis can estimate indirect and direct effects and the proportion mediated, which is a statistical estimate of how much of the total effect works through the mediators of interest.

Inverse odds weighting

For the mediation model presented in Figure 10, we had multiple mediators that potentially affect one another: a time-to-event setting, a rare event, and a binary outcome[195-197]. We needed to accommodate a method that could handle all factors: both continuous and binary/count mediators and potential mediator–mediator interactions. In short, we included weights in a Cox regression model. This was conducted using *inverse odds weighting*

(IOW)[197]. Three distinct sets of mediators measured at inclusion to HUNT2 were covered: 1) *health behaviors* (smoking and alcohol use) and *educational attainment*; 2) *cardiovascular risk factors* (BMI, systolic blood pressure (mmHg) and non-HDL cholesterol (mmol/L)); and 3) *comorbidities*, which comprised self-report of cardiovascular disease (history of myocardial infarction, angina pectoris, and/or stroke), diabetes, cancer history, lung disease (asthma or chronic obstructive pulmonary disease), and standardized measurements of kidney disease; eGFR < 60 ml/min/1.73 m^2 . The three sets all mirror known risk factors for BSI and sepsis[15,18-20]. The method is also robust regarding unmeasured common causes of the included mediators[197].

The inverse odds weights were obtained from a model where the exposure was regressed on all mediators of interests, with age as a covariate. The total effect was calculated using Cox regression, the natural direct effect was estimated by including the weights in the Cox regression model, and the natural indirect effect was calculated by subtracting the direct effect from the total effect[197,198]. In our analysis, the total effect is best interpreted as the total association between exposure (sex) and outcome (first-time BSI), the natural indirect effect is the proportion of excess BSI risk in men mediated by conventional BSI risk factors, while the natural direct effect is the proportion of excess BSI risk in men not associated with these factors. The proportion mediated is the percent of the total association that is mediated through the risk factors.

Sequential mediation approach

The natural indirect effect was estimated with a *sequential mediation approach* using three models. We could not estimate the natural indirect effect of individual mediators separately, as it may not be appropriate when the mediators affect one another or when single mediator–outcome confounders may be affected by exposure. This approach assumes that the cardiovascular risk factors and the comorbidities are causal descendants of the health behaviors and educational attainment. In Model 1, we assessed health behaviors and education, in Model 2 we added BMI, systolic blood pressure, and non-HDL cholesterol, and in Model 3 comorbidities were included in the complete set of mediators. The sequential approach further implies that Model 3 reflects the best interpretation of the mediation analyses, as all mediators and age were included[196].

We performed bootstrapping based on 1,000 replications to derive CIs for all mediation parameters[199]. Bootstrapping relies on data-driven simulations to make statistical inferences and the core mechanism is sampling with replacement[200]. We present the natural direct and indirect effects as HRs with a 95% confidence interval (CI). The CIs are reported as percentile-based CIs, as the percentile method has been demonstrated to be valid in the multiple mediation approach. The proportion mediated on the log scale was calculated using the formula ($\ln HR_{NIE}$ /$\ln HR_{TOTAL}$).

All statistical analyses for Paper 2 were performed using Stata version 16 and 17.

Paper 3: Mendelian Randomization

Observational studies are prone to unmeasured and/or residual confounding and reverse causation and we wanted to explore iron status in the context of severe infections. A novel GWAS on iron status had identified novel genetic variants associated with iron homeostasis biomarkers, including sex-disaggregated data[164]. We performed two-sample MR analyses on risk of sepsis and hospitalization due to COVID-19, including sex-stratified analyses for the COVID-19 outcomes.

The main analysis was the inverse-variance-weighted (IVW) method which assumes all genetic instruments to be valid[201]. We applied weighted median, weighted mode, and MR Egger regressions as complementary analyses. The weighted median orders MR estimates produced by each SNP by their magnitude, weighted for their precision, and gives an overall MR estimate based on the median value, with standard errors estimated by bootstrapping. This method allows for 50% of the genetic instruments to be invalid[202]. The weighted mode assumes that the most common causal effect is consistent with the true causal effect and allows some invalid instruments without biasing the MR estimate[203]. MR Egger allows directional pleiotropic effects to some extent, where some SNPs could be acting on the outcome through another pathway than the exposure of interest, but at the cost of statistical power[204]. A consistent effect across the methods is less likely to represent a false positive or negative estimate. To further investigate potential pleiotropic effects of the included SNPs, we used PhenoScanner to investigate other biological traits where the SNPs have been identified. We also used leave-one-out plots to assess whether the MR estimates were driven strongly by a single SNP.

All analyses were performed in R version 4.2.1.

Ethics

The HUNT Study was approved by the Regional Committee for Medical and Health Ethics of Central Norway (REK-Midt) and by the Norwegian Data Protection Authority. Each HUNT Study participant signed a written consent regarding screening, follow-up, and the use of data and blood samples for research purposes. They also consented to linking their data to other registries. For participants in HUNT2, a new consent form including the performance of genetic research was obtained in 2002 and included all surviving HUNT2 participants (*n*=61,426), of whom 1,185 (1.9%) withdrew their consent. This re-consent was approved by the REK-Midt and the Norwegian Data Protection Authority[175]. The REK also approved the linkage of blood culture information in the HNT HF Sepsis Registry to HUNT2 (REK no 2012/153) and the use of data for Papers 1 and 2 was approved by the Nord-Trøndelag Hospital Trust.

UK Biobank has approval from the Northwest Multi-Centre Research Ethics Committee as a Research Tissue Bank (www.ukbiobank.ac.uk/learn-more-about-uk-biobank/about-us/ethics). All summary data used in this work are publicly available and they were obtained with relevant participant consent and ethical approval.

Summary of Results

PAPER 1

Association of iron status with the risk of bloodstream infections: results from the prospective population-based HUNT Study in Norway

In this prospective cohort study, we followed 61,852 HUNT2 participants for a median follow-up of 14.8 years, 1,738 (2.9%) of whom experienced at least one episode of BSI, and 370 (0.6%) of whom died from BSI. Participants who experienced a BSI during follow-up were older and more likely to have comorbid illnesses. Participants with indices of low iron status had increased risk of BSI and BSI mortality. In age- and sex-adjusted analyses, the risk of BSI for participants with low serum iron was 72% (95% CI 34–121%), for low TSAT the risk of BSI was 48% (95% CI 12–96%), and for high TIBC the risk of BSI was 46% (95% CI 6–101%) higher compared with participants in the middle quintile of the iron biomarkers. There were no clear associations between indices of high iron status and increased risk of BSI, and no associations with risk of BSI for the groups within the 2.5th and 97.5th percentiles (Table 2).

In analyses of BSI mortality, we found similar associations as those observed for first-time BSI, but precise estimates were precluded by a low number of BSI-related deaths. Age- and sex-adjusted BSI mortality was higher in participants with low serum iron with an HR of 1.52 (95% CI 0.86–2.66), low TSAT with an HR of 1.41, 95% CI 0.75–2.67) and high TIBC with an HR of 1.67, 95% CI 0.76–3.68). The results did not attenuate after adjustments for BMI and comorbidities.

Multiple sensitivity analyses were performed to ascertain our results. First, the results remained essentially similar after adjustments for BMI and chronic illnesses and after omitting cancer-related BSI (*n*=368). Second, to account for potential unmeasured residual confounding, sensitivity analyses were performed in which we excluded BSI events in the first two years of follow-up; the results remained similar, showing that the increased risk of BSI in iron-deficient subjects was not only present shortly after the time of diagnosis (i.e., measurement of low iron status). Third, the associations were also similar when we excluded BSI events in participants diagnosed with rheumatic illnesses and inflammatory bowel disease. Fourth, cumulative incidence curves enabled us to account for competing risk of death, and they confirm that the excess risk of BSI in participants with low iron status continued throughout the follow-up period for all iron indices: low serum iron, low TSAT, and high TIBC. Interestingly, for serum iron and TIBC, the cumulative incidence was highest for both low and high iron status.

Table 2. Associations of indices of iron status with risk of bloodstream infection

Indices of iron status	Risk of any BSI, adjusted for age and sex				Risk of any BSI, adjusted for age, sex, BMI, and comorbidities[a]		Risk of non-cancer-related BSI[b], adjusted for age, sex, BMI, and comorbidities[a]		
	Years at risk	No. BSI	HR	95% CI	HR	95% CI	No. BSI	HR	95% CI
Serum iron (µmol/L)	786,527	1738					1370		
Low ≤ 6	27,125	83	1.72	1.34–2.21	1.71	1.33–2.20	71	1.79	1.36–2.35
7–12	177,840	418	1.08	0.93–1.27	1.06	0.90–1.24	334	1.03	0.87–1.23
13–15	164,480	383	1.01	0.87–1.19	1.01	0.86–1.19	294	0.96	0.80–1.15
16–17	108,973	246	1.00	Reference	1.00	Reference	199	1.00	Reference
18–21	163,237	344	0.97	0.83–1.15	0.98	0.84–1.16	272	0.97	0.81–1.16
22–31	128,313	236	0.91	0.76–1.09	0.93	0.78–1.12	178	0.88	0.72–1.08
High ≥ 32	16,555	28	1.06	0.72–1.57	1.06	0.72–1.57	22	1.05	0.68–1.63
Transferrin saturation percentage (TSAT)									
Low ≤ 9	24,676	59	1.48	1.12–1.96	1.45	1.10–1.91	48	1.40	1.03–1.91
10–20	184,421	432	1.11	0.96–1.28	1.08	0.93–1.24	342	1.02	0.87–1.19
21–25	164,506	357	0.95	0.82–1.10	0.94	0.81–1.09	278	0.88	0.75–1.04
26–30	147,447	345	1.00	Reference	1.00	Reference	284	1.00	Reference
31–36	128,719	275	0.92	0.79–1.08	0.94	0.80–1.10	210	0.88	0.74–1.06
37–52	115,412	236	0.90	0.77–1.07	0.93	0.79–1.10	183	0.89	0.74–1.07
High ≥ 53	21,344	34	0.84	0.59–1.20	0.86	0.60–1.22	25	0.78	0.52–1.18
Total iron binding capacity (TIBC) (µmol/L)									
High ≥ 82	21,553	43	1.46	1.06–2.01	1.36	0.99–1.87	35	1.34	0.94–1.91
69–81	128,767	225	0.95	0.80–1.12	0.92	0.78–1.09	183	0.93	0.77–1.12
64–68	139,549	283	0.94	0.80–1.10	0.93	0.80–1.09	208	0.86	0.72–1.03
60–63	151,071	346	1.00	Reference	1.00	Reference	274	1.00	Reference
56–59	152,182	345	0.92	0.79–1.07	0.93	0.81–1.09	283	0.97	0.82–1.15
47–55	171,521	428	0.92	0.79–1.05	0.95	0.82–1.09	337	0.95	0.81–1.11
Low ≤ 46	21,881	68	1.00	0.77–1.30	1.04	0.80–1.35	50	0.98	0.72–1.32

BSI = bloodstream infection; BMI = body mass index; HR = hazard ratio; CI = confidence interval.

a) Comorbidities: cardiovascular events, chronic renal disease, diabetes, and lung disease.

b) The outcome is any first-time BSI except cancer-related BSI, indicated by a cancer diagnosis within five years prior to BSI or two years after.

PAPER 2

Explaining sex differences in risk of bloodstream infections: mediation analysis in the population-based HUNT Study in Norway

We carried out a prospective cohort study in HUNT2, to assess the impact of sex as a risk factor for first-time BSI, BSI mortality, and BSI caused by the most frequent infecting bacteria, *S. aureus, S. pneumoniae*, and *E. coli*. Further, we examined whether sex differences in health behaviors and education attainment, cardiovascular risk factors, and comorbidities could explain the observed sex differences in risk of first-time BSI. We included 64,040 HUNT2 participants, of whom 46.8% were men. The median age at inclusion in HUNT2 was similar for men and women (48.6 and 48.7, respectively). During a follow-up period of 16.8 years, 1,840 individuals (2.9%) experienced any first-time BSI and 396 (0.6%) died within 30 days after a BSI episode. Both men and women who experienced a BSI had higher comorbidity burden and were older (with a median age at inclusion of 67.4 for men and 68.0 for women) than participants who did not experience a BSI during follow-up.

Men had a 41% higher risk of first-time BSI (95% CI 28–54%), and an 87% higher risk of dying from a BSI (95% CI 53–128%) compared with women. We also found that men had a substantially higher risk of a BSI caused by *S. aureus* with HR 2.09 (95% CI 1.28–2.54) in men versus women. The HRs with 95% CIs were derived from Cox regression analyses. We additionally present cumulative incidence curves for the three most common infecting bacteria in Figure 14, showing that men had higher incidence of *S. aureus*, especially after the first seven years of follow-up, whereas *E. coli* had higher cumulative incidence among women, starting from the early years of follow-up. For *S. pneumoniae*, the sex differences on cumulative incidence were not as evident throughout follow-up.

Figure 13:

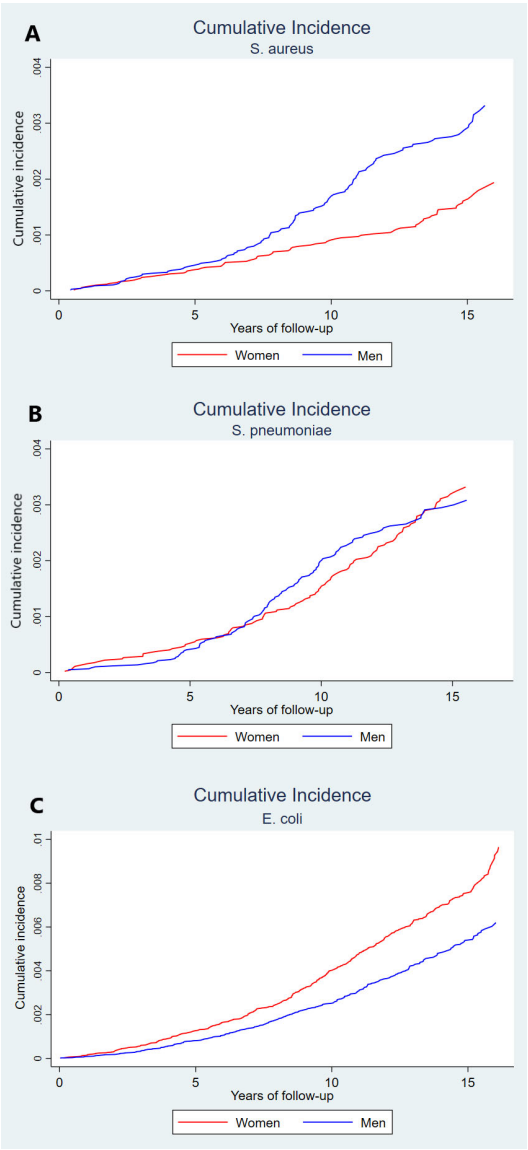


Figure 13:
Sex differences in cumulative incidence of first-time BSI caused by A) *S. aureus*, B) *S. pneumoniae*, and C) *E. coli*. Adjusted for mean age of the population, 49.9 years.

Illustration prepared in Stata version 17 using Graph Editor and in Adobe Illustrator by R.M. Mohus

Results of the mediation analyses

For the mediation analyses, we present the total effect, natural direct effect, and indirect effects of sex on risk of first-time BSI. Compared with women, men had an estimated HR of 1.40 (95% CI 1.24–1.55) for first-time BSI which is in line with the Cox regression results. Using a mediation approach, we explored this association into natural direct effects and indirect effects, varying with the models included and presented in Table 3. The important finding in these analyses is the proportion mediated by the included mediators, where behavioral risk factors and education mediated 10% (Model 1), after adding the cardiovascular risk factors the proportion mediated was reduced to 5% (Model 2), whereas the whole set of mediators, including comorbidities, jointly mediated 34% of the total effect (Model 3), as illustrated in Figure 14.

Table 3: Mediation analyses using inverse odds weighting showing the associations between sex and BSI

	Risk of first-time BSI
Model 1	
Mediation by behavioral riskfactors[1] and education	**HRs (95% CI[a]) [b]**
Total effect	1.40 (1.24–1.55)
Natural direct effect	1.36 (1.18–1.57)
Natural indirect effect	1.04 (0.97–1.07)
Proportion mediated[c]	10%
Model 2	
Mediation by behavioral risk factors[1], education, and cardiovascular risk factors[2]	**HRs (95% CI[a]) [b]**
Total effect	1.40 (1.24–1.55)
Natural direct effect	1.38 (1.19–1.58)
Natural indirect effect	1.02 (0.92–1.07)
Proportion mediated[c]	5%
Model 3	
Mediation by behavioral risk factors[1], education, cardiovascular risk factors[2], and comorbidity risk factors[3]	**HRs (95% CI[a]) [b]**
Total effect	1.40 (1.24–1.55)
Natural direct effect	1.25 (1.05–1.47)
Natural indirect effect	1.12 (1.02–1.17)
Proportion mediated[c]	34%

BSI = bloodstream infection; HRs = hazard ratios; 95% CI = 95% confidence intervals.
1) Smoking, alcohol use, and educational attainment at baseline.
2) Systolic blood pressure, non-high-density lipoprotein cholesterol and body mass index.
3) Cardiovascular disease, chronic kidney disease, diabetes, history of cancer, or chronic lung disease.
a) Percentile-based bootstrap CIs are reported.
b) Estimates are adjusted for age as a covariate.
c) Proportion mediated: (ln HR_{NIE}/ln HR_{TOTAL}).

Figure 14: Results of mediation analyses

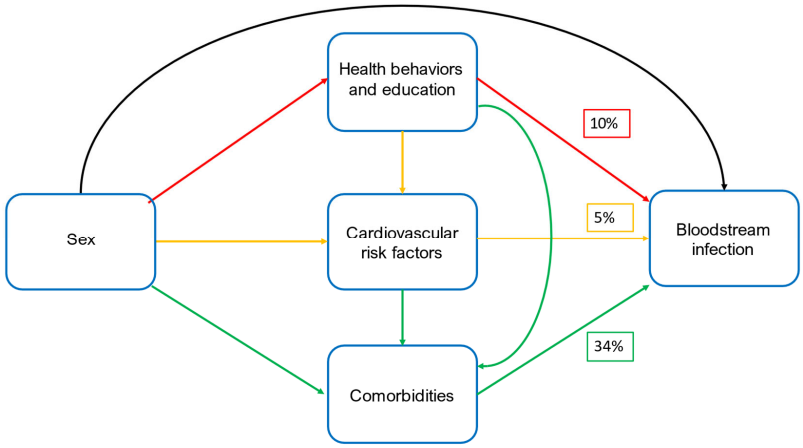


Figure 14:
Red arrows represents Model 1, proportion mediated by health behaviors and education was 10%.
Yellow arrows represent Model 2, proportion mediated jointly by health behaviors, education, and cardiovascular risk factors which was 5%.
Green arrows represent Model 3, proportion mediated jointly by health behaviors, education, cardiovascular risk factors and comorbidities which was 34%.
Model 1) Smoking habits, alcohol use, and education level.
Model 2) Systolic blood pressure, non-high-density lipoprotein cholesterol, and body mass index.
Model 3) Cardiovascular disease, chronic kidney disease, diabetes, history of cancer, and chronic lung disease.

Illustration prepared in PowerPoint and Adobe Illustrator by R.M. Mohus.

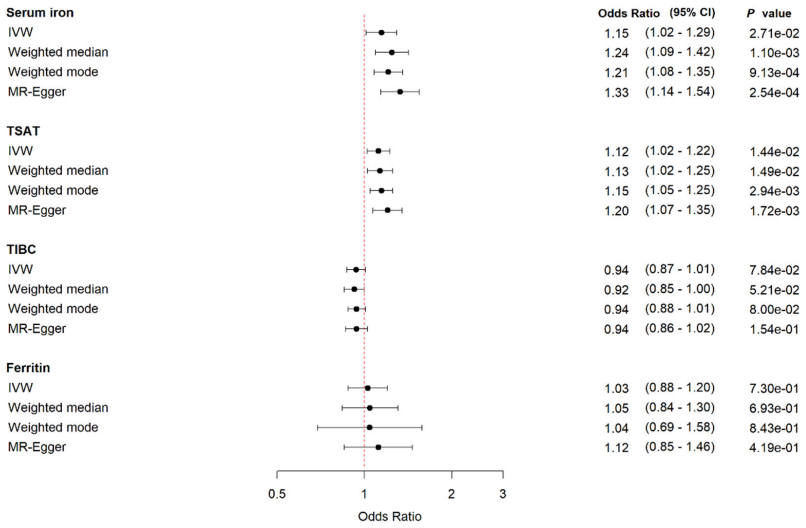
PAPER 3

Iron status and the risk of sepsis and severe COVID-19: A two-sample Mendelian randomization study

MR results—sepsis

Genetically predicted higher serum iron levels were associated with higher risk of sepsis, with an odds ratio (OR) of 1.15 (95% CI, 1.02–1.29, *p* value 0.027) for each standard deviation (7.76 µmol/L) increase in serum iron; for TSAT, an OR of 1.12 (95% CI 1.02–1.25, *p* value 0.014) per standard deviation (13.25%) increase in TSAT. For TIBC, the direction of the MR estimate showed a tendency of lower TIBC being related to increased risk of sepsis (i.e., low levels of TIBC indicate high iron status), with an OR of 0.94 (95% CI 0.87–1.01, *p* value 0.09). For ferritin, the MR estimates yielded inconclusive results. The complementary MR methods supported the findings (Figure 15).

Figure 15: Forest plot with MR estimates for risk of sepsis



CI = confidence interval; TSAT = transferrin saturation; TIBC = total iron binding capacity

MR results for being hospitalized with COVID-19

We found evidence of a relationship between genetically predicted higher levels of serum iron and risk of being hospitalized with COVID-19 compared to non-hospitalized COVID cases, with an OR of 1.29 (95% CI 0.97–1.72, *p* value 0.08). MR estimates derived from weighted median, weighted mode, and MR Egger regressions showed similar point estimates, but lower precision. For the other iron biomarkers, TSAT showed the same tendency as serum iron, but the estimate was uncertain with an OR of 1.15 (95% CI 0.95–1.40, *p* value 0.148). For TIBC and ferritin, the estimates were inconclusive. Taking advantage of sex-disaggregated summary-level data for both iron status and COVID-19 outcomes, we conducted sex-stratified MR analyses. There was indication of a more pronounced risk in women of being hospitalized with COVID-19 compared to non-hospitalized COVID-19 cases, with an OR of 1.63 (95% CI 0.94–2.86, *p* value 0.09); nevertheless, the estimates had low precision. The corresponding result in men was an OR of 1.21 (95% CI 0.92–1.62, *p* value 0.17). There was no clear evidence that genetically predicted levels of iron homeostasis biomarkers were associated with a risk of being hospitalized with COVID-19 compared with the non-hospitalized population.

Discussion

This thesis aimed to investigate and uncover potential risk factors for severe infections, in particular the role of iron status and sex on bloodstream infections, sepsis, and COVID-19. The main findings were:

- Low iron status measured with serum iron, TSAT, and TIBC was associated with increased risk of first-time BSI in a prospective cohort study of HUNT2 participants with almost 17 years of follow-up.
- Cumulative incidence of BSI was higher throughout follow-up in participants with low iron status for all iron indices. For serum iron and TIBC, cumulative incidence was also higher in participants with high iron status.
- Analyses of BSI mortality showed similar associations, with higher risk in participants with low iron status but low number of BSI-related deaths precluded precise estimates.
- Using Mendelian randomization, we showed that higher genetically predicted iron status was associated with increased risk of sepsis. We also identified a tendency of high iron status related to risk of being hospitalized with COVID-19 compared to non-hospitalized COVID-19 cases.
- Male sex was associated with a 40% higher risk of a first-time BSI and an 87% higher risk of dying from a BSI.
- Mediation analyses estimated that 34% of the observed higher risk of first-time BSI in men was mediated by known BSI risk factors.
- Men had a two-fold higher risk of *S. aureus* BSI and 30% higher risk of *S. pneumoniae* BSI.
- Cumulative incidence of BSI and cumulative mortality were higher in men. For the specific bacteria, men had higher incidence of *S. aureus* after the first seven years of follow-up, whereas women had higher cumulative incidence of *E. coli* BSI.

Throughout my work on this thesis, I have traveled back in time to the origin of life in the sulfur vents where iron established a role as one of the most important elements for cellular life; I have also explored the biology of the sexes. This journey would reveal that iron and sex matter in severe infections. When I first began this work, the overall aim was to identify potential modifiable risk factors for BSI and sepsis. Working with different epidemiological studies, I stumbled into indisputable sex differences, realizing that the most obvious biological factor was often neglected and not accounted for in biological research, including in severe infections. Conducting a PhD during a pandemic—caused by SARS-CoV2 with a potentially severe sepsis, often accompanied by ARDS and multi-organ failure—gave me a sudden opportunity and obligation to explore risk factors in this context.

In this thesis, I present iron status and sex as important risk factors using an array of epidemiological approaches and methods. In all scientific studies there will be potential sources of error that could influence the results. In observational studies, the main sources of error are random errors that reduce the precision of the associations,

and systematic errors that reduce the validity of the results[153]. The potential sources of error in this project will be discussed in the following sections. I will also discuss my findings related to previous studies in the field, strengths and limitations of my results, and implications of my findings.

METHODOLOGICAL CONSIDERATIONS

Random error

In epidemiological studies, the observed associations may be due to variability in the data or chance, which is termed *random error*. Random error in epidemiological studies could arise from any factors that randomly affect the data, such as biological variation, subject sampling, and the ways in which variables are collected and measured. This variability in data may lead to error in the estimate. High precision of the estimates indicates that there is little possibility for random error. Large sample sizes will reduce the risk of random error and generate a more precise estimate[153]. In Papers 1 and 2, the precision of the results and measure of uncertainty due to random error were assessed using 95% CIs. In Paper 3, the precision of the results was assessed using both 95% CIs and *p* values.

Paper 1 and Paper 2

The study population in Paper 1 included a large sample size with 61,852 HUNT2 participants, and 1,738 participants had at least one episode of BSI. The large sample size allowed us to perform sensitivity analyses in subgroups of the sample. In the analyses of first-time BSI, the point estimates for low iron status were quite precise with narrow 95% CIs. For high iron status, there were few cases, which precluded precise estimates. The same is true for BSI mortality, with 370 deaths: the point estimates indicate a risk in participants with low iron status but with wide 95% CIs.

In Paper 2, we included 64,040 participants from the same population, with 1,840 episodes of BSI, 396 BSI-related deaths, and 212 *S. aureus* BSI, 232 *S. pneumonia* BSI, and 684 *E. coli* BSI episodes, respectively. The point estimate for first-time BSI was precise, with a narrow 95% CI; we also found the point estimates for BSI-related death and the specific bacteria to be precise, but with a wider 95% CI due to a lower number of events. For the mediation analyses, we conducted a percentile-based bootstrap for the 95% CI, and our estimates in the three models are quite precise.

Paper 3

In Paper 3, we used summary data from large GWASs, which is necessary to identify genetic variants at genome-wide significance level (i.e., *p* value of the associations $<5 \times 10^{-8}$). The genetic studies exploited in our MR analyses have large sample sizes. In UK Biobank, the sample size is almost 500,000 and the COVID-19 HGI more than 1,000,000 participants. The exposure GWAS cover iron biomarker measurements in ~130,000 to 250,000 individuals. Large sample sizes are the preferred method to increase precision in GWAS and MR studies and reduce the chance that the associations arise from spurious relationships[205].

The precision of the MR estimates depends on the precision with which the associations between the genetic variants for each of the exposures and outcomes have been assessed (i.e., the size of the standard error of the estimated associations). In our study, some of the MR estimates were imprecise, with some of the 95% CIs including 1, and thus we could not draw causal conclusions on the relationship between iron status and COVID-19 outcomes. For sepsis, the MR estimates suggested a causal relationship between high iron status measured by genetically predicted levels of serum iron and TSAT. We conducted several sensitivity analyses and our main MR estimates were similar when using IVW, weighted median, weighted mode, and MR Egger methods[201-204]. The consistent results along several MR methods affirmed our estimates, including various strategies to detect and account for the potential pleiotropy[191]. Taken together, the overall conclusions of our study were less likely to be affected by bias due to pleiotropy.

Systematic error

Systematic error in epidemiological studies may lead to incorrect results. The main sources of systematic error are selection bias, information bias, and confounding. For a study to be valid, it must be free of systematic error. Systematic errors are not affected by increasing sample size. Little systematic error reflects high internal validity of the study[153].

Confounding—*confusion of effects*

Confounding reflects the potential for distortion of the association effects by a factor or several factors associated with both the outcome and the exposure, but not affected by them. Confounding may lead to underestimation or overestimation, or even cause an association in the opposite direction of the true effect[206]. Misclassification of the confounders may lead to residual confounding, and there may also be unknown or unmeasured confounding.

The identification of confounders in Paper 1 was based on subject matter knowledge gained via literature review, as recommended in modern epidemiological research[153]. Age and sex are directed towards both iron status and BSI events and BSI mortality. Age is also strongly associated with risk of infections[2,25,40]. At a population level, iron status tends to decrease with increasing age: the reasons for this are multifactorial, and include chronic inflammation, medications, and poor diet[207]. We included a wide range of comorbidities associated with both iron status and BSI risk[15,18,73] and the associations remained similar in the adjusted analyses. For cancer, we found the baseline variable in HUNT2 ("cancer history") too uncertain, and a cancer event during follow-up could also have led to a substantial change in risk of BSI and change in iron status. We therefore chose not to adjust for cancer history (from the baseline questionnaire in HUNT2) in the analyses. Instead, we carried out a sensitivity analysis where we omitted cancer-related BSIs from the outcome, using complete information from the Cancer Registry. The HR remained similar, with slightly wider CIs. The same applied to rheumatic illnesses and inflammatory bowel disease. For these categories of diseases, we chose to perform another sensitivity analysis on BSI cases at Levanger Hospital, with complete information on these diagnoses from reviewing participants'

patient records. The associations remained essentially the same after omitting BSI cases with known rheumatic or inflammatory bowel disease.

To explore whether unknown pre-clinical illnesses could affect the associations, we conducted sensitivity analyses where we started follow-up two years after baseline measurements in HUNT2; the results remained similar, indicating that the increased risk in iron-deficient subjects was not present shortly after diagnosis. The cumulative incidence curves also confirm that the excess risk of BSI in iron-deficient subjects continued throughout follow-up.

A key limitation of observational studies is that they are prone to bias due to confounding; meticulous considerations are therefore needed to develop statistical models that can adjust for confounders. Confounding is removed from the estimates and adjusted models reflect the best estimates of the true associations. MR studies, to some extent, can overcome the limitations of unmeasured confounding by using genetic variants closely related to the exposures as instrumental variables. Because genetic variants are distributed randomly at conception, the risk of confounding (e.g., from comorbidities) is greatly reduced[159]. Considering the opposite effects observed regarding iron status in Paper 1 (low iron status associated with risk of BSI) and Paper 3 (higher iron status associated with risk of sepsis), this might indicate that either 1) there are residual confounding that we could not identify in Paper 1, or 2) the MR methods applied in Paper 3 could not perfectly harbor the non-linear relationship between iron status and risk of severe infections, or 3) a combination of these effects.

Selection bias

Selection bias may arise from the procedures used to select subjects to a study and from factors that influence study participation, or if individuals included in the study population are not a representative sample of the target population[153].

Selection bias in HUNT2

Papers 1 and 2 are prospective cohort studies, where inclusion in the study population was decided by participation in the HUNT2 study, with an overall participation rate of 69.5%, which is considered a very high participation rate in a population health survey[208]. However, the participation rate differed between age groups, with the lowest participation rate in the youngest age group (20–29 years; 49% participation rate) and highest in the group aged 60 to 69 (85.6%). There were also sex differences in participation, with an overall higher participation rate in women (53.2%). The representativeness in men was lowest in the youngest age group. The HUNT2 non-participation study, which was conducted shortly after the completion of HUNT2, showed that health-related mechanisms were unlikely reasons for not participating in the young age group. Health conditions attributed to non-participation in the age groups >70 years[175]. In cohorts where only a small proportion of the eligible population participated, there is a risk of selection bias and that factors not associated with the outcome studied may falsely appear to be associated with the outcome because they associate with study participation.

The high participation rate in HUNT2, and that all BSI events reported in our studies occurred after inclusion in HUNT2, make substantial selection bias unlikely.

Another source of selection bias is missing data. If there is an association between not giving information about an exposure (for example iron biomarker measurements) and risk of BSI, this could lead to selection bias. In our study population, we had almost complete iron status in HUNT2 participants (65,184). In total, information on iron status or important covariates were missing in 2,197 (3.4%) of the HUNT2 participants. We handled missing data by excluding participants with one or more missing variables from the study cohort. We chose this method because we considered the overall missingness to be limited, and when we performed similar analyses in a model with complete cases, the changes in HR in the adjusted model remained the same. This indicates that the adjusted HRs in these models were due to statistical adjustments and not due to the exclusion of participants with missing data[209].

For Paper 2, we had low missingness, and the Cox regression models and cumulative incidence were performed on the complete study cohort. For the mediation analyses, we excluded participants with missing data, and the changes in HRs remained the same in models with complete cases[209]. An alternative approach would have been multiple imputation, but we chose not to perform this as the missingness was relatively low.

In Papers 1 and 2, the loss of follow-up was minor due to the completeness of the HNT HF Sepsis Registry and the high residential stability of the Nord-Trøndelag county, where the net out-migration is only 0.3%. The HUNT Databank is regularly updated with information on residential status and death from Statistics Norway, enabling censoring of participants during follow-up.

Selection bias in UK Biobank, COVID-19 HGI, and iron-status GWAS

In MR, selection bias arises if individuals included in the study population are not representative of the population under investigation, or if participation in either the exposure GWAS or outcome GWAS was associated with their exposure or outcome[160]. In our MR study, the GWAS used for identifying novel iron-related SNPs consists partly of blood donors from Denmark and the INTERVAL study in the United Kingdom[164]. Blood donors are regularly screened for iron depletion and health conditions that could affect iron status, with the possibility of missing individuals with iron deficiency or iron overload disorders. This could potentially introduce selection bias and thereby dilute the effect seen in MR analysis[210]. We performed a two-sample MR analysis, meaning that the included outcome GWASs relied on correct case definitions and identification. Both the exposure and outcome GWASs must be ancestrally homogenous to avoid bias due to population stratification, and the same causal process must operate in both data sets[201]. Sepsis as a phenotype has proven to be heterogeneous depending on the causal agent, with bacterial, viral, or fungal pathogens acting differently on the host immune functions[211]. Timing and correct treatment of infections before they evolve to sepsis, further access to organ-supportive treatment in ICUs, and severity of sepsis might also be different[2].

Sepsis summary-level data were obtained from UK Biobank, defined as explicit sepsis in accordance with Rudd et al[2]. UK Biobank is subject to the "healthy volunteer" effect (i.e., fewer self-reported health conditions, lower all-cause mortality), which is considered a selection bias and not necessarily representative of the UK population[177]. Adding the fact that the UK Biobank only achieved a 5% response rate, it is not representative of the UK adult population and there is the potential of selection bias with potentially misleading genotypic associations[212]. We used data from the COVID-19 HGI, an international collaboration to rapidly assemble summary-level data from COVID-19 cases around the world, including data from population studies[185]. During the COVID-19 pandemic, limited hospital resources and capacity might have influenced hospitalizations, and this could also have influenced the data being reported to this collaboration.

Weak instrumental bias

The introduction of weak instruments could lead to biased effect estimates. We assessed the instrumental variables (i.e., the SNPs used in our MR study) using the F statistic and a threshold of F ~10 has typically been used to define a weak instrumental variable. An F statistic ~10 ensures that the relative bias will be at least < 10% at least 95% of the time, regardless of the numbers of IVs used in the analysis[213]. For our included SNPs, the F statistic range was 22 to 1288 so substantial bias due to weak instruments was unlikely[214].

Survival bias

Some argue that performing population studies of outcomes more frequent in older persons, might lead to survival bias[215]—a phenomenon that arises if the population studied consists of a non-random subset of individuals who have survived long enough to be included. The iron-status GWAS included blood donors from the UK INTERVAL study with a mean age of 41 and 45 years[216], from the Danish Blood Donor Study with a mean age of 25 and 30 years[217] (for women and men, respectively), and from Iceland, where the median age was 47.4 years[164]. On the other hand, the outcome GWASs for sepsis and sex-stratified COVID-19 included participants in UK Biobank aged 40–69 years at inclusion[176]. The mean age in the COVID-19 HGI was 55 years[185]. The genetic associations of the exposure were thus estimated in a younger population than the outcomes studied. This could be problematic if iron status is linked to survival, and indeed there are indications that iron status is linked to longevity; an MR study found an association between higher proxied iron status and reduced life expectancy[218]. We are not aware of the extent to which this may have affected the results in our studies. As sepsis and severe COVID-19 have a greater impact on older adults, some participants with genetic phenotypes related to higher iron status might not have survived to experience an episode of sepsis or COVID-19, most likely diluting the associations in our study.

Information bias

Information bias can arise because the information collected about and from study participants is erroneous. For example if the classification of disease, the exposure of interest, or covariates included in the statistical model is erroneous. In HUNT2, all participants were measured by specially trained nurses to ensure that all participants

had clinical parameters measured with the same procedures, reducing the likelihood for measurement bias. Laboratory measurements included non-fasting serum samples which were analyzed at a single laboratory, ensuring similar handling of the samples. The laboratory measurements reported in this thesis included iron biomarkers (Paper 1), creatinine (Papers 1 and 2), and non-HDL cholesterol (Paper 2). After completion of the HUNT surveys, if there were issues concerning subsequent quality control of the data, impossible values were removed from the HUNT Databank.

If the measurement error is not dependent on other variables, the misclassification is *non-differential*. When the misclassification differs according to the value of other variables, the misclassification is *differential.* Non-differential misclassification of binary exposures results in bias towards the null. Differential misclassification can exaggerate or underestimate the effect[153].

Potential misclassification in Papers 1 and 2

BSI events were prospectively recorded in the HNT HF Sepsis Registry, reducing the risk of differential misclassification of exposure variables based on outcome measures. BSI was defined as a positive blood culture; subjects who experienced a BSI after participation in HUNT2 but who were not diagnosed due to non-performance of blood cultures, or to antibiotic treatment prior to blood culture collection were not registered as cases in the two studies. Investigating BSI is dependent on clinicians' suspicion and decision to submit blood cultures for testing, thus there is a chance that some cases go undetected. This would be considered a non-differential misclassification, and may have reduced the effect estimate of the studied associations. Since BSI is a rare event in the population, we believe that this is a minor problem in the studies. For Paper 2, we cannot rule out whether the clinical presentation of infections is different in men and women and if this could result in disproportionate blood culture sampling, depending on sex. However, we have no reason to believe that the threshold for taking blood cultures would differ systematically between the sexes.

We did not include patients with positive blood cultures due to potential skin contamination[178], which could have introduced information bias. The inclusion of blood cultures with clinical significance were verified in the studies of *S. aureus* and *S. pneumococcus,* where ~98% of the cases met the SIRS criteria of being septic[166,167], and the exclusion criteria remained the same throughout the study period.

Self-reported information

For the HUNT2 papers, self-reported cardiovascular disease, lung disease, and diabetes were included as covariates. In paper 2, we also included education level, smoking, alcohol use, and cancer history. The validity of self-reported information relies on subject memory, which is prone to recall bias[219]. For self-reported diabetes in HUNT, a validation study was performed and found high concordance between the patient administered questionnaires and medical files of general practitioners[220]. People also tend to underreport smoking and alcohol use[221], so there is a possibility that the alcohol consumption among HUNT2 participants was underreported.

However, the HUNT2 questionnaires have been described as giving participants a feeling of anonymity compared to a diagnostic interview, as they were filled in and returned by mail[175].

Long follow-up and potential changes in exposure values and covariates

The long period of follow-up of nearly 17 years from inclusion in HUNT2 to the BSI event for some participants poses both advantages and challenges. BSI is a rare condition, and the long follow-up allowed us to include around 1,800 BSI cases in the association analyses. However, participants' iron status might have changed during the follow-up due to medical conditions or later identification and correction. However, nutritional surveys among adults in Norway conducted in the same period as our study indicate that iron intake was below the recommended daily amount[222], suggesting that iron deficiency was stable at the population level. This is also supported by a population study from Portugal that found high prevalence of iron deficiency and that iron deficiency was largely underdiagnosed[48].

We believe that participants' biological sex would not have changed during follow-up. However, there is the potential that some of the covariates used in the analyses changed during follow-up as the population aged. We believe that this constitutes a non-differential bias, which would likely have led to underestimation of the associations.

Competing risk bias

A competing risk is an event that precludes the occurrence of the primary event of interest[223]. For survival analyses, it is important to be aware of potential competing risks and to what extent they might bias risk estimates. Ignoring competing risks might lead to upward-biased Cox regression results. In both Papers 1 and 2, we prepared cumulative incidence curves taking death as a competing risk into account. When studying a cohort that ages during follow-up, and the incidence of the outcome of interest increases with increasing age, death from other causes than BSI is considered a competing event. For both studies, the numbers of competing events were approximately 10,000 during follow-up. The directions of cumulative incidence supported the Cox regression results, but the cumulative incidence curves had some differences from the Cox regression results. In Paper 1, for serum iron and TIBC, they showed higher incidence of BSI for both low and high iron status. In Paper 2, for sex differences in BSI caused by *S. pneumoniae*, the cumulative incidence curves showed no clear evidence of an increased risk in men compared to women, whereas the HRs pointed towards an increased risk in men (HR 1.36, 95% CI 1.05–1.76). For sex differences in BSI caused by *E. coli*, the female predominance was obvious in the cumulative incidence curves, but not in the Cox regression results. This reflects the importance of using different analytical approaches to investigate a relationship in a time-to-event setting with possible competing risks[224].

External validity

External validity refers to generalizability; to what extent our results apply to other populations[153]. The HUNT studies provide a good basis for evaluating the health status and serves as a source of prospective health data

to evaluate risk factors for future disease in the adult population in Nord-Trøndelag region. The adult population in Nord-Trøndelag is fairly representative of Norway regarding age distribution, morbidity and mortality, economy, industry, sources of income, and geography which is mostly rural and sparsely populated and there are no large cities in the Nord-Trøndelag region[175]. In the non-participation study conducted shortly after HUNT2, non-participants had lower socioeconomic status and higher over-all mortality then participants, which may indicate that this study population is not completely representative of the overall population[174]. In HUNT2 the participation rate differed in age groups with lower participation rates among young adults. However, the non-participation study showed that health related issues were not the reasons for not participating among young adults[174]. Norway has a public well-fare system with equal access to health care. Incidence of BSI varies according to the population studied[20,22,25,37,38], the same applies for the prevalence of iron deficiency which is more common in disadvantageous subpopulations[46,48,49,79]. In HUNT2, subjects with high iron status were scheduled for further examinations and those diagnosed with hereditary hemochromatosis were followed closely for this condition thereafter. This may have affected the results in persons with high iron status. However, we believe that the relationship between iron status and risk of BSI with underlying biological explanations examined in paper 1 are probably generalizable to any population where iron deficiency is prevalent. The same applies to sex differences assessed in Paper 2, but with the precaution that access to health care might be different and sex dependent in some populations and cultures[138,139]. The population-based design add to the generalizability of our studies, as the optimal means of defining the epidemiology of infectious diseases, compared to non-population-based cohorts[36]. For Paper 1 and 2 including participants from HUNT2 with the possibility of prospective follow-up of blood culture positive BSIs in the HNT HF Sepsis Registry, gave us a unique possibility to investigate risk factors in this setting.

For the results of our MR study, we think the relationship between genetically predicted iron status and risk of sepsis and COVID-19 are generalizable, but the actual associations might be limited to populations of European decent due to the nature of GWASs, where different allele frequencies, linkage disequilibrium and population stratification come into effect[225].

Mediation

A mediator is a variable that lies in the path between the exposure and the outcome and could partly explain the effect of the association. Mediation analysis is important for understanding causal relationship and identifying possible intervention points. The conflicting results from previous studies concerning sex differences in incidence of sepsis and BSI could be due to sex differences in risk factors known to affect BSI risk[15,19,20], or they could be caused by biological factors related to sex[125]. Sex cannot be confounded, as our biological sex is randomly determined at conception and no other factors can influence this process. In a traditional analysis of a causal relationship or association, a mediator should not be adjusted for[153]. Using mediation analyses, we can assess the proportion of the total effect of the association between sex and BSI that works through the mediator(s) of interest.

We applied the IOW method, which is known to be robust, adding multiple mediators en bloc; this allowed us to conduct mediation analyses in a time-to-event context[196,197]. According to VanderWeele and Vandsteelandt, assessing one mediator at a time will generally fail, if the mediators affect one another[196]. We included possible mediators in a sequential order decided by their mutual influence on each other and the outcome of interest. Based on subject matter knowledge, we considered the potential pathways of the included mediators, and arrived at three sets of mediators that would best meet the model assumptions. This entails that the three models must be causal descendants of each other and that the model with the complete set of mediators likely reflects the best modeling of the associations[196].

The mediators were measured at inclusion to HUNT2. For health behaviors, more men reported smoking, and they reported higher alcohol use. We also observed higher prevalence of obesity among women in HUNT2. Health behaviors and education attainment, mediated 10%; adding cardiovascular risk factors to health behaviors and education, the proportion mediated lowered from 10 to 5%, which indicates some interactions or common pathways for these mediators[196]. This result might be due to some of the mediators included in Model 2 being more common or harmful among women, or that the mediators might reduce the risk of BSI. The complete model with all mediators included accommodates the assumptions required[195,196], explaining 34% of the excess BSI risk in men.

STRENGTHS AND LIMITATIONS

Major strengths with the thesis

This thesis provides a thorough review of the existing literature and knowledge on iron status and sex as risk factors for severe infections. We had access to a large population-based cohort study (HUNT2) with a high participation rate and baseline information on a vast range of health-related topics, including clinical examinations and laboratory measurements. The uniqueness of HUNT2 comes from the opportunity to link participants with prospectively recorded information on BSI events in the HNT HF Sepsis registry, the linkage to the Norwegian Population Registry with information on dates of death and migration, and the possibility to include data from the Cancer Registry. The long-term follow-up enabled us to include a substantial number of BSI events with the opportunity to conduct subgroup analyses. The linkage to microbiological records with information on bacterial species gave us the opportunity both to assess the most common infecting bacteria, and to exclude bacteria associated with contamination. This assures that the majority of positive blood cultures in this material most likely represent serious infections which has been published in other work in the group[22,166,167]. Further strengths include the use of several epidemiological methods to assess and explore the associations, and the ability to perform sensitivity analyses to obtain our results.

Strengths of the studies included in this thesis

Paper 1

We had information on potential confounding factors with the possibility of thorough adjustments in the analyses. In the main analyses, we adjusted for baseline information on comorbidities, BMI, age, and sex as they could influence both iron status and the risk of BSI. To further exploit potential confounding by undiagnosed or pre-clinical diseases we performed a sensitivity analysis, in which we excluded BSI events during the first two years of follow-up. The association with increased risk of BSI in iron-deficient subjects remained unchanged. Taken together with cumulative incidence, our findings indicate that iron deficiency could be persistent in our subjects, supported by population studies on iron status and nutritional surveys in Norway at the same time[48,222]. Additionally, we performed different sensitivity analyses to explore whether a diagnosis of cancer or cancer treatment and rheumatic or inflammatory bowel disease could affect the associations. The rich baseline information in HUNT2, information from the Cancer Registry, and the HNT HF Sepsis Registry on potential confounders enabled us to explore the association. Using cumulative incidence, we acknowledge competing risk factors and we show that the increased risk of BSI in iron-deficient subjects is evident throughout a follow-up period of nearly 17 years. The overall conclusion is that low iron status was associated with increased risk of BSI after adjustments for potential confounding factors and competing risks.

Paper 2

With the conflicting results regarding sex differences in infectious diseases in previous studies, we wanted to examine in depth the association between sex and BSI. Mediation analyses enabled us to measure/quantify the effects of health behaviors, education level, cardiovascular risk factors, and comorbidities on the relationship and provide important insight on sex differences in risk of BSI. Adding information on the specific bacteria causing BSI from the HNT HF Sepsis Registry, we identified that men had a substantial increased risk of BSI caused by *S. aureus*, and women had a higher cumulative incidence of *E. coli.* Using cumulative mortality, we also showed that men had higher BSI-related mortality compared to women throughout the study follow-up. As BSI incidence rises with increasing age for both sexes, we adjusted all analyses for age. We were also able to conduct age-stratified analyses to assess whether menopause could alter women's risk of BSI, and found that BSI incidence was low in the young age groups for both sexes, but surges more pronounced in men as they aged. There was no marked difference for women at the age of menopause.

For both studies, we were able to study BSI incidence and mortality in a large population without the referral bias seen in single-institution studies of BSI. The complete identification of all BSI events enabled an accurate estimation of incidence and mortality in the population, with an opportunity for risk factor identification[36] and for Paper 2 using this information in mediation analyses.

Paper 3

Major strengths included the use of large GWASs summary data for iron status, sepsis, and severe COVID-19. Large sample sizes were important for drawing causal conclusions using genetic data[160]. We used summary data from European descendants to reduce confounding due to population stratification[151,152]. Observational studies measuring iron status at time of infection might be biased by the acute phase response leading to iron depletion and hyperferritinemia, which we avoided using an MR framework. Due to the MR methods' use of genetic instruments, the possibility of confounding was limited. MR carries the potential to exploit life-long effects of the exposure on the outcomes studied. Although, when investigating the risk of sepsis and severe COVID-19—infectious diseases that depend on individuals being exposed and infected by the pathogens—not all individuals carrying the genetic susceptibility will have the disease.

Our main MR estimates were similar using IVW, weighted median, and weighted mode methods, and we used various strategies to detect and account for potential pleiotropy. The slight difference in estimation and CIs between the different MR methods were expected and most likely do not represent actual differences[156]. Using sex-stratified summary data for both iron status and COVID-19 outcomes, we were able to investigate potential sex differences in the associations between iron status and risk of COVID-19. However, the precision of our estimates could not support a biological difference between men and women.

Limitations in the included studies

Paper 1

Serum iron is prone to diurnal variation and becomes higher after eating an iron-rich meal. We lack measurements of ferritin, which is widely considered a standardized assessment of global body iron stores, but largely influenced by inflammation[226]. Hemoglobin was not measured in HUNT2, leaving us unable to diagnose iron deficiency anemia in our subjects. However, hemoglobin concentration becomes abnormal only in long-standing iron deficiency and is influenced by a wide range of medical conditions[226]. Some studies have shown that TSAT or TIBC alone is an alternative diagnostic test for iron deficiency[227,228]. Neither serum iron, TSAT, TIBC, nor ferritin are single biomarkers reflecting overall systemic iron status. Iron biomarker measurements have not been repeated in the consecutive HUNT surveys and iron status was measured up to 15 years before the outcome. We do not have any iron status measurements at the time of BSI. Iron status could have changed during the follow-up period, possibly due to later identification and correction by supplementation. However, any potential misclassification of iron indices caused by diurnal variation, non-fasting blood sampling, or the single measurement would most likely be non-differential, meaning that it is not related to later risk of BSI, and is therefore likely to have led to an underestimation of the associations. In nutritional surveys among adults from Norway in the same period as our study, the intake of iron was below the recommended daily amount[222], suggesting that iron deficiency was stable at the population level. The histograms of the iron biomarker measurements in HUNT2 also confirm the impression that the population was in the lower range of the normal

range of the iron biomarkers. This is supported in a population study from Portugal, where the prevalence of iron deficiency was high and largely underdiagnosed[48].

Paper 2

The mediators were only measured once at inclusion to HUNT2 and could have changed during the 17 years of follow-up. This potential misclassification would most likely be non-differential and lead to underestimation of the mediating effect[219]. Another concern is the subjective assessment of some mediators, and dichotomized mediators are more prone to possible mediator misclassification. This could lead to underestimation of the indirect effect and overestimation of the direct effect[195]. We lack information on immunosuppressive medication and use of statins before or at time of infection, and these are known risk factors for BSI and BSI mortality[168,229]. Investigating BSI is dependent on clinicians' suspicion and decision to submit blood cultures for testing, hence the chance of some undetected cases. Further, we cannot rule out whether the clinical presentation of infections is different in men and women, and whether this may result in disproportionate blood culture sampling depending on sex.

Paper 3

Iron status fluctuates during our lifetime, during periods of higher demand and need (e.g., pregnancy and growth), in situations with increased losses (i.e., blood loss or critical illness), and due to chronical medical disorders[46,207]. Genetically predicted iron status does not perfectly reflect this time-varying exposure[230]. During infection and inflammation, iron status changes substantially, further exacerbated by tissue destruction and cell death. The U-shaped risk relationship that has been proposed for the extremes of iron status[101,111] might cause an attenuated association when evaluated as linear in two-sample MR methods. Non-linear MR methods could be more suitable to explore this U-shaped relationship, but require large GWAS with both measurements of iron biomarkers as well as the outcomes of interest[162].

Bias due to overfitting could be a concern when summary-level data are used and the two data sets overlap. We were not able to identify the number of participants in the INTERVAL study that also contributed to UK Biobank, but we believe this only applies to a small number of individuals. The same holds for the Danish and Icelandic participants who contributed to the iron GWAS, as Denmark and Iceland also delivered data to the COVID-19 HGI. Studies show that overlap between exposure and outcome GWAS do not bias the results as much as previously thought[160]. Unfortunately, we were not able to find sex-disaggregated summary-level data on sepsis and were unable to assess potential sex differences in the associations.

DISCUSSION OF FINDINGS

Iron status as a risk factor for severe infections

In a large, population-based cohort we showed that iron deficiency was associated with increased risk of a future BSI. In an MR framework, we further elucidated the role of genetically predicted iron status and showed that

higher iron status was related to increased risk of sepsis. We also found a tendency for the same relationship with higher risk of being hospitalized with COVID-19. Why do we have different findings from the two studies? Do they contradict each other?

Previous studies have indicated a U-shaped risk profile of iron status[100,101]. To best explore this hypothesis, in Paper 1, we categorized our iron indices into quintiles using the middle quintile as a reference, and at the extreme ends ≤ 2.5th percentile or ≥ 97.5th percentile. A reason we observed no clear association between high iron status and risk of BSI in our observational study could be because the low number of BSI cases in the high iron status categories precluded precise estimates of the associations. Another reason could be that all subjects with high iron status in HUNT2 were scheduled for further examinations, and those diagnosed with hereditary hemochromatosis were followed closely for this condition thereafter. Nevertheless, the cumulative incidence curves showed that both high and low serum iron and TIBC had the highest and second-highest incidence of BSI during the follow-up period. This uncertainty was a motivation to perform an MR study. Initially we performed a non-linear MR in the genotyped HUNT2 population, but low numbers of genotyped HUNT2 participants with iron status measurements left us without power to identify any relationship (results not shown or published). Instead, we acknowledged the increasing numbers of GWAS with publicly available summary data from large consortia. The MR approach can help overcome the limitations of traditional epidemiological studies and potentially enable causal inferences on the effect of iron status on risk of sepsis and severe COVID-19. In contrast with the observation study, our MR study suggested that genetically predicted higher iron levels were associated with an increased risk of sepsis and a tendency towards a higher risk of being hospitalized due to COVID-19.

We believe that the two studies elucidate the complex role of iron in the context of severe infections. Both studies have their strengths and limitations, and the findings add weight to an increasing body of evidence linking iron status to risk of infections. Iron is a crucial element in a wide range of cellular processes in almost all living creatures (including pathogens), and it lies at the center of a constant battle between host and invaders[86]. During infections, iron is sequestered into stores, and iron absorption is limited—a process mainly induced by increasing levels of hepcidin[100]. However, adequate iron levels are needed to maintain immune functions, and iron depletion may reduce important immune functions[88,231]. Viewing our results within this context, immune defense mechanisms may be relatively more depressed than the ability of bacteria to sequester iron in low iron environments, resulting in a net increased risk of BSI in iron-deficient individuals. For iron overload situations, during acute infections, the person's ability to cope with the acute iron load due to tissue and cell destruction may overwhelm the iron acquisition system and render the individual both vulnerable to the toxicity of free iron but also to pathogens.

Iron deficiency[232], iron overload[50], and iron supplementation and fortification programs without adequate infection surveillance have been linked to increased risk of infections[95,96]. A recent meta-analysis found evidence of increased infection risk in patients with iron deficiency anemia who had intravenous iron supplementation versus oral or no iron supplementation[233]. There are indications of better immune functions after fortification programs

with reduced burden of respiratory infections in children[234]. Better adaptive immune responses in iron-repleted children have led to iron supplementation in advance of vaccinations[235]. The suboptimal antibody response to some vaccines in iron-deficient individuals has led to ongoing trials to investigate iron supplementation prior to COVID-19 vaccination[236] (ClinicalTrials.gov Identifier: NCT04915820). There are several plausible pathophysiologic explanations for an increased risk of BSI at low iron levels. Reduced T-cell function[89,91], reduced bactericidal activity of macrophages[88], and decreased ability to produce inflammatory cytokines[90,231] have been shown in iron-depleted individuals.

Iron overload alters the chemotactic and phagocytic properties of neutrophils and their ability to kill invading pathogens. Patients with hemochromatosis are more prone to infections by various pathogens, including *E. coli*, V*ibrio vulnificus*, *Vibrio cholera*, *Klebsiella* species, *Listeria monocytogenes*, and *Shigella* species[50]. Elevated iron levels have been associated with a poorer prognosis in patients with sepsis[237,238]. Excess iron is also associated with increased morbidity of and mortality from hepatitis C[239], and changes in iron metabolism might exacerbate the development of AIDS from HIV infection[240]. Interestingly, many viruses target important proteins in the iron metabolism process to capture iron[239].

MR has the potential to exploit life-long effects of the exposure on the outcomes studied. Iron status fluctuates throughout the lifespan, affected by periods of higher demands and low nutritional supplementation[46]. MR estimates can be interpreted as the lifetime effect of the exposure—in our study, the lifetime effect of iron status[160]. When investigating risk of sepsis and severe COVID-19, which are infectious diseases that depend on individuals being exposed to and infected by the pathogens, not all individuals carrying the genetic susceptibility will eventually have the disease. This is not only a challenge when using the MR framework, but also for observational studies of infectious diseases, like those reported in Papers 1 and 2.

Explaining sex differences in bloodstream infections

We show that sex matters in BSI. Sex differences in response to pathogens are evolutionarily well preserved. Men and women have different genetic makeups, anatomies, and sex hormones, all of which directly alter and determine how we protect ourselves and fight infections[125]. Reports on a wide range of different pathogens show that they exhibit large sex differences in the incidence, intensity, and severity of infections[129,241]. For COVID-19, a substantial sex disparity with a male predominance was observed in many countries among hospitalized cases and mortality[242-244]. For sepsis and BSI, the current literature investigating sex differences is scarce and the results are conflicting depending on the population studied[2,52,55,121-123]. Our study advances the existing literature by examining a population-based cohort in contrast with previous studies on sex difference in BSI and sepsis that have mainly been performed in ICU cohorts, typically in selected patient groups, and not in the general population.

Only a few studies have conducted a population-based assessment of BSI epidemiology. Uslan et al. found higher incidence of BSI among men, with a substantial difference in the incidence of *S. aureus* BSI[124]. Laupland

et al. conducted a population-based study in Canada and found an overall higher incidence among men, but the sex differences were age-dependent, with higher incidence in the young age (<10 years) and older (>50 years), whereas in the age between 10 to 49 years, women were more at risk[20]. Kontula et al. identified sex differences in BSI incidence and mortality in Finland in the period from 2004-2018 with a male predominance in infants and among persons ≥40 years of age, among persons aged 20-29 years the BSI incidence was higher among females[25]. They also identified that gram positive bacteria were the most common cause of BSI in men, whereas gram negative bacteria were more common in women[25]. We found that the sex differences in BSI risk are evident after predicted age of menopause, indicating that alterations in both innate and adaptive immune functions with age may be sex specific. Women face an abrupt decline during menopause, whereas men have a steady decline from the second decade of life[118]. Nevertheless, the Global Burden of Disease Study, which included results from 195 countries and comprised all age groups, found higher sepsis incidence among women, while sepsis mortality was higher in men[2]. This indicates that the sex disparities also depend on high- versus low-income countries, with local challenges like poverty, nutrition, health care facilities, and access to health care[138,139,245].

In our study we were not able to identify any sex differences in the age-group younger than 50 years as there were very few cases of BSI, and children were not included in HUNT2. Interestingly, in a retrospective study of 142 prepubertal children with severe sepsis, they found stronger inflammatory response in girls, suggesting that sex hormones are not the only origin of the sex differences observed[246].

In our work to assess sex differences in BSI, we were surprised that the most obvious biological factors have not been explored more deeply in relation to severe infections. A substantial number of observational research articles on severe infections adjusted for sex—even identifying sex bias—but did not assess the reasons behind this[19,20,25,40,52,124,245,247]. This is pervasive as ignoring sex (and gender) differences by just controlling for them statistically, leave researchers, clinicians and public health authorities unaware of how sex and gender contribute to global health outcomes[247].

The rich baseline information from HUNT2 allowed us to investigate how known BSI risk factors mediated the association between sex and BSI. As noted earlier, we used the IOW method, which is known to be robust, using multiple mediators en bloc; this enabled us to implement causal mediation analyses in a time-to-event context. This sequential approach allowed us to examine the excess risk in men mediated through health behaviors and education, cardiovascular risk factors, and comorbidities. The complete model reflects the best modeling of the associations, and the proportion mediated was 34%. Interventions to reduce modifiable risk factors in the population will likely reduce the burden of BSI, particularly in aging men with a high burden of known risk factors. This is an important finding that deserves further investigation.

We found a substantial higher risk of BSI due to *S. aureus* in men, and we show male predominance of *S. pneumonia* BSI, whereas women had higher cumulative incidence of *E. coli*. Higher incidence of *S. aureus* in men have been reported earlier[124,248]. This is an important finding as preventive measures like eradication or temporary suppression could lower the risk of invasive infections[249]. For *S. pneumoniae* male predominance

have been observed before, and is considered a major cause of morbidity and mortality in elderly[167,250]. Immunization programmes which was introduced in Norway for children in 2006 and elderly and risk-groups from 2013, have shown promising effects on incidence of invasive pneumococcal disease[167]. Higher incidence of *E. coli* BSI in women have been attributed to anatomical differences between men and women, including urethra length, but also by sex-based variation in testosterone and estrogen. *E. coli* BSI usually arises as a complication of focal infections in the urinary or gastrointestinal tract, but occasionally cause primary bacteriemia[251]. Interestingly, a female predominance has been observed in cases of hemolytic-uremic syndrome caused by shiga-toxin producing *E. coli*[252].

BSI mortality was higher in men than in women, which is in line with another population-based study of BSI[253]. Even so, there is conflicting evidence, with some ICU studies reporting higher sepsis-related mortality in women[54,122]. These conflicting reports might relate to the cohort studied[36,253], sex differences in treatment[54,56], or sex differences in immune responses[129,254]. Sex affects the shape of immune responses and this is attributed to genetic, hormonal, and environmental factors[125,129,254]. The human X-CHR encodes a number of critical genes involved in the regulation of immune functions[147]. Efforts to map the entire genome have also identified genes on the Y-CHR with associations with immune functions[148].

RESEARCH IN CONTEXT

Epidemiological methods are the cornerstone in "translating" knowledge from basic research in biology and physiology into hypotheses for preventive measures and treatments of diseases. Using population-based observational data with strict control and awareness of potential bias, we have the opportunity to reveal important relationship between risk factors and outcomes of interest. We supply the scientific society with tools to further place this knowledge into clinical settings and improve the health of populations, but also the individuals who have the diseases being studied. Our observational and genetic studies fit very well into this category, and I would like to place our work and results in this context.

There is substantial knowledge on iron's role in our metabolism, and the effect of iron levels on important functions within our immune system is well established, but neither is completely understood. The discovery of hepcidin in the late 90s placed iron metabolism as a central part of our immune system[100]. Iron status is also linked to other diseases—often those in which inflammation cascades are activated (e.g., cardiovascular diseases, chronic pulmonary disease, cancer, renal failure, diabetes type 2 and obesity)[255-259] and iron status is linked to longevity[218]. Anemia of inflammation is prevalent in patients with prolonged activation of the immune system and is related to higher hepcidin levels during a state of inflammation. Hepcidin reduces intestinal iron uptake and cause iron retention into iron stores, in particular iron sequestration into macrophages, resulting in iron-restricted erythropoiesis (i.e., functional iron deficiency)[73].

Nevertheless, iron status at a population level is rarely investigated, and the limited assessment of iron status in populations is worrisome. Even in the HUNT Study, iron status has only been measured once (in HUNT2), despite the finding that about 10% of HUNT2 participants had clinically relevant low iron status. Iron deficiency is the most common nutritional deficiency, and successive measurements of iron status were not prioritized in HUNT3 or HUNT4. This is of great concern when iron-status-related disorders rank as one of the leading causes of disadvantage and disability in the world, according to the Global Burden of Disease Study[49].

Likely because of the scant number of population-based studies with iron measurements, there were no population studies that had examined the associations between iron status and BSI (or sepsis) before our study. Some studies examined iron status at hospitalization or ICU admission with sepsis[260]. Tacke et al. showed that iron status deviations were linked to short- and long-term survival in septic ICU patients, with septic patients having low serum iron and low TSAT showing better prognosis. They also identified that patients admitted to the ICU with TSAT >55% displayed poor short- and long-term survival[104]. In line with this, high iron status was identified as an individual risk factor of 90-day mortality in septic ICU patients[238], and another study found reduced survival in septic patients admitted to ICU with evidence of high iron status measured at admission[237]. Septic patients face a katabolic state with decreased iron consumption on the one hand, but increased iron release from destroyed tissues and erythrocytes on the other. Free iron leads to the production of reactive oxygen species and further exaggerates the development of multi-organ failure[238]. Relating this to the MR results, which show higher genetically predicted iron status and increased risk of sepsis and hospitalization due to COVID-19, this might indicate that individuals with high iron status are less able to handle the acute iron load seen in sepsis or severe COVID-19.

The challenge when investigating iron status as a risk factor for severe infections is the profound physiological changes in iron metabolism at the time of infection. Because of this physiological change in iron homeostasis, we cannot compare the results from population-based studies with studies where iron status was measured at the time of infection. It could be that long-standing iron deficiency renders an individual at risk for infections, and further at increased risk of developing a BSI or sepsis, due to suppressed or altered immune functions. At the same time, low iron status measured at hospitalization could be the best physiological response to the infectious threat. Patients with high iron status at hospitalization might have indications of a more severe implication of their immune functions (as they are not able to withhold iron from invading pathogens), leaving those patients more at risk of a severe course or death. Studies on sepsis and COVID-19 may indicate such associations[104,111,237,238]. Many pathogens become more virulent when iron is made freely available[261], a process seen in iron overload but also due to diverse pathological mechanisms of sepsis, such as lysis of red blood cells, alteration of pH, hypoxia, and tissue destruction. The same altered physical conditions may affect the ability of polymorphs to kill bacteria: the phagocytic cells become overwhelmed, and the bacteria outgrow the phagocytic capacity[262].

In line with this, the results of our MR study indicate that genetically predicted higher iron status was associated with increased risk of sepsis and showed a tendency towards higher risk of being hospitalized due to COVID-19.

In sex-stratified analyses, we found evidence of a more pronounced effect of high serum iron being related to COVID-19 hospitalization in women, but we could not suggest a biological difference between the sexes due to low precision. We replicated the findings from a previous MR on sepsis that had used other genetic instruments as exposures[263]. The findings add weight to the importance of strictly controlling iron status in the context of severe infections.

Sex is an important biological factor which we encounter in epidemiology, pathophysiology, and pharmacology. Sex modifies major causes of death and morbidity, but what we clinicians know about the diagnosis, treatment, and prevention of disease originates from research performed on male cells, male mice, and men[56]. The human X-CHR encodes a number of critical genes involved in the regulation of the immune system. It is becoming increasingly clear that sex extensively influences the host immune responses, but this sexual dimorphism is underappreciated, and sex bias is a major challenge in clinical trials[56]. In sepsis, females are systematically underrepresented in clinical trials[264]. A recent systematic review of the effects of sex on sepsis treatment such as antibiotics and fluids in animal models, only identified two relevant articles[265]. There are no preclinical studies investigating sex differences in the effect or dosage of important vasopressors used in sepsis. Highlighting the scarcity of studies investigating the effect of sex on foundational therapies for sepsis and BSI.

In our study, we show that sex disparities in BSI cannot be explained by comorbidity burden, health behaviors, and/or educational level alone. The clinical implications of our findings largely depend on elucidating the underlying mechanisms. Sex affects the shape of immune responses, which are attributed to genetic, hormonal, and environmental factors. Sex hormones are important modulators of immune functions and responses; testosterone and progesterone are immunosuppressive, while estradiol is immunoenhancing. Few studies have investigated human sex hormones' effects in severe infections and sepsis. There are indications of salutary effects of estrogens following circulatory shock conditions as seen in sepsis[266]. Despite experimental studies indicating beneficial effects of estrogens, clinical studies are challenging to conduct as they will have to consider the phase of estrous cycle, menopause and current medications, nevertheless estrogens provide a potential therapeutic target for sepsis[267]. Interestingly, in COVID-19, where men were more prone to a severe course of infection, the use of antiandrogens in men has shown promising results regarding severity[268,269]. Furthermore, a review of the health records of post-menopausal women with regular use of estradiol, found that the fatality risk of COVID-19 was reduced by more than 50%[270].

Concluding remarks

The studies included in this thesis have identified iron status as a risk factor for BSI, sepsis, and severe COVID-19, and sex as a risk factor for BSI and BSI mortality. We have explored the risk factors using different epidemiological approaches. The strengths and limitations of the applied methods have been discussed and addressed with sensitivity analyses where possible and appropriate.

The studies provide foundational observation of iron status in the context of severe infections and existing sex differences. BSI, sepsis, and COVID-19 represent a global burden of disease. Our studies contribute important information for researchers, clinicians, and policymakers concerning the epidemiology, possible preventive efforts, management, and future research in this field. They provide evidence that iron status increases the risk of severe infections. The controversies regarding iron supplementation demonstrate how delicate iron homeostasis is, and the challenges we meet concerning infections. For sex differences in BSI, we argue that the most important message from our study is to acknowledge this sexual dimorphism, and that sex bias is a major challenge in many clinical trials[56], including studies of BSI and sepsis[264,265]. We propose increased efforts to improve knowledge about sex differences, not only in the epidemiology of severe infections, but also in managing and treating men and women with infections differently—to potentially save more people's lives.

FUTURE PERSPECTIVES

Iron matters in severe infections. In the vast puzzle of identifying and targeting risk factors for severe infections, we still need epidemiological studies to guide large clinical studies in sepsis. Iron status in the context of infections is truly a double-edged sword, where iron supplementation and treatment with iron chelators must be balanced against harmful effects. The most important message from our studies is to include iron status measurements in population studies. Access to large population-based studies, including the GWAS consortia, will potentially reveal new knowledge about this essential trace element for several conditions.

Sex matters in severe infections. Recognition, awareness, and knowledge of sex differences in severe infections is important. There is substantial need for targeted research on how to address the observed sex differences in BSI and sepsis to achieve a longer and healthier life for both men and women.

When planning future studies in sepsis, one should remember the global burden of disease, where both iron status and sex differences vary depending on the population studied. In low-income countries, iron deficiency is of great concern, and globally, women have higher incidence of sepsis[2]. Women in low-income countries still face a high burden of infection during, pregnancy and birth. These are important factors not taken into account by the studies included in this thesis.

Being male or female is an important variable affecting health and disease throughout the life course. Future studies should focus on how virulence factors and host factors such as sex hormones, genetics, and epigenetics play a role in the sex disparities in infectious disease.

To make progress in sepsis research, we must continue systematically elucidating the underlying mechanisms behind our observations; this is of utmost importance for public health leaders, researchers, and clinicians, as they can help identify individuals particularly at risk and instigate preventive actions. Here, my hope is that we disentangle the risk factors for severe infections and understand why patients develop sepsis.

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Papers 1 to 3

«i vårt hus er det mange rom

og du har flytta inn i det mørkeste

her sitter jeg og holder deg i hånda

og lar hendene snakke

jeg later som om hendene vet noe om deg

de sier de vet at det må gå over

jeg later som om hendene mine

er fulle av noe

som kan få deg til å lyse

hendene mine forstår noe

som jeg ennå ikke har forstått

de holder glasset mot munnen din

de gjør det ikke vanskelig

å mate et voksent menneske

de får det til å se enkelt ut

å holde andres gråt i hendene

de følger en oppskrift

jeg ikke kan huske å ha lært

hvordan kan jeg ha unngått å få med meg dette

som hendene har vist om bestandig

Jeg legger hodet ditt fra meg og går ut

i hagen, der åpner jeg ansiktet alene»

- Lina Undrum Mariussen

«Finne deg der inne og hente deg ut»

Paper 1

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ORIGINAL

Association of iron status with the risk of bloodstream infections: results from the prospective population-based HUNT Study in Norway

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Abstract

Purpose: As iron is essential for both immune function and microbial growth, alterations in iron status could influence the risk of infections. We assessed the associations of iron status with risk of bloodstream infections (BSIs) and BSI mortality.

Methods: We measured serum iron, transferrin saturation (Tsat) and total iron-binding capacity (TIBC) in 61,852 participants in the population-based HUNT2 study (1995–97). Incident BSIs (1995–2011) were identified through linkage with the Mid-Norway Sepsis Register, which includes prospectively registered information on BSI from local and regional hospitals. We assessed the risk of a first-time BSI and BSI mortality with the iron indices using Cox proportional hazards regression analysis.

Results: During a median follow-up of 14.8 years, 1738 individuals experienced at least one episode of BSI, and 370 died within 30 days after a BSI. In age- and sex-adjusted analyses, BSI risk was increased among participants with indices of iron deficiency, serum iron ≤ 2.5th percentile (HR 1.72, 95% CI 1.34–2.21), Tsat ≤ 2.5th percentile (HR 1.48, 95% CI 1.12–1.96) or TIBC ≥ 97.5th percentile (HR 1.46, 95% CI 1.06–2.01). The associations remained similar after adjusting for comorbidities and exclusion of BSI related to cancer, rheumatic illnesses and inflammatory bowel disease. BSI mortality showed similar associations.

Conclusion: Indices of severe iron deficiency are associated with an increased risk of a future BSI.

Keywords: Bacteraemia, Sepsis, Iron, Epidemiology, Population based

Introduction

Bloodstream infections (BSIs) cause sepsis and critical illness and are major causes of morbidity and mortality worldwide [1, 2]. Due to its large impact on global health, there have been increased initiatives to identify modifiable risk factors for BSI [1, 3]. So far, we know that the risk of acquiring BSI depends on both host factors such as social and demographic factors (e.g., age, nutrition, lifestyle and poverty) [1, 4] as well as biologic factors such as host genetic susceptibility, chronic medical disorders (e.g., cancers, obesity, diabetes) [3, 5] and the ability to mount an adequate immune response to the invading bacteria [6]. Moreover, differences in virulence and

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antimicrobial susceptibility of infecting microbes also influence the incidence and mortality of BSI [1].

Recent advances in adaptive and innate immunity have demonstrated an essential role of systemic and cellular iron-regulating mechanisms in protecting hosts from infection [7]. On the other hand, most microbes also depend on iron for their pathogenicity, and some bacteria (e.g., *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas*) have evolved the ability to scavenge iron from host iron-binding proteins such as transferrin [7]. Iron is tightly bound to transferrin to control the balance between the host's need for iron in cellular metabolism and restricting invading bacteria from obtaining iron [7]. Disturbances in this delicate homeostasis between free iron in serum and transferrin-bound iron could clearly influence the risk of BSI and sepsis [8].

Most studies of the association between disturbances in iron metabolism and risk of infections have been performed in children with iron deficiency anemia in developing countries. While some studies have shown an increased risk of infections such as respiratory tract infections [9], others have found that iron-deficient individuals seem to be less prone to infections such as malaria [10]. There is evidence to suggest a U-shaped relationship indicating that both low and high iron levels could increase infection risk [7, 11]. In the adult western population, few studies exist on the association between iron levels and susceptibility to infections. As most of these studies have used indirect markers of iron levels in serum (e.g., anemia, hypochromasia), there is also a lack of studies on the association between free iron in serum and transferrin-bound iron (i.e., iron status) and the risk of infections.

In this era of rising antibiotic resistance, we need new measures to prevent severe infections [12]. To the best of our knowledge, no study has examined the associations of iron status with the risk and mortality of BSI in a long-term follow-up in the general population. The large Norwegian population-based HUNT2 study cohort has prospective follow-up data on blood culture-positive infections over 15 years used as a specific indicator of sepsis [13, 14]. In this cohort of 61,852 adults, we assessed the association of iron status with risk of BSI and BSI mortality [15].

Take-home message

Bloodstream infections and iron deficiency represent an important burden of disease. Our study assessed iron status and risk of bloodstream infections in the HUNT2 survey with 61,852 individuals and 15 year follow-up, showing increased risk of BSI among individuals with low iron status.

Methods

Study population

The second Nord-Trøndelag Health Survey (HUNT2, 1995–1997) invited all inhabitants ≥ 20 years old ($n = 93{,}865$) in Nord-Trøndelag county to a clinical examination that included non-fasting blood sampling, and the participants completed questionnaires covering a range of health-related topics [16] (http://www.ntnu.edu/hunt/databank). A total of 65,236 (69.5%) persons participated. Iron status measurements were performed as a population-screening for hereditary hemochromatosis, and the study revealed an estimated hereditary hemochromatosis prevalence of 0.7% [19]. No routine follow-up was carried out in pathologically low iron indices in the HUNT2 study, but later diagnostic and/or treatment cannot be ruled out. For this study, we selected all participants in HUNT2 with baseline measurements of serum iron, transferrin saturation percentage (Tsat) and total iron-binding capacity (TIBC). Using the 11-digit unique personal identification number of Norwegian citizens, the HUNT2 study cohort was linked to the Mid-Norway Sepsis Register with prospectively recorded information on all BSI events at Levanger and St. Olavs hospitals from 1 January 1995, and Namsos hospital was included in the register from 1 September 1999. The HUNT study database is regularly updated with information on site of residence and vital status from the Norwegian population register. Nord-Trøndelag county in central Norway has a population of 130,000 where 70% is served by Levanger hospital and 30% by Namsos hospital, and the tertiary referral center is St. Olavs hospital in Trondheim. The population is stable (net out-migration 0.3% per year) and ethnically homogeneous (97% Caucasians) [17]. Among the 65,236 HUNT2 participants, prior to start of follow-up (which was the entry date in HUNT2 between August 1995–June 1997 for residents having Levanger as their primary hospital and 1 September 1999 for residents having Namsos as their primary hospital) [17], 47 (0.07%) had a positive blood culture, 1140 (1.8%) migrated or died, and 2197 (3.4%) had incomplete information on iron status or comorbidities, leaving 61,852 participants for analysis (see Supplementary Figure 1).

Laboratory measurements

Fresh non-fasting serum samples were analyzed at the Central Laboratory at Levanger hospital using a Hitachi 911 Autoanalyser (Mito, Japan). Iron concentrations were measured after reduction of transferrin with ascorbic acid, complexed with bathophenanthrolin and

quantitated colorimetrically (Boehringer, Germany). TIBC was calculated from serum transferrin analyzed by immunoturbidimetric methodology from DAKO A/S, Denmark. The method was calibrated against the international standard CRM 470. Transferrin saturation percentage was calculated as 100 × (serum iron/2 × TIBC)%. Serum creatinine was analyzed using the Jaffé method (Roche Diagnostics, Germany). eGFR was estimated from recalibrated creatinine values using the Modification of Diet in Renal Disease (MDRD formula), as previously described [18].

Outcome ascertainment

Participants were followed up for BSI identified at the two hospitals (Levanger and Namsos) in Nord-Trøndelag county or at the tertiary referral center, St. Olavs hospital in Trondheim. We used BSI mortality as an indicator of risk of severe BSI and defined death from BSI as death occurring within 30 days after detection of a BSI. Outcome variables were first-time BSI and death from BSI. In participants with multiple positive blood cultures, a new episode of BSI was defined as positive blood culture > 30 days after the previous one. Blood cultures solely with microorganisms commonly associated with skin contamination such as coagulase negative *Staphylococcus* species, *Corynebacterium* species and *Propionibacterium* species were not considered as BSI [19].

Covariates

HUNT2 participants self-reported a range of chronic illnesses including cardiovascular disease (history of myocardial infarction, stroke and/or angina), lung disease (asthma or chronic obstructive pulmonary disease) and diabetes. Chronic kidney disease was defined as estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m^2. Body mass index (BMI) was calculated as weight (kg) divided by the squared value of height (m^2) and categorized as recommended by WHO (< 18.5, 18.5–24.9, 25–29.9, 30.0–34.9, 35.0–39.9 and ≥ 40.0 kg/m^2). We retrieved information on cancer diagnoses from the Cancer Registry of Norway for all patients with BSI (from 1 January 1953 until 1 January 2014). Information on rheumatic illnesses (e.g., rheumatoid arthritis, ankylosing spondylitis) and inflammatory bowel disease (e.g., ulcerative colitis, Crohn's disease) was retrieved from medical records in patients with BSI. This information had been retrieved for 1216 BSI patients (68%); 1145 were BSI patients at Levanger hospital, and 111 of them had this diagnosis. Thus, the prevalence of rheumatic illness was 0.09%, which correlates with other prevalence studies [20, 21].

Statistical analyses

Levels of serum iron, Tsat and TIBC were categorized into values ≤ 2.5th percentile (low) or ≥ 97.5th percentile (high); the values in between were categorized into quintiles. The HUNT study population is representative of the Norwegian adult population, and we therefore based our iron values on the distribution in the entire cohort. For each category of iron indices, we used the Stata *stcompadj* command to estimate the age-adjusted cumulative incidence of first-time BSI, accounting for death as a competing risk. For each outcome, we used Cox regression analysis to estimate hazard ratios (HRs) with 95% confidence intervals (CIs) by categories of the iron indices using the middle quintile as reference. In the analysis of BSI risk, participants were followed until their first BSI, migration out of Nord-Trøndelag county, death or end of follow-up at 31 December 2011, whichever occurred first. In the analyses of BSI mortality, participants were followed until migration out of Nord-Trøndelag county, death or 31 December 2011, whichever occurred first. The proportional hazards assumption was examined using log-log plots and tests of Schoenfeld residuals. In a first model we adjusted for age (using attained age as the time scale) [22] and sex (by stratification). In a second model we additionally adjusted for BMI and chronic illnesses as these conditions may increase the risk of BSI [3], can cause "anemia of chronic disease" and altered iron status [23]. In a separate analysis, we omitted potentially cancer-related BSI from the outcome definition, indicated by a cancer diagnosis within 5 years prior to a BSI or 2 years after. Both cancer and cancer treatment could confound iron status and BSI risk. In a sensitivity analysis, we excluded the first 2 years of follow-up after HUNT2 participation from the follow-up to reduce possible confounding by prevalent but unknown disease at the time of serum measurements. Another sensitivity analysis was performed to investigate the impact of rheumatic illnesses and inflammatory bowel disease. We omitted BSI potentially related to this group of diseases from the outcome definition in 1145 cases of BSI at Levanger hospital, and Cox regression models were performed solely in participants having Levanger as their primary hospital ($n = 43{,}280$). All analyses were performed with Stata version 15.1 (Statacorp, Texas, USA).

Ethics

This study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (REK no 2012/153), and all participants signed an informed consent.

Results

Among 61,852 participants, 1738 (2.9%) experienced at least one episode of BSI during median follow-up of 14.8 years, and 370 (0.6%) persons died from BSI. This corresponds to an incidence rate of 221/100,000 person-years and mortality rate of 47/100,000 person-years. Participants who experienced BSI were more likely to be male, older and obese (Table 1).

Table 1 Baseline characteristics of the study population at inclusion to HUNT 2, *n* = 61,852

	***n* (%)**
BSI during follow-up	1738 (2.8)
Sex (female)	32,847 (53.1)
	Median (IQR)
Age	49.8 (36.3–63.2)
	Mean (SD)
BMI kg/m^2	26.3 (4.1)
Comorbidities	
Cardiovascular disease	4567 (7.4)
Chronic renal disease	2612 (4.2)
Lung disease	2157 (3.5)
Diabetes	1734 (2.8)
Serum iron (µmol/l), mean 16.5 (SD 6.3)	
Low ≤ 6	2202 (3.5)
7–12	14,169 (22.9)
13–15	12,950 (20.9)
16–17	8510 (13.8)
18–21	12,749 (20.6)
22–31	9975 (16.1)
High ≥ 32	1297 (2.1)
Transferrin saturation percentage (Tsat), mean 27.4 (SD 11.3)	
Low ≤ 9	1974 (3.2)
10–20	14,634 (23.7)
21–25	12,906 (20.9)
26–30	11,572 (18.7)
31–36	10,040 (16.2)
37–52	9050 (14.6)
High ≥ 53	1676 (2.7)
Total iron-binding capacity (TIBC) (µmol/l)[a], mean 61.5 (SD 9.2)	
High ≥ 82	1685 (2.7)
69–81	10,068 (16.3)
64–68	10,855 (17.6)
60–63	11,782 (19.1)
56–59	11,955 (19.33)
47–55	13,664 (22.1)
Low ≤ 46	1843 (3.0)

BSI bloodstream infection, *IQR* interquartile range, *BMI* body mass index, *SD* standard deviation

[a] High values of TIBC indicate iron deficiency

In age- and sex-adjusted analyses, BSI risk was increased among participants with indices of iron deficiency: low serum iron (HR 1.72, 95% CI 1.34–2.21), low Tsat (HR 1.48, 95% CI 1.12–1.96) or high TIBC (HR 1.46, 95% CI 1.06–2.01). The associations remained essentially similar after adjustments for BMI and chronic illnesses and also after exclusion of cancer-related BSI ($n = 368$) from the outcome definition (Table 2). In a sensitivity analysis where we omitted BSI-related rheumatic illnesses and inflammatory bowel disease ($n = 111$) from the outcome definition, the associations remained essentially similar (Supplementary Table 1). The results were also similar when we started follow-up 2 years after baseline (Supplementary Table 2), showing that the increased risk of BSI in iron-deficient subjects is not only present shortly after the time of diagnosis. The cumulative incidence curves also confirm that the excess risk at indices of iron deficiency continued throughout the follow-up period (Fig. 1). There was no association between indices of high iron levels and increased risk of BSI and no association between differences in iron status between the 2.5th and 97.5th percentiles and BSI risk (Table 2).

Analyses of BSI mortality showed similar associations as those we observed for a first-time BSI, but the low number of BSI deaths precluded precise estimates. Thus, age- and sex-adjusted BSI mortality was increased among participants with low serum iron (HR 1.52, 95% CI 0.86–2.66), low Tsat (HR 1.41, 95% CI 0.75–2.67) and high TIBC (HR 1.67, 95% CI 0.76–3.68), and the associations did not attenuate after adjustment for BMI and comorbidities (Table 3).

Discussion

In this large population-based cohort, indices of iron deficiency were associated with increased risk of a future BSI. Interestingly, individuals who were iron depleted continued to have an increased risk of BSI during a 15-year follow-up, even after adjusting for chronic medical disorders and excluding BSIs that occurred in relation to malignancies and chronic inflammatory disorders. To the best of our knowledge, our study is the first to show an increased risk of future BSI in individuals with iron depletion not related to cancers or chronic illnesses.

Former studies on the association between iron status and risk of infection have indicated a U-shaped risk profile where both iron deficiency and excess iron are linked to increased infection risk [7, 11]. To explore this hypothesis, we categorized our iron indices into quintiles using the middle quintile as reference and in the extreme ends ≤ 2.5th percentile or ≥ 97.5th percentile. A reason why we see no clear association between high iron status and risk of infection in our study could be that the low number of BSI cases in the high iron status categories precluded precise estimates of the associations between

Table 2 Associations of indices of iron status with risk of bloodstream infection

Indices of iron status	Risk of first-time BSI, adjusted for age and sex				Risk of first-time BSI, adjusted for age, sex, BMI and comorbidities[a]		Risk of non-cancer related BSI[b], adjusted for age, sex, BMI and comorbidities[a]		
	Years at risk	No. BSI	HR	95% CI	HR	95% CI	No. BSI	HR	95% CI
Serum iron (μmol/l)	786527	1738					1370		
Low ≤ 6	27125	83	1.72	1.34–2.21	1.71	1.33–2.20	71	1.79	1.36–2.35
7–12	177840	418	1.08	0.93–1.27	1.06	0.90–1.24	334	1.03	0.87–1.23
13–15	164480	383	1.01	0.87–1.19	1.01	0.86–1.19	294	0.96	0.80–1.15
16–17	108973	246	1.00	Reference	1.00	Reference	199	1.00	Reference
18–21	163237	344	0.97	0.83–1.15	0.98	0.84–1.16	272	0.97	0.81–1.16
22–31	128313	236	0.91	0.76–1.09	0.93	0.78–1.12	178	0.88	0.72–1.08
High ≥ 32	16555	28	1.06	0.72–1.57	1.06	0.72–1.57	22	1.05	0.68–1.63
Transferrin saturation percentage (Tsat)									
Low ≤ 9	24676	59	1.48	1.12–1.96	1.45	1.10–1.91	48	1.40	1.03–1.91
10–20	184421	432	1.11	0.96–1.28	1.08	0.93–1.24	342	1.02	0.87–1.19
21–25	164506	357	0.95	0.82–1.10	0.94	0.81–1.09	278	0.88	0.75–1.04
26–30	147447	345	1.00	Reference	1.00	Reference	284	1.00	Reference
31–36	128719	275	0.92	0.79–1.08	0.94	0.80–1.10	210	0.88	0.74–1.06
37–52	115412	236	0.90	0.77–1.07	0.93	0.79–1.10	183	0.89	0.74–1.07
High ≥ 53	21344	34	0.84	0.59–1.20	0.86	0.60–1.22	25	0.78	0.52–1.18
Total iron-binding capacity (TIBC) (μmol/l)									
High ≥ 82	21553	43	1.46	1.06–2.01	1.36	0.99–1.87	35	1.34	0.94–1.91
69–81	128767	225	0.95	0.80–1.12	0.92	0.78–1.09	183	0.93	0.77–1.12
64–68	139549	283	0.94	0.80–1.10	0.93	0.80–1.09	208	0.86	0.72–1.03
60–63	151071	346	1.00	Reference	1.00	Reference	274	1.00	Reference
56–59	152182	345	0.92	0.79–1.07	0.93	0.81–1.09	283	0.97	0.82–1.15
47–55	171521	428	0.92	0.79–1.05	0.95	0.82–1.09	337	0.95	0.81–1.11
Low ≤ 46	21881	68	1.00	0.77–1.30	1.04	0.80–1.35	50	0.98	0.72–1.32

BSI bloodstream infection, *BMI* body mass index, *HR* hazard ratio, *CI* confidence interval

[a] Comorbidities: cardiovascular events, chronic renal disease, lung disease and diabetes

[b] The outcome is first-time BSI except cancer-related BSI, indicated by a cancer diagnosis within 5 years prior to BSI or 2 years after

iron excess and increased risk of BSI. Moreover, all subjects with high iron status in HUNT2 were scheduled for further examinations, and those diagnosed with hereditary hemochromatosis were followed closely at the hospital for this condition thereafter.

In line with our findings, a systematic review reported that five out of six studies found higher occurrence of infections among patients with iron deficiency [24]. There are several plausible pathophysiologic explanations for an increased risk of BSI at low iron levels. Recent work has shown that iron plays a crucial role in the hypoxia-inducible-factor (HIF) transcription factor/prolyl hydroxylase domain pathway. HIFα induces a number of aspects of host immune function, from boosting phagocyte microbicidal capacity to driving T cell differentiation and cytotoxic activity [25]. Indeed, reduced T cell function [26], reduced bactericidal activity of macrophages [27] and decreased ability to produce inflammatory cytokines [28] have been shown in iron-depleted persons.

Our study suggests that the immune defense mechanisms may be relatively more depressed than the ability of bacteria to sequester iron in low iron environments, resulting in a net increased risk of BSI in iron-deficient individuals.

Major strengths of our study include its large size, the population-based design and long-term follow-up. Information on potential confounding factors such as chronic illnesses and malignancies, as well as linkage to microbiologic records, also add strength to our study. Moreover, in a sensitivity analysis we showed that the increased risk of BSI among iron-depleted subjects still was present after excluding the BSI events during the first 2 years of follow-up, which reduces the possibility that confounding from preclinical disease may explain the association and indicates that iron deficiency could be persistent in our subjects.

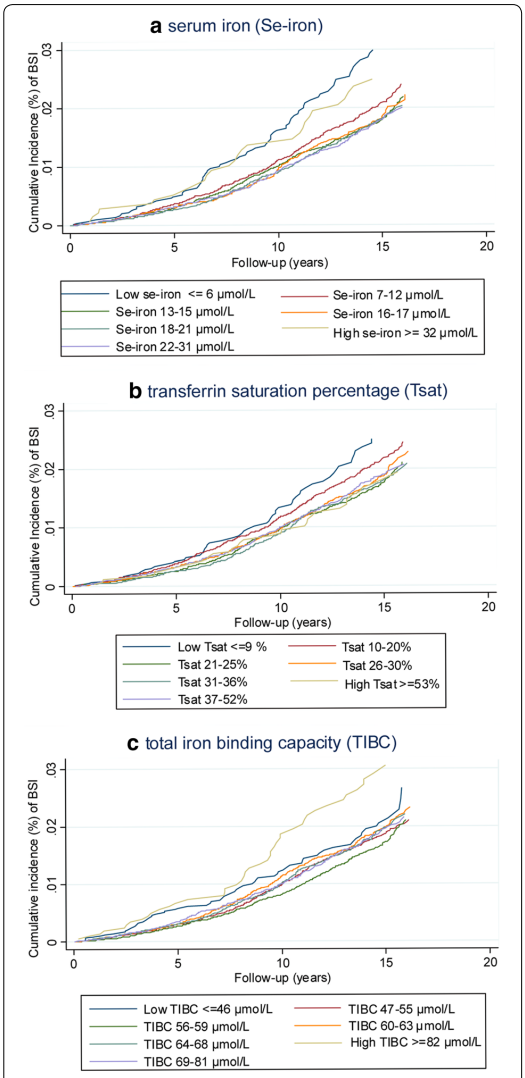

Fig. 1 Age-adjusted cumulative incidence of BSI associated with levels of serum iron (**a**), transferrin saturation percentage (**b**) and total iron-binding capacity (**c**), estimated for age 49.8 years (the mean age of the total population)

Iron status in the context of infection risk must take in account anemia of chronic disease, counting for 20% of anemias in the elderly (≥ 65 years) [29]. We have therefore adjusted for these confounding factors by adjusting for chronic illnesses, age and BMI, as these factors could influence both BSI risk and iron status [3, 23]. By retrieving data from the Cancer Registry of Norway, we also obtained valid and essentially complete information on cancer diagnoses among BSI cases. Importantly, the associations between low iron status and increased BSI risk remained similar after these adjustments. Also in sensitivity analyses leaving out patients with rheumatic illnesses and inflammatory bowel, we obtained similar results to the main analysis.

Linkage to microbial records with information on bacterial species enabled us to exclude pathogens commonly associated with contamination of blood cultures. The majority of positive blood cultures in this material are thus likely to represent serious infections. Although we did not have clinical information about the course of infection for all participants, a review of medical records of the patients with *S. aureus* and *Streptococcus pneumoniae* BSI in this cohort has shown that ~ 98% met the 2001 sepsis criteria [13, 14]. Thus, we find it likely that the patients with BSI clinically could be characterized as septic.

There are some limitations of our study. One is that our definition of BSI mortality as any death occurring within 30 days after detection of a BSI could be confounded by other causes of death not being related to the event of BSI. The lack of information on ferritin and hemoglobin concentrations is also a limitation. Ferritin is widely accepted as a standardized assessment of global iron stores, but is largely influenced by inflammation [30]. Hemoglobin concentration becomes abnormal only in long-standing iron deficiency and is influenced by a wide range of medical conditions [30]. Some studies have shown that Tsat or TIBC alone is an alternative diagnostic test for iron deficiency [31, 32]. We believe that our study has proved that serum iron, Tsat and TIBC, although prone to diurnal variation and fasting status, are useful for studying the association between iron metabolism and the future risk of BSI.

Another limitation is the one single measurement of the iron indices in our study and that we do not have measurements at the time when BSI was acquired. As iron status was measured up to 15 years before the outcome, it could have changed during the follow-up period, possible by later identification and correction by supplementation. However, any potential misclassification of iron indices caused by diurnal variation, non-fasting blood sampling or the single measurement would most likely be non-differential, i.e., not related to later risk of BSI, and is therefore likely to have led to underestimation of the associations. In nutritional surveys among adults from Norway during the same period as our study, the intake of iron was below the recommended daily amount [33], suggesting that iron deficiency was stable at the population level. A recent population study from

Table 3 Associations of indices of iron status with BSI mortality[a]

Indices of iron status	BSI mortality, adjusted for age and sex				BSI mortality, adjusted for age, sex, BMI and comorbidities[b]		Non-cancer related BSI mortality[c], adjusted for age, sex, BMI and comorbidities[b]		
	Years at risk	No. BSI deaths	HR	95% CI	HR	95% CI	No. BSI deaths	HR	95% CI
Serum iron (µmol/l)	786527	370					222		
Low ≤ 6	27125	16	1.52	0.86–2.66	1.54	0.89–2.69	7	1.06	0.47–2.39
7–12	177840	99	1.11	0.80–1.54	1.07	0.77–1.48	64	1.05	0.70–1.58
13–15	164480	89	1.00	0.72–1.40	1.00	0.72–1.39	54	0.94	0.62–1.43
16–17	108973	58	1.00	Reference	1.00	Reference	37	1.00	Reference
18–21	163237	62	0.75	0.52–1.07	0.77	0.54–1.10	36	0.72	0.45–1.14
22–31	128313	42	0.69	0.47–1.04	0.72	0.48–1.07	22	0.63	0.37–1.07
High ≥ 32	16555	4	0.70	0.25–1.19	0.70	0.25–1.93	2	0.62	0.15–2.56
Transferrin saturation percentage (Tsat)									
Low ≤ 9	24676	11	1.41	0.75–2.67	1.39	0.73–2.61	3	0.64	0.20–2.07
10–20	184421	102	1.24	0.92–1.67	1.18	0.88–1.59	68	1.29	0.88–1.89
21–25	164506	79	0.97	0.71–1.32	0.94	0.69–1.29	46	0.92	0.61–1.38
26–30	147447	76	1.00	Reference	1.00	Reference	45	1.00	Reference
31–36	128719	64	0.97	0.70–1.35	1.00	0.72–1.40	37	1.01	0.65–1.56
37–52	115412	33	0.57	0.38–0.85	0.59	0.39–0.89	21	0.67	0.40–1.13
High ≥ 53	21344	5	0.59	0.24–1.45	0.60	0.24–1.49	2	0.44	0.11–1.83
Total iron-binding capacity (TIBC) (µmol/l)									
High ≥ 82	21553	7	1.67	0.76–3.68	1.47	0.67–3.25	6	1.81	0.76–4.33
69–81	128767	49	1.32	0.90–1.93	1.26	0.86–1.85	31	1.20	0.75–1.93
64–68	139549	67	1.36	0.95–1.93	1.34	0.94–1.91	33	0.98	0.62–1.57
60–63	151071	57	1.00	Reference	1.00	Reference	38	1.00	Reference
56–59	152182	77	1.21	0.86–1.70	1.25	0.94–1.81	47	1.14	0.74–1.75
47–55	171521	102	1.24	0.90–1.72	1.30	0.94–1.81	61	1.16	0.77–1.75
Low ≤ 46	21881	11	0.90	0.47–1.72	0.95	0.50–1.81	6	0.75	0.32–1.79

BSI bloodstream infection, *BMI* body mass index, *HR* hazard ratio, *CI* confidence interval

[a] Death from BSI was defined as death within 30 days after an episode of BSI

[b] Comorbidities: cardiovascular events, chronic renal disease, lung disease and diabetes

[c] The outcome is BSI mortality except cancer-related BSI mortality, indicated by a cancer diagnosis within 5 years prior to BSI or 2 years after

Portugal found a high prevalence of iron deficiency and that it was largely undiagnosed [34].

Although our study adds weight to the growing body of evidence linking iron deficiency and risk of infections, we should be careful recommending iron supplements in individuals with mild-to-moderate iron deficiency to prevent infections. While there are studies suggesting that iron supplementation may decrease the risk for some infections [35], a recent systematic review showed increased susceptibility to infections with intravenous versus oral or no iron administration [36]. The controversies shown in iron supplemental studies demonstrate how delicate iron homeostasis is and that iron supplement should be given with caution.

In summary, we show an increased risk of BSI in individuals with low iron status in a 15-year follow-up study. As iron deficiency and BSI represent an important burden of disease globally, our findings suggest increased research on the effect of identifying and correcting iron deficiency to prevent BSI and sepsis.

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00134-018-5320-8) contains supplementary material, which is available to authorized users.

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Compliance with ethical standards

Conflicts of interest
Nothing to declare.

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Supplementary information

Randi Marie Mohus et al. *Intensive Care Med (2018) 44:1276-1283*

Supplementary table 1. Associations of indices of iron status with risk of BSI after omitting rheumatic and inflammatory illnesses[a]

Indices of iron status	Risk of BSI adjusted for age and sex				Risk of BSI adjusted for age, sex, BMI, and comorbidities[b]	
	Years at risk	No. BSI	HR	95% CI	HR	95% CI
Serum iron (µmol/L)	584772	1145				
Low ≤ 6	19712	51	1.64	1.20 - 2.25	1.63	1.19 - 2.23
7-12	131799	278	1.09	0.90 - 1.32	1.06	0.87 - 1.29
13-15	120479	239	0.97	0.80 - 1.19	0.97	0.80 - 1.19
16-17	81621	164	1.00	Reference	1.00	Reference
18-21	122119	233	0.98	0.80 - 1.20	0.99	0.81 - 1.21
22-31	96493	161	0.93	0.75 - 1.16	0.95	0.76 - 1.18
High ≥ 32	12545	19	1.04	0.65 - 1.68	1.04	0.64 - 1.67
Transferrin saturation percentage (Tsat)						
Low ≤ 9	18182	40	1.62	1.16 - 2.28	1.58	1.12 - 2.22
10-20	136822	286	1.17	0.98 - 1.39	1.13	0.94 - 1.35
21-25	120795	230	0.98	0.81 - 1.17	0.96	0.80 - 1.16
26-30	109876	221	1.00	Reference	1.00	Reference
31-36	96910	187	0.97	0.80 - 1.18	0.98	0.81 - 1.19
37-52	86138	159	0.94	0.77 - 1.15	0.97	0.79 - 1.19
High ≥ 53	16046	22	0.85	0.55 - 1.31	0.85	0.55 - 1.32
Total iron binding capacity (TIBC) (µmol/L)						
High ≥ 82	16025	29	1.52	1.03 - 2.25	1.43	0.97 - 2.11
69-81	95427	152	0.99	0.81 - 1.22	0.97	0.79 -1.20
64-68	104560	198	1.01	0.83 - 1.22	1.01	0.83 - 1.22
60-63	112714	227	1.00	Reference	1.00	Reference
56-59	112726	220	0.89	0.74 - 1.08	0.92	0.76 - 1.10
47-55	127424	270	0.87	0.73 - 1.04	0.90	0.75 - 1.07
Low ≤ 46	15893	49	1.10	0.81 - 1.50	1.15	0.84 - 1.57

BSI: bloodstream infection, BMI: body mass index, HR: Hazard ratio, CI: Confidence interval.

a) The Cox model was performed on participants having Levanger as their primary hospital, *n*=43,280

b) Comorbidities; cardiovascular events, chronic renal disease, lung disease and diabetes.

Supplementary Table 2. Associations of indices of iron status with risk of bloodstream infections, delayed entry date with two years[a]

Indices of iron status	Risk of first-time BSI, adjusted for age and sex				Risk of first-time BSI, adjusted for age, sex, BMI and comorbidities[b]	
	Years at risk	No. BSI	HR	95% CI	HR	95% CI
Serum iron (µmol/L)	701246	1633				
Low ≤ 6	24197	78	1.73	1.34 - 2.25	1.73	1.34 - 2.24
7-12	158364	392	1.08	0.92 - 1.28	1.06	0.90 - 1.24
13-15	146935	359	1.01	0.85 - 1.18	1.00	0.85 - 1.18
16-17	97114	233	1.00	Reference	1.00	Reference
18-21	145532	326	0.97	0.82 - 1.15	0.98	0.83 - 1.16
22-31	114370	221	0.90	0.75 - 1.08	0.92	0.76 - 1.10
High ≥ 32	14731	24	0.96	0.63 - 1.46	0.96	0.63 - 1.47
Transferrin saturation percentage (Tsat)						
Low ≤ 9	22007	56	1.52	1.14 - 2.03	1.47	1.12 - 1.98
10-20	164291	402	1.10	0.96 - 1.28	1.07	0.93 - 1.24
21-25	146960	335	0.95	0.81 - 1.10	0.93	0.80 - 1.09
26-30	131462	325	1.00	Reference	1.00	Reference
31-36	114660	265	0.94	0.80 - 1.11	0.96	0.82 - 1.13
37-52	102868	219	0.89	0.75 - 1.06	0.91	0.77 - 1.09
High ≥ 53	18995	31	0.81	0.56 - 1.18	0.83	0.58 - 1.20
Total iron binding capacity (TIBC) (µmol/L)						
High ≥ 82	19238	39	1.45	1.03 - 2.02	1.34	0.97 - 1.88
69-81	114966	210	0.95	0.80 - 1.13	0.92	0.77 - 1.09
64-68	124463	267	0.94	0.80 - 1.11	0.98	0.79 - 1.10
60-63	134745	325	1.00	Reference	1.00	Reference
56-59	135752	331	0.94	0.80 - 1.09	0.96	0.82 - 1.11
47-55	152680	400	0.91	0.79 - 1.06	0.95	0.82 - 1.09
Low ≤ 46	19400	61	0.98	0.75 - 1.29	1.02	0.78 - 1.35

BSI: Bloodstream infection, BMI: Body mass index, HR: Hazard ratio, CI: Confidence interval.

a) The first two years after HUNT2 participation were excluded from the follow-up time

b) Comorbidities; cardiovascular events, chronic renal disease, lung disease and diabetes.

Paper 2

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Explaining sex differences in risk of bloodstream infections using mediation analysis in the population-based HUNT study in Norway

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Previous studies indicate sex differences in incidence and severity of bloodstream infections (BSI). We examined the effect of sex on risk of BSI, BSI mortality, and BSI caused by the most common infecting bacteria. Using causal mediation analyses, we assessed if this effect is mediated by health behaviours (smoking, alcohol consumption), education, cardiovascular risk factors (systolic blood pressure, non-HDL cholesterol, body mass index) and selected comorbidities. This prospective study included 64,040 participants (46.8% men) in the population-based HUNT2 Survey (1995–1997) linked with hospital records in incident BSI. During median follow-up of 15.2 years, 1840 (2.9%) participants (51.3% men) experienced a BSI and 396 (0.6%) died (56.6% men). Men had 41% higher risk of first-time BSI (95% confidence interval (CI), 28–54%) than women. Together, health behaviours, education, cardiovascular risk factors and comorbidities mediated 34% of the excess risk of BSI observed in men. The HR of BSI mortality was 1.87 (95% CI 1.53–2.28), for BSI due to *S. aureus* 2.09 (1.28–2.54), *S. pneumoniae* 1.36 (1.05–1.76), *E. coli* 0.97 (0.84–1.13) in men vs women. This study shows that men have higher risk of BSI and BSI mortality than women. One-third of this effect was mediated by potential modifiable risk factors for incident BSI.

Bloodstream infection (BSI) is a major global burden and may lead to sepsis which constitutes up to 60% of the global mortality burden[1,2]. The risk of acquiring BSI depends on the bacterial virulence, host characteristics, geographical location, and biological factors[1,3–8]. Epidemiological studies indicate a male predominance in BSI

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and sepsis. Nevertheless former studies on sex differences in incidence and mortality of BSI and sepsis have given conflicting results with increased risk in women[1], increased risk in men[6,9], increased mortality in women[10], or increased mortality in men[1,11,12]. Importantly, disparities in immune function between sexes may arise from differences in biological characteristics such as anatomy and hormonal status, medical conditions, health behaviours, lifestyle, and exposure to different pathogens[13,14]. Most previous studies on sex differences in BSI and sepsis have been performed in small and selected cohorts, mainly from the intensive care unit (ICU)[6,10,11], and there are limited population-based studies[9,15,16] which better account for selection bias[17]. Studies on severe infections and sepsis tend to *adjust* for sex in their analyses[18] but the mechanisms behind the observed sex differences are unexplored[19]. Little is known whether conditions that are known to increase BSI risk, like health behaviours[4] cardiovascular disease risk factors or comorbidity[20,21], contribute to the observed difference in risk of BSI between men and women. Such knowledge may help identify targets for intervention to reduce BSI and sepsis risk.

To assess the impact of sex as a risk factor for first-time BSI, BSI mortality, and BSI caused by the most common infecting bacteria, *Staphylococcus (S.) aureus, Streptococcus (S.) pneumoniae* and *Escherichia (E.) coli* we used data from the Norwegian HUNT study linked with prospectively recorded BSI episodes. Further, we examined if sex differences in health behaviours and education attainment, cardiovascular risk factors and selected comorbidities, which reflect known risk factors for BSI[4,20,21] may explain the observed sex difference in risk of first-time BSI. We applied sequential mediation analysis[22] using inverse-odds weighting[23] to explore their potential mediating effect on the associations between sex and BSI.

Methods

Study population. The HUNT Study is a population-based health study conducted in the Nord-Trøndelag region in Norway and consists of four consecutive surveys inviting the total adult population approximately every 10th year. The second survey (HUNT2, 1995–1997), invited all adult inhabitants ≥ 20 years ($n = 93{,}898$) to a clinical examination and a comprehensive self-report of health-related topics. Of these, 65,237 (69%) chose to participate. The HUNT study database is regularly updated with information on date of migration and death from the National Registry. More details on the HUNT study are published elsewhere[24]. For the purpose of the present study, we excluded 47 (0.07%) participants who had a prior positive blood culture and 1150 (1.8%) who migrated or died before start of follow-up. A total of 64,040 participants were eligible for analyses (Supplemental Fig. S1).

Measures. The exposure is sex as registered in the National Registry. The two main outcomes were first-time BSI and BSI mortality. The participants were followed for incident BSI by linkage to the Nord-Trøndelag Hospital Trust (HNT HF) Sepsis Registry using the personal identification number of Norwegian citizens[25]. All BSIs were confirmed at the microbiology laboratories at Levanger Hospital which provided all microbiology services in the Nord-Trøndelag region or at St. Olavs Hospital. Details about HNT HF sepsis registry are included in the supplemental material. We defined BSI mortality as death occurring within 30 days after detection of any BSI. In secondary analyses we assessed first-time BSI caused by the most common bacteria *E. coli*, *S. aureus* and *S. pneumonia,* and performed age-stratified analyses.

Mediators are variables that are causally located between exposure and outcome variables, and that partly explain the effect of the exposure on outcome[22,26]. Mediation analysis can assess indirect and direct effects and estimate the proportion of the total effect that works through the mediator of interest (i.e. proportion mediated)[27]. We used three distinct sets of mediators measured at inclusion to HUNT2; (1) health behaviours (smoking and alcohol use) and educational attainment; (2) cardiovascular risk factors (body mass index (BMI, kg/m^2), systolic blood pressure (mmHg) and non-high-density lipoprotein cholesterol (non-HDL cholesterol, mmol/L); (3) comorbidities defined by self-report of cardiovascular disease (history of myocardial infarction, angina pectoris, and/or stroke), diabetes, cancer history, lung disease (asthma or chronic obstructive pulmonary disease) and standardised measurements of kidney function (estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2). The three sets of mediators reflect known risk factors for BSI[4,20,21]. For some of the included mediators there are reports of sex differences in prevalence, pathophysiology and outcomes[28,29]. The proposed diagram for the relationship between sex and risk of BSI is shown in Fig. 1. The aim of the analysis was to examine to which extent sex differences in risk of BSI may be related to these mediating factors. Details about the measurements and categorisation of mediators are included in the Supplemental Material.

Statistical analyses. We used Cox proportional hazard regression to estimate the hazard ratios (HRs) with 95% confidence intervals (95% CI) of a first-time BSI and of BSI mortality in men compared to women. Attained age was used as the time scale. Start of follow-up was defined by the availability of data in the sepsis registry. For patients referred to St. Olavs hospital, the tertiary referral centre, BSI information was included depending on their primary hospital. Participants contributed person-years from inclusion date in HUNT2 except for participants having Namsos as their primary hospital, they contributed from 1 September 1999.

In the analysis of BSI risk, participants were followed until their first BSI. For BSI-mortality participants were followed until death within 30 days of any BSI episode. For both analyses participants were censored at time of migration out of Nord-Trøndelag, death of all causes, or end of follow-up set to 31 December 2011, whichever occurred first. The proportional hazards assumption was examined by visual inspection of log–log plots and tests of Schoenfeld residuals. Using Stata *stcompadj,* we estimated cumulative incidence and mortality from start of follow-up to first-time BSI and BSI mortality, accounting for death by all causes as a competing risk and we provide cumulative incidence and cumulative mortality curves to illustrate changes during follow-up. As supplemental analyses we assessed subhazard ratios taking death as a competing event into account for first-time BSI and BSI mortality.

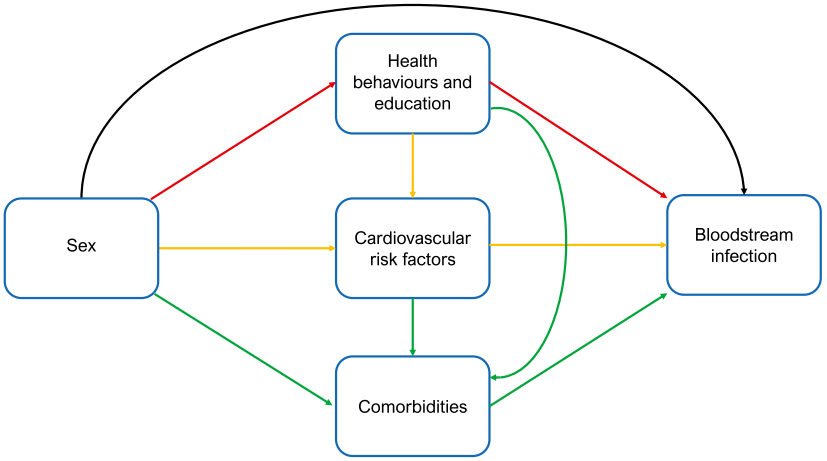

Figure 1. Mediation analysis. Diagram of the direct and indirect (i.e., mediated) effects of *sex* on *bloodstream infection*. The black arrow represents the natural direct effect of the association. Red arrows represent model 1, proportion mediated by health behaviours and education attainment. Yellow arrows represent model 2, proportion mediated jointly by health behaviours, education, and cardiovascular risk factors. Green arrows represent model 3, proportion mediated jointly by health behaviours, education, cardiovascular risk factors and comorbidities. Model 1) Smoking, alcohol use and educational attainment. Model 2) Systolic blood pressure, non-high-density lipoprotein cholesterol and Body Mass Index. Model 3) Cardiovascular disease, chronic kidney disease, diabetes, history of cancer, and chronic lung disease.

In secondary analyses we estimated hazard ratios and cumulative incidence of first-time BSI caused by the most common infecting bacteria. Further, we conducted age-stratified analyses on risk of first-time BSI; < 50, 50 to < 65, 65 to 79, and ≥ 80 years to address whether menopause affects women's risk of BSI, and to assess sex differences in BSI risk with advancing age. To examine the associations between the mediators included in Model 2 and risk of first-time BSI, we conducted sensitivity analyses using Cox regression for BMI, systolic blood pressure and non-HDL cholesterol.

For the mediation analysis we used an *inverse odds weighting (IOW)* procedure[23]. IOW is a counterfactual method that enables a decomposition of the *total effect* of the exposure (sex) on the outcome (first-time BSI) into a *natural direct effect (NDE)* from exposure on outcome, and a *natural indirect effect (NIE)* through multiple mediators[22,23]. The method accommodates multiple mediators simultaneously and is robust to unmeasured common causes of the mediators[22].

The inverse odds weights were obtained by regressing the exposure on all mediators of interest with age as a covariate. In our analysis, the total effect is interpreted as the total association between sex and first-time BSI, the NIE is the proportion of excess BSI risk in men mediated by the risk factors, whereas the NDE is the proportion of excess BSI risk in men not associated with these factors. The proportion mediated is the percent of the total association that is mediated through the risk factors. We did not estimate the NIE of individual mediators separately as it may not be appropriate when the mediators affect each other or when single mediator-outcome confounders may be affected by exposure[22,30]. Instead, we estimated the NIE with a sequential approach using three models. In model 1, we assessed education attainment and health behaviours (smoking and alcohol use); in model 2, we added the cardiovascular risk factors (BMI, systolic blood pressure and non-HDL cholesterol) to address potential preclinical disease; and in model 3, the selected comorbidities (cardiovascular, diabetes, cancer history, lung, and kidney disease) were included to the complete set of mediators. This approach assumes that the cardiovascular risk factors and further the comorbidities are causal descendants of the health behaviours and educational attainment. The sequential approach further implies that model 3 reflects the best interpretation of the mediation analyses as all mediators and age are included[22].

We performed bootstrapping based on 1000 replications to derive percentile-based CIs for all mediation parameters[31], and the NDE and NIE are presented as HRs with 95% CIs. The proportion mediated on the log scale was calculated using the formula ($\ln HR_{NIE}$ /$\ln HR_{TOTAL}$)[23]. All statistical analyses were performed using Stata version 17.0. A detailed description of the IOW analyses is included in Supplemental Table S1.

Ethical approval. The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (REK no 2012/153 and REK no 94135), and by the HUNT data access committee. Participation in HUNT 2 was voluntary, and informed written consent to data collection and linking their data to other registers was obtained from all participants. All methods were performed in accordance with the Declaration of Helsinki.

	Men	Women
Total population n (%)	29,962 (46.8)	34,087 (53.2)
First-time BSI n (%)[1]	943 (51.3)	897 (48.7)
BSI mortality n (%)[2]	224 (56.6)	172 (43.4)
Age (mean, IQR)	48.6 (36.5–62.9)	48.7 (36.2–64.2)
Smoking		
Current (%)	8334 (27.8)	9726 (28.5)
Prior (%)	9422 (31.4)	6516 (19.1)
Never (%)	10,668 (35.6)	15,230 (44.7)
Alcohol use		
< 1 unit/2 weeks (%)	8448 (28.2)	16,069 (47.3)
1–7 units/2 weeks (%)	14,258 (47.6)	14,484 (42.6)
8–14 units/2 weeks (%)	4602 (15.4)	1861 (5.5)
≥ 15 units/2 weeks (%)	1643 (5.5)	272 (0.8)
Education		
< 10 years (%)	20,625 (68.9)	22,259 (65.3)
10–12 years (%)	2302 (7.7)	3436 (10.1)
> 12 years (%)	5650 (18.9)	6412 (18.8)
BMI (kg/m^2)		
< 18.5 (%)	118 (3.9)	349 (1.0)
18.5–24.9 (%)	10,498 (35.0)	14,736 (43.2)
25–29.9 (%)	14,757 (49.3)	12,345 (36.2)
30–34.9 (%)	3674 (12.3)	4640 (13.6)
35–39.9 (%)	496 (1.7)	1236 (3.6)
≥ 40 (%)	74 (0.3)	344 (1.0)
Systolic blood pressure (mmHg) median (IQR)	137 (127–150)	131 (118–149)
Non-HDL cholesterol (mmol/L) median (IQR)	4.5 (3.7–5.3)	4.3 (3.5–5.3)
Comorbidities		
Cardiovascular disease[3] (%)	2918 (9.7)	2014 (5.9)
Chronic kidney disease (%)	979 (3.3)	1802 (5.3)
Diabetes (%)	895 (3.1)	970 (2.9)
Cancer history (%)	878 (2.8)	1413 (4.1)
Chronic lung disease[4] (%)	1183 (4.0)	1011 (3.0)

Table 1. Baseline characteristics of the study population at inclusion in HUNT2, n = 64,040. *BSI* bloodstream infection, *n* numbers, *IQR* interquartile range, *BMI* body mass index, *HDL* high-density lipoprotein. [1]Percentage of total first-time BSI in both sexes. [2]BSI mortality was defined as all-cause mortality within 30 days after a BSI. Percentage of BSI mortality on both sexes. [3]History of myocardial infarction, angina pectoris and/or stroke. [4]History of chronic obstructive pulmonary disease or asthma.

Results

Population characteristics. During a median follow-up of 15.2 years (IQR 12.3–15.5 years), among 64,040 participants (46.8% men), 1840 (2.9%) experienced a BSI and 396 (0.6%) died within 30 days after a BSI episode. The median age at inclusion was similar for both sexes. Both men and women who experienced a BSI were older (median age at inclusion 67.4 and 68.0 respectively), and they had a higher comorbidity burden than participants who did not have a BSI during follow-up (Table 1).

Risk of BSI and BSI mortality. Men were more likely to experience a first-time BSI and to die from a BSI compared to women. Men had 1.41 (HR, 95% CI 1.28–1.54) times the risk of first-time BSI, and had 1.87 (HR, 95% CI 1.53–2.28) times the risk of dying from a BSI (Table 2) compared with women. In analyses by the most common infecting bacteria, men had a 2.09-fold (HR, 95% CI 1.28–2.54) risk of BSI caused by *S. aureus*, and 1.36 (HR, 95% CI 1.05–1.76) increased risk of *S. pneumonia*. The corresponding result for *E. coli* did not show higher risk in men with HR of 0.97 (95% CI 0.84–1.13) (Table 3).

The above findings are illustrated by graphing the age-adjusted cumulative incidence of first-time BSI and cumulative BSI mortality in Fig. 2. The cumulative incidence of BSI was higher among men than women after the first five years of follow-up. For BSI mortality the sex difference was apparent after the first 2.5 years of follow-up and during follow-up the sex differences in mortality increased. The subhazards obtained for first-time BSI and BSI mortality, provide the same direction of the associations as the Cox regression analyses (Supplemental Table S2). We additionally present cumulative incidence curves for the three most common infecting bacteria in Fig. 3A–C. Men had higher cumulative incidence of *S. aureus*, especially after the first seven years of follow-up, whereas *E. coli* had higher cumulative incidence among women.

	Risk of first-time BSI adjusted for age[1]				BSI mortality[2] adjusted for age[1]			
	Years at risk	No. BSI	HR	95% CI	Years at risk	No. BSI deaths	HR	95% CI
Women	436,758	897	1.0	Reference	472,012	172	1.0	Reference
Men	373,915	943	1.41	1.28–1.54	404,723	224	1.87	1.53–2.28

Table 2. Associations of sex with risk of bloodstream infection and BSI mortality. *BSI* bloodstream infection, *HRs* hazard ratios, *95% CI* 95% confidence intervals, *No.* numbers. [1]Cox regression analyses were adjusted with age as the underlying scale. [2]BSI mortality was defined as all-cause mortality within 30 days after a bloodstream infection.

		Risk of *S. aureus* BSI[1]			Risk of *S. pneumoniae* BSI[1]			Risk of *E. coli* BSI[1]		
	Years at risk	No. BSI	HR	95% CI	No. BSI	HR	95% CI	No. BSI	HR	95% CI
Women	436,758	83	1.0	Reference	113	1.0	Reference	399	1.0	Reference
Men	373,915	129	2.09	1.28–2.54	119	1.36	1.05–1.76	285	0.97	0.84–1.13

Table 3. Associations of sex with risk of bloodstream infections caused by the most common bacteria. *BSI* bloodstream infection, *HRs* hazard ratios, *95% CI* 95% confidence intervals. [1]Cox regression analyses were adjusted for age as the underlying scale.

The age-stratified analyses at estimated menopause (< 50 years) did not reveal any clear sex difference in risk of BSI before menopause. However, the youngest age-group had few episodes of BSI. In the older age-groups we observed an increased incidence of BSI in both men and women, where men show a substantially increased risk of BSI as they age compared to women (Supplemental Table S3).

Mediation analyses. In Table 4 we present the total effect, the natural direct and indirect effects of sex on risk of first-time BSI. Compared with women men had an estimated HR of 1.40 (95% CI 1.24–1.55) for first-time BSI. Behavioural risk factors and education mediated 10% (model 1), after adding the cardiovascular risk factors the proportion mediated was reduced to 5% (model 2), whereas the whole set of mediators, including comorbidities, jointly mediated 34% of the total effect (model 3).

To examine the reduction in proportion mediated from 10 to 5% in model 2, in sensitivity analyses, we observed an increased risk of BSI in persons with low BMI (< 18.5) and in persons with increasing BMI compared to the normal BMI group (18.5–24.4). For systolic blood pressure we did not observe any risk difference, and for non-HDL cholesterol the HR suggested a protective effect but with imprecise estimates (Supplemental Table S4).

Discussion

In this large Norwegian population-based study with a follow-up of more than 15 years, male sex was associated with 41% higher risk of BSI and 87% higher risk of dying from a BSI. An estimated 34% of the increased risk of BSI in men was mediated by known BSI risk factors. We additionally found that men had 2.09 times the risk of BSI caused by *S. aureus* compared to women. These findings add weight to the observed male preponderance seen in severe infections and point out modifiable BSI risk factors that are targets for preventive measures to reduce the burden of BSI.

There are few population-based studies on sex differences in the epidemiology of BSI and to our knowledge, no previous studies have performed mediation analysis to explain the sex differences of BSI. We used the IOW method which is known to be robust using multiple mediators en bloc and the rich baseline information from HUNT2 allowed us to implement mediation analyses in a time-to-event context[22,23]. The sequential approach enabled us to examine if the observed excess risk in men was mediated through different known risk factors for BSI. For many medical conditions men and women differ regarding incidence, the underlying pathophysiology and responses to therapy[28,29]. For health behaviours, more men reported smoking, and they reported higher alcohol use. We also observed higher prevalence of obesity among women in HUNT2. Adding cardiovascular risk factors to health behaviours and education, the proportion mediated lowered from 10 to 5%, which indicates some interactions or common pathways for these mediators[22]. This result might be due to some of the mediators included in model 2 being more frequent or harmful in women, or the mediators might reduce the risk of BSI. The complete model with all mediators included accommodates the assumptions required, and most likely reflects the best modelling of the associations[22,30] explaining 34% of the excess BSI risk in men. Interventions to reduce modifiable risk factors in the population will likely reduce the burden of BSI, particularly in older men with high burden of known BSI risk factors.

The population-based design ensures that all BSI occurring in residents of a defined geographical area are included, which is an advantage over ICU cohorts[17]. Our results are supported by one study including 1051 patients, showing that men had higher risk of BSI. Like our study they described that BSI incidence increases by age, and men had twice the rate of *S. aureus* BSI[15]. Another population-based study comprising 9266 patients with BSI admitted to ICU found that male sex is a risk factor for BSI[9]. A recent study restricted to persons aged ≥ 65, found that men were at increased risk of BSI compared to females (incidence rate ratio 1.44, 95% CI 1.32–1.59)

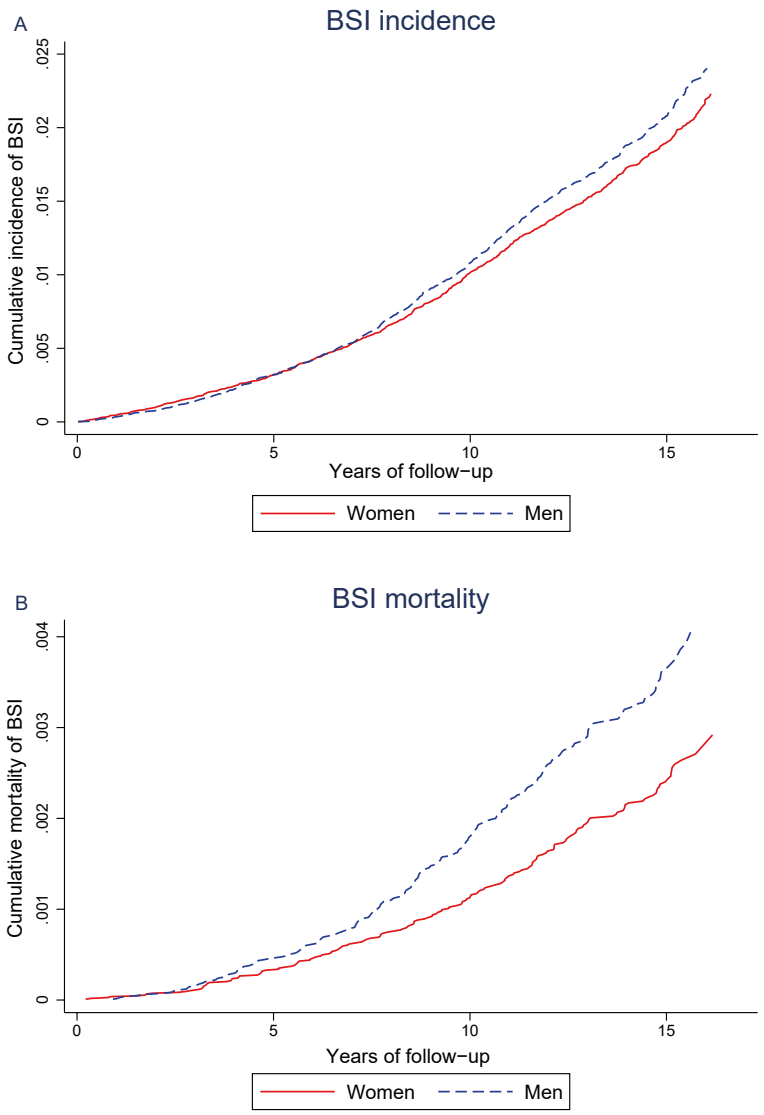

Figure 2. Sex differences in cumulative incidence and mortality of BSI. Age-adjusted sex difference in cumulative incidence of BSI (**A**), and in cumulative mortality (**B**), estimated for age 49.99 (the mean age of the total population). Note: due to the variation in incidence of different outcomes the scale of the Y-axis is not uniform across the panels.

and the sex difference was most pronounced in the oldest patients, similar to our results[16]. On the other hand, the Global Burden of Disease Study found that age-standardised sepsis incidence was higher among women, while sepsis-related mortality was higher among men[1]. This study included results from 195 countries and comprised all age groups. They found higher sepsis incidence in low-income countries, and the pattern of sepsis incidence and mortality varied according to location, which is not directly comparable to our study population.

We identified higher BSI mortality in men which is in line with a recent study of infection related death in UK Biobank[12]. Conversely, some ICU studies report higher sepsis-related mortality in women[10,29]. A recent meta-analysis evaluating the associations between sex and mortality in critically ill adults showed inconclusive results[30]. This conflicting evidence concerning sex differences in mortality is most likely due to the heterogeneity of BSI and sepsis depending on the aetiology and the cohort studied[17], but may also be affected by sex differences in immune responses[13,14,32] and differences in treatment[10,18].

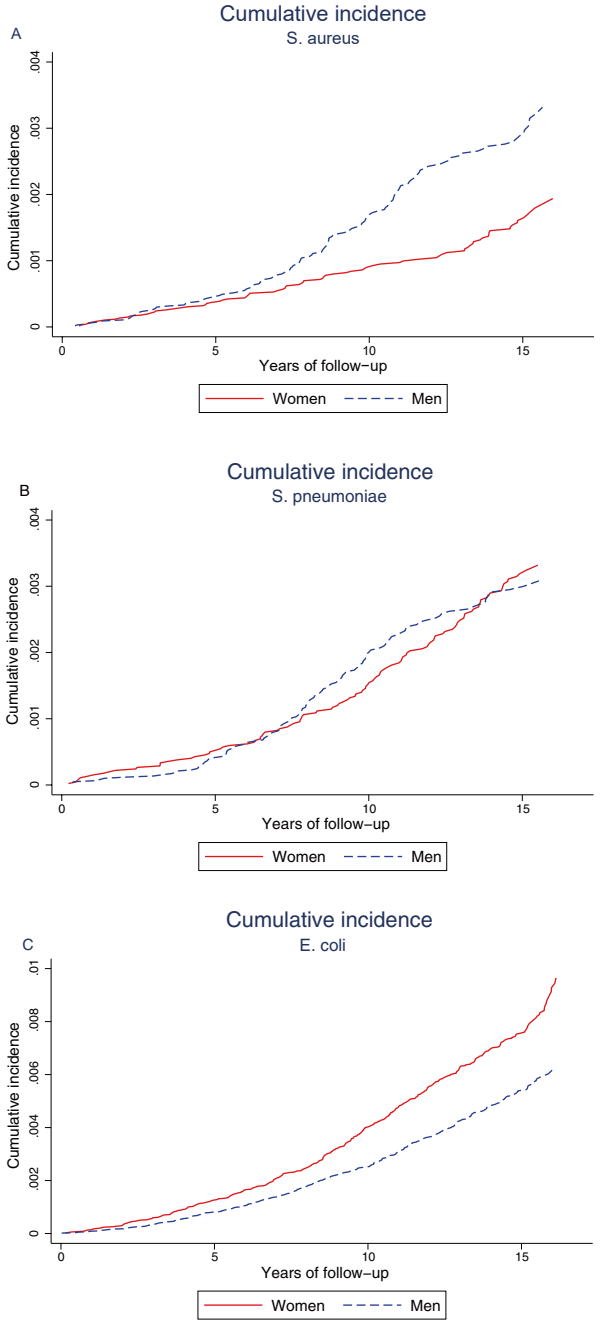

Figure 3. Sex differences in cumulative incidence of BSI caused by the most common bacteria. Age-adjusted sex difference in cumulative incidence of *S. aureus* (**A**), *S. pneumoniae* (**B**), and *E. coli* (**C**), estimated for age 49.99 (the mean age of the total population). Note: due to the variation in incidence of different bacteria the scale of the Y-axis is not uniform across the panels.

Mediation by behavioural risk factors[1] and education	Risk of first-time BSI HRs (95% CI[a])[b]
Model 1	
Total effect	1.40 (1.24–1.55)
Natural direct effect (NDE)	1.36 (1.18–1.57)
Natural indirect effect (NIE)	1.04 (0.97–1.07)
Proportion mediated[c]	10%
Mediation by behavioural risk factors[1], education, and cardiovascular risk factors[2]	**HRs (95% CI[a])[b]**
Model 2	
Total effect	1.40 (1.24–1.55)
Natural direct effect (NDE)	1.38 (1.19–1.58)
Natural indirect effect (NIE)	1.02 (0.92–1.07)
Proportion mediated[c]	5%
Mediation by behavioural risk factors[1], education, cardiovascular risk factors[2] and comorbidity risk factors[3]	**HRs (95% CI[a])[b]**
Model 3	
Total effect	1.40 (1.24–1.55)
Natural direct effect (NDE)	1.25 (1.05–1.47)
Natural indirect effect (NIE)	1.12 (1.02–1.17)
Proportion mediated[c]	34%

Table 4. Mediation of the associations between sex and BSI by behavioural risk factors, educational attainment, cardiovascular risk factors and comorbidities. *BSI* bloodstream infection, *HRs* hazard ratios, *95% CI* 95% confidence intervals. [1]Smoking, alcohol use and educational attainment at baseline. [2]Systolic blood pressure, non-high-density lipoprotein cholesterol and Body Mass Index. [3]Cardiovascular disease, chronic kidney disease, diabetes, history of cancer, or chronic lung disease. [a]Percentile-based bootstrap CIs are reported. [b]Estimates are adjusted for age as a covariate. [c]Proportion mediated: ($\ln HR_{NIE}/\ln HR_{TOTAL}$).

The second most common infecting agent in our study was *S. aureus* which was far more common in men. *S. aureus* is associated with superficial infections of soft tissues with the potential for invasive infections and is the most important cause of BSI-associated death[33]. Previous studies indicate higher probability of nasal colonization in men, which is a risk factor for invasive *S. aureus* infections[34]. Other studies show that testosterone levels and use of hormone contraceptives among females alter nasal colonization, indicating that sex hormones affect the immune response to *S. aureus*[35,36]. The higher prevalence of *S. aureus* colonization in men is of particular interest, as preventive measures like eradication or temporary suppression could lower the risk of invasive infections which is especially important in hospitalized patients[37].

In a sensitivity analysis we found that the sex differences in BSI risk are evident after predicted age of menopause indicating that alterations in both innate and adaptive immune functions with age may be sex specific. Aging is associated with chronic inflammation and a generally reduced immune function. Sex hormone levels in men and women change with age. Women face an abrupt decline during menopause, whereas men have a steady decline from second decade of life[38]. As in former studies, our study points out that advancing age is a risk factor for developing and dying from BSI and elderly men are at particular risk[9,15,16].

Major strengths of our study include its large size, the population-based design, long-term follow-up and linkage to microbiological records which represent the gold standard to identify BSI within a population[17]. Our definition of BSI as a laboratory verified positive blood culture, excluding blood cultures solely with microorganisms associated with skin contamination, ascertains the accuracy of the outcome studied. In addition, reviews of medical records of patients with *S. aureus* and *S. pneumoniae* BSI in this cohort showed that ~ 98% met the 2001 sepsis criteria[32,39]. We were able to study BSI incidence and BSI mortality in a large population without the potential referral bias seen in single institution studies of BSI. The complete ascertainment of all BSI, together with the rich baseline measurements of known BSI risk factors in HUNT2, allows for an accurate estimation of incidence and mortality in the population, with the potential of risk factor identification and mediation analyses.

There are some limitations of our study that merit attention. First, we lack information on immunosuppressive medication which are known risk factors for BSI and BSI mortality[40]. We did not have information on the clinical course after detection of a BSI. Therefore, we did not perform mediation analyses on risk of BSI mortality as in-hospital factors such as correct and timely antibiotics and resuscitation measures strongly influence mortality[41]. Second, investigating BSI is dependent on clinician's suspicion and decision to submit blood cultures for testing, with the chance of some undetected cases. Further, we cannot rule out if the clinical presentation of infections is different in men and women, and that this could result in disproportionate blood culture sampling depending on sex. Another concern would be if the clinical presentation of infection led to disproportionate and sex dependent hospital admissions. Third, the mediators were only measured once at inclusion to HUNT2 and could have changed during the 16 years of follow-up. This potential misclassification would most likely lead to underestimation of the mediating effect. Forth, the subjective assessment of some mediators, and dichotomised mediators, are more prone to possible mediator misclassification. This could lead to underestimation of the

indirect effect and overestimation of the direct effect[22]. Fifth, we were not able to assess the mediators individually, as this could have violated the model assumptions[22,30].

Despite these limitations, our study provides a foundational observation of the existing sex differences in BSI epidemiology and adds important information for clinicians, researchers and policymakers concerning BSIs. Our results suggest that sex disparities in BSI cannot be explained fully by the mediating factors investigated. Sex affects the shape of immune responses attributed to genetic, hormonal, and environmental factors[13,14,32]. The human X-chromosome encodes a number of critical genes involved in the regulation of immune functions[32]. It is clear that sex extensively influences the host immune responses, but this sexual dimorphism is underappreciated, and sex bias is a major challenge in clinical trials[18]. Sex hormones act as important modulators of immune functions and responses; testosterone and progesterone are immunosuppressive, while oestradiol is immunoenhancing[14]. Few human studies have investigated sex hormones' effects in severe infections and sepsis. Interestingly, in covid-19 where men are more prone to a severe course, the use of antiandrogens in men have shown promising results on severity[42]. Furthermore, in a review of health records in post-menopausal women with regular use of oestradiol, the fatality risk of covid-19 is reduced by more than 50%[43].

Future perspectives of our results include the need for targeted research on how these sex differences could be addressed to achieve a longer and healthier life for both men and women. Additional work should focus on how health behaviours, education level, cardiovascular risk factors and comorbidities play a role in the sex disparities seen in severe infectious diseases. Knowledge of mediating factors together with recognition of sex differences in severe infections are important for public health leaders, researchers, and clinicians as it can inform preventive actions and identify individuals especially at risk[19].

Conclusion

Our study has shown that men have an increased risk of BSI and BSI mortality. Using mediation analyses we estimated that 34% of the increased risk of BSI is mediated through known BSI risk factors. As BSI represents an important global burden of disease, our study serves as a catalyst for additional investigations by establishing the presence of sex differences and mediating risk factors. This will potentially lead to targeted management strategies to prevent BSI and sepsis in both men and women.

Data availability

Data is available from the authors upon reasonable request and by application to HUNT Research Centre. https://hunt-db.medisin.ntnu.no/hunt-db/.

Code availability

A detailed description of the IOW analyses is included in Supplemental Material.

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Author contributions
R.M.M., L.T.G., M.K.M., K.V.L., S.E.Å., T.N., J.K.D. and E.S. conceived and designed the study. T.N., B.O.Å., L.G., A.T.D., T.R., J.K.D. and E.S. supervised the study. R.M.M., B.O.Å., Å.A. and L.T.G. contributed in the acquisition of data for statistical analyses. R.M.M. analyzed the data, prepared tables, and figures. All authors interpreted the results, drafted the manuscript, contributed to the discussions, and revised the manuscript critically for important intellectual content. All authors have read and approved the final version of manuscript and agreed to be accountable for all aspects of the work.

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Competing interests
The authors declare no competing interests.

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Supplementary Information

Explaining sex differences in risk of bloodstream infections using mediation analysis in the population-based HUNT study in Norway

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Supplemental to methods

Study population and The Nord-Trøndelag Hospital Trust Sepsis Registry

The HUNT study database is regularly updated with information on date of migration and death from the National Registry. The Nord-Trøndelag region in Norway has a population of 130,000, where approximately 70% of the population is served by Levanger hospital and 30% is served by Namsos hospital. The tertiary referral centre is St. Olavs hospital in Trondheim. The population is stable with a net out-migration of 0.3% per year, and ethnically homogeneous (97% Caucasians) [1].

The Nord-Trøndelag Hospital Trust (HNT HF) Sepsis Registry has prospectively recorded information on all clinically relevant BSI events at Levanger Hospital from 1 January 1995, and Namsos Hospital was included in the registry from 1 September 1999. The microbiology laboratory at Levanger Hospital exclusively provided all

microbiology services for the two hospitals in Nord-Trøndelag region [2]. In addition, all HUNT2 participants with a positive blood culture recorded at St. Olavs hospital were included in the registry from 1 January 1995 to assure completeness of the study cohort. For all analyses using first-time BSI or first-time BSI caused by *E. coli, S. aureus or S. pneumoniae* as the outcome the first registered date of a positive blood culture from either of the microbiology labs at Levanger or St. Olavs hospitals decided the episode. BSI mortality was based on information on every BSI event with information on dates of positive blood cultures from both microbiology labs. BSI mortality was defined as all cause death within 30 days after a BSI episode. Data from all hospitals were available through 2011. Blood cultures solely with microorganisms associated with skin contamination such as coagulase negative *Staphylococcus* species*, Corynebacterium* species and *Cutibacterium* species were not considered as BSI [3].

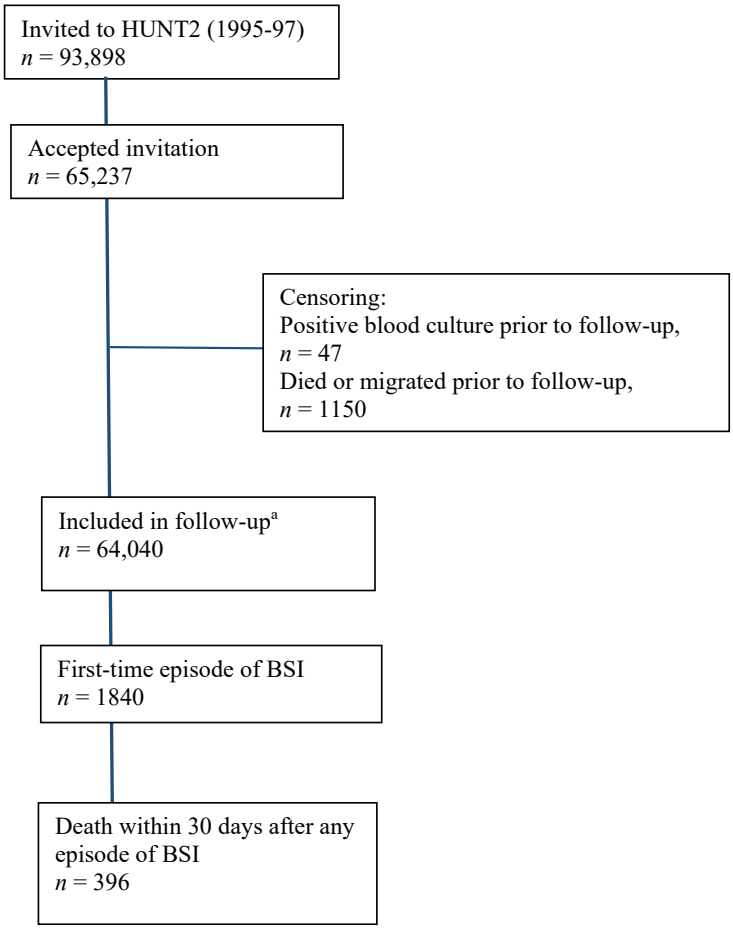
Mediators

Health behaviours were defined by smoking status and alcohol use. Smoking was defined from several questions on past and current smoking; as "current smoking" (smoking tobacco daily), "prior smoking" (any prior daily tobacco smoking) or "never smoked". Alcohol use as "never drink alcohol", "1−7 units of alcohol in two weeks", "8−12 units alcohol in two weeks" or "more than 15 units in two weeks".

Educational attainment was categorized as <10 years, 10−12 years and >12 years of schooling.

Cardiovascular risk factors were defined as body mass index (BMI), blood pressure, cholesterol and estimated glomerular filtration rate (eGFR). BMI was calculated as weight (kg) divided by the squared value of height (m^2), measured by trained nurses at the clinical examination at inclusion in HUNT2 with the participants wearing light clothing and no shoes. BMI was categorized as recommended by WHO (<18.5, 18.5−24.9, 25−29.9, 30−34.9, 35−39.9, ≥ 40 kg/m^2). Systolic blood pressure was measured three times at 1-minute intervals using an automatic oscillometric method (Dinamap, Critikon, Florida, USA) after a person had come to rest, with cuff size adjusted to arm circumference. We used the mean of the second and third blood pressure measurements. Serum total and high-density lipoprotein (HDL) cholesterol were analysed using enzymatic colorimetric methods (Boeheringer Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total and HDL cholesterol. The creatinine values used in the eGFR calculation were measured in non-fasting serum blood samples drawn by trained nurses and analyzed at the Central Laboratory at Levanger Hospital. eGFR was estimated from recalibrated creatinine values using the Modification of Diet in Renal Disease (MDRD)-formula [4].

Supplemental Figure S1: Flow Chart of Study Recruitment and Follow-up



a) Follow-up for residents belonging to Levanger hospital and for patients referred to St. Olavs hospital (tertiary referral center): From entry date in HUNT2.
a) Follow-up for residents belonging to Namsos hospital: From 1 September 1999.

Supplemental Table S1: Procedure of estimating mediation parameters using the IOW method* [5,6]

Steps	Procedure	Stata code
	Preparing the data	*User written program to estimate mediation parameters Capture program drop IOW Program IOW, rclass Capture drop predprob inverseodds wt_iow
Step 1: Exposure model	The exposure model is run by regressing the exposure on all mediators and age as a covariate using logistic regression	*model 1 logit Sex i. smoking i. alc i. edu_cat age *model 2 logit Sex i. smoking i. alc i. edu_cat systBP non_HDL i. BMI age *model 3 logit Sex i. smoking i. alc i. edu_cat systBP non_HDL i. BMI i. LungDis i. CardDis i. diabetes i. cancer i. RenalDis age
Step 2: Create inverse odds weights	Based on the logistic regression models in step 1, the inverse odds weights are created by estimating the inverse of the predicted odds for each observation in the exposed group. The exposed and unexposed groups are then reweighted as follows: exposed = inverse odds, unexposed = 1	*obtain predicted probability for each individual based on the above regression models: predict predprob, p *calculate each individual's inverse odds from the predicted probability: gen inverse odds = ((1-preprob)/predprob) gen wt_iow = 1 if sex==0 replace wt_iow = inverseodds if sex==1
Step 3: Total effect model	The total effect of the exposure is estimated by using Cox regression model	stset eof, id(PID) failure(bacteriemia) origin(birthyear) enter(Enterdate) scale(365.25) stcox sex matrix bb_total = e(b) scalar b_total =bb_total [1,1] return scalar b_total=bb_total [1,1]
Step 4: Natural direct effect model	The direct effect model is similar to the total effect model, but includes the inverse odds weight constructed from the mediators, instead of controlling for the mediators themselves.	*Estimate the direct effect of sex on BSI by means of a weighted Cox proportional hazards model with the weights (pweight=wt_iow) achieved in step 2: stset eof [pweight=wt_iow], id(PID) failure(bacteriemia) origin(birthyear) enter(Enterdate) scale(365.25) stcox sex matrix bb_direct = e(b) scalar b_direct = bb_direct [1,1] return scalar b_direct = bb_direct [1,1]
Step 5: Natural indirect effect model	The indirect effect is estimated by subtracting the direct effect from the total effect.	return scalar b_indirect=b-total-b_direct
Step 6: Proportion mediated	The proportion mediated is estimated using the formula: $\ln HR_{NIE} / \ln HR_{TOTAL}$	return scalar b_mediated=((b_indirect)/b_total) end
Step 7: Estimate standard errors	The standard errors and confidence intervals are estimated by bootstrapping.	bootstrap r(b_indirect) r(b_direct) r(b_total) r(b_mediated), seed (32222) reps(1000):IOW estat bootstrap, all

*) Stata 17.0 was used in all statistical analyses.

Supplemental Table S2: Age [1]-stratified analyses of the associations of sex with risk of bloodstream infection

Age group	Sex	Years at risk	Risk of first-time BSI		
			No of BSI	HR	95% CI
< 50 years	Women	243943	162	1.00	Reference
	Men	215523	144	0.99	0.79 – 1.24
≥ 50 – < 65 years	Women	107835	228	1.00	Reference
	Men	97375	263	1.31	1.10 – 1.57
≥ 65 – < 80 years	Women	74200	432	1.00	Reference
	Men	55512	455	1.53	1.34 – 1.75
≥ 80 years	Women	10778	75	1.00	Reference
	Men	5503	81	2.17	1.58 – 2.97

BSI: Bloodstream infection HR = hazard ratio. 95% CI = 95% confidence intervals. No. = Numbers
1) Age at inclusion in HUNT2

Supplemental Table S3: Subhazards[1] of the association between sex and first-time BSI and BSI mortality defining death by other causes than BSI as a competing risk event

	Years at risk	No. competing events	Subhazard ratio	95% CI
First-time BSI	810674	10805	1.20	1.10 – 1.32
BSI mortality	876744	10805	1.52	1.25 – 1.86

BSI: Bloodstream infection, No. = Numbers, 95% CI = 95% confidence intervals.
1) Using Stata command stcrreg.

Supplemental Table S4: Association between BMI groups and risk of first-time BSI adjusted for age[1] and sex[2]

BMI	Years at risk	No. BSI	HR	95% CI
<18.5	5270	15	1.75	1.04 – 2.92
18.5 - 25	320605	523	Reference	
25 – 29.9	348436	813	1.03	0.93 – 1.16
30 – 34.9	103977	339	1.31	1.14 - 1.50
35 – 39.0	21402	90	1.87	1.49 – 2.34
≥40	5188	33	3.09	2.17 - 4.41
Systolic blood pressure[3]	802764	1805	1.00	0.999 – 1.001
Non-HDL cholesterol[3]	803177	1809	0.97	0.93 – 1.01

1) Adjusted for age as underlying time-scale.
2) Stratification by sex.
3) Additionally adjusted for BMI.

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Paper 3

Iron status and the risk of sepsis and severe COVID-19: A two-sample Mendelian randomization study

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Abstract

Introduction: Observational studies have indicated an association between iron status and risk of sepsis and severe COVID-19. However, these findings may be affected by residual confounding, reverse causation.
Methods: In a two-sample Mendelian randomization study using inverse variance weighted method, we estimated the effect of genetically-predicted iron biomarkers (serum iron, transferrin saturation (TSAT), total iron binding capacity (TIBC) and ferritin) on risk of sepsis and risk of being hospitalized with COVID-19. For the COVID-19 outcomes we additionally conducted sex-stratified analyses. Weighted median, Weighted mode and MR Egger were used as sensitivity analyses.

Results: For risk of sepsis, one standard deviation increase in genetically-predicted serum iron was associated with odds ratio (OR) of 1.14 (95% confidence interval [CI] 1.01 to 1.29, *P*=0.031). The findings were supported in the analyses for transferrin saturation and total iron binding capacity, while the estimate for ferritin was inconclusive. We found a tendency of higher risk of hospitalization with COVID-19 for serum iron; OR 1.29 (CI 0.97–1.72, *P*=0.08), where sex stratified analyses showed OR 1.63 (CI 0.94–2.86, *P*=0.09) for women and OR 1.21 (CI 0.92–1.62, *P*=0.17) for men. Sensitivity analyses supported the main findings and did not suggest bias due to pleiotropy.
Conclusions: Our findings suggest a causal effect of genetically-predicted higher iron status and risk of hospitalization due to sepsis and indications of an increased risk of being hospitalized with COVID-19. These findings warrant further studies to assess iron status in relation to severe infections, including the potential of improved management.

Key words: Iron status, Iron metabolism, Sepsis, COVID-19, Mendelian randomization, Sex differences

Introduction

Iron is an essential element to various physiological processes, including immune function, metabolism, and erythropoiesis (1, 2). Deviations in iron status (e.g., iron deficiency or iron overload) can have considerable health implications and iron status deviations show substantial sex differences with women more at risk of iron deficiency (1, 3). Iron status can be assessed clinically by using serum iron, transferrin saturation (TSAT), total iron binding capacity (TIBC) and ferritin (3, 4). A growing body of evidence has demonstrated an essential role of systemic and cellular iron-regulating mechanisms in protecting hosts from infections, and most pathogens depend on iron for their pathogenicity (2). Observational studies have indicated an association between iron status and risk of severe infections, where both low iron status (5, 6) and high iron status (7-9) have been linked to increased risk (10, 11). Studies related to COVID-19 found evidence that iron deficiency measured at hospitalization (12), or low serum iron and TSAT but high ferritin (13), were linked to severe COVID-19. On the other hand, excess serum iron, TSAT and lower TIBC (i.e. indication of iron overload) and hyperferritinemia have been associated with critical illness from COVID-19 (14, 15). In a study examining nutritional status in European populations, there were indications of low iron status linked to higher mortality from COVID-19 (16). There is evidence of sex differences in incidence and outcomes of COVID-19 infection (17-19). Few studies have evaluated sex differences in iron

status at time of infection. In a small study iron status were lower in female patients when measured at hospitalization due to COVID-19 (15).

A key limitation of observational studies is that they are prone to bias due to confounding and reverse causation. Mendelian randomization (MR) studies can overcome these limitations by using genetic variants associated with the exposures as instrumental variables. Because genetic variants are distributed randomly at conception, the risk of confounding (e.g. from lifestyle factors) and reverse causation (i.e. that the disease affects levels of the exposure) is greatly reduced (20). A recent MR study found a positive association between genetically-predicted high levels of iron biomarkers and risk of sepsis (21), but a more recent set of genetic instruments for iron status has since been published (22). No study has evaluated the role of iron status on the risk of COVID-19 in an MR framework and there is a lack of studies assessing sex differences (23) using sex-stratified MR analyses.

Leveraging data from large genome-wide association studies (GWAS), we aimed to evaluate the association between genetically-predicted iron status biomarkers and risk of being hospitalized with sepsis or COVID-19. In addition, by using sex specific summary-level data on iron status and COVID-19 outcomes, we assessed sex differences in the associations between genetically-predicted iron status and risk of hospitalization due to COVID-19.

Methods

We performed a two-sample MR study to estimate the effect of genetically-predicted markers of iron status on risk of sepsis and COVID-19 outcomes. None of the iron biomarkers reflect iron status perfectly and iron status in populations is challenging to assess (3, 4). Ferritin is widely used to assess global iron stores but is heavily influenced by inflammation (3, 4). Serum iron is a measure of the fraction of iron that circulates which is readily available and most of it is bound to transferrin. Serum iron is subject to diurnal variation and is affected by fasting status. By measuring the total number of binding sites for iron atoms on transferrin, we calculate the TIBC. TSAT reflects the amount of binding sites on transferrin occupied with iron (calculated as [Serum iron]/[TIBC] %). The normal

range is narrow, which is attributed to lower physiological variation than the other iron biomarkers. Low serum iron, low TSAT, low ferritin and high TIBC reflect low iron status. Elevated serum iron, TSAT and ferritin and low TIBC indicate high iron status. The iron in circulation turns over very quickly, especially during infection and inflammation and in clinical conditions with tissue destruction or repeated transfusions (4).

Genetic instruments for iron status

The exposure of interest was iron status and we ran the analyses for the four iron biomarkers serum iron, TSAT, TIBC and ferritin. The genetic instruments for the iron biomarkers were collected from a GWAS published in 2021 of 246,139 participants of European ancestry (22). The selected single nucleotide polymorphisms (SNPs) used as instruments were strongly associated (p-value <5e-8) with at least one iron biomarker (assumption 1 of MR studies), they should share no common cause with sepsis or COVID-19 (assumption 2), and should only affect the outcome through the risk factor (assumption 3) (20). F statistic above 10 was required for sufficient strength to limit bias due to weak intrumental variables (24). To reduce possible bias due to population stratification, both exposure and outcome cohorts included individuals of European ancestry. Independence between SNPs were ensured by using the LD-reference panel of European populations in 10,000 kb windows and $R^2 <$ 0.01 that is included in the TwoSampleMR (version 0.5.6) package in R (25), and we adjusted for correlation between SNPs using MendelianRandomization (version 0.6.0) in R (version 4.2.1) (26). Sex-specific effects for each biomarker were extracted from the same iron status GWAS using similar precautions for correlation between SNPs (22). We estimated R^2 in the TwoSampleMR package and calculated F-statistics using the formula F= ([n-k-1]/k)([R^2/1- R^2]) (24). The included numbers of SNPs with F-statistics and explained variance of the iron biomarkers is presented for all and separately for men and women, in Supplemental Table S1.

Genetic susceptibility to sepsis and COVID-19

The genetic susceptibility to sepsis was collected from the IEU OpenGWAS with summary-level data obtained from the UK Biobank which included 10,154 sepsis cases, defined as explicit sepsis (27), and 454,764 controls (25, 28). For COVID-19, we used data from the COVID-19 Host Genetics

Initiative (HGI), which is an international collaboration to facilitate COVID-19 genetics research, release 5 (18 Jan 2021). We evaluated two different COVID-19 outcomes: Hospitalized COVID-19 patients (n=4,829) compared with non-hospitalized COVID-19 patients (n=11,816), and hospitalized COVID-19 patients (n=9,986) compared with population-based controls (n=1,877,672) (29). Additionally, we used the sex specific summary-level data on the two COVID-19 outcomes from UKBiobank only, using the NHLBI GRASP catalogue (18 Jun 2021). As with the non-stratified analyses, we used two different COVID-19 outcomes: Hospitalized COVID-19 cases compared with non-hospitalized COVID-19 patients (female cases: n=1,181, controls n=7,586; male cases: 1,703, male controls: n=6,081), and hospitalized with COVID-19 compared to non-hospitalized population (female cases =1,181, controls =248,118; male: cases =1,703, controls =208,248) (30). Unfortunately, we were not able to find sex-stratified summary-level on sepsis.

MR analyses

The main analysis was the inverse variance weighted (IVW) method which assumes all genetic instruments to be valid (31) and a p-value of 0.05 was used for statistical significance. Three sensitivity analyses were conducted: weighted median, weighted mode, and MR Egger regression. The weighted median orders MR estimates produced by each SNP by their magnitude weighted for their precision and gives an overall MR estimate based on the median value with standard errors estimated by bootstrapping. This method allows for some of the IVs to be invalid (32). The weighted mode assumes that the most common causal effect is consistent with the true causal effect and allows some invalid instruments without biasing the MR estimate (33). MR Egger allows directional pleiotropic effects where some SNPs could be acting on the outcome through another pathway than the exposure of interest, but at the cost of statistical power (34). A consistent effect across these three sensitivity analyses and the IVW analysis suggests that pleiotropy did not bias the IVW estimate.

We used leave-one-out analyses to evaluate whether the IVW estimates were strongly driven by single SNPs (31). Additionally we used PhenoScanner version 2 (35) to check if any of the genetic instruments had important pleiotropic associations. All summary data used in this work are publicly available and with relevant ethical approvals (22, 28, 29), and follow recommendations of reporting

MR studies according to STROBE-MR guidelines (36).

Results

Sepsis

Genetically-predicted serum iron and TSAT levels were associated with risk of sepsis: Odds ratio (OR) 1.15 (95% confidence interval (CI) 1.01 –1.29, *P*= 0.03) for each standard deviation (SD) (7.76 µmol/L) increase in serum iron; and OR 1.12 (95% CI 1.02 – 1.23, *P*=0.01) per SD increase in TSAT (13.25 %) (Figure 1). The direction of effect for TIBC showed evidence of lower TIBC (i.e. indicating increased iron status) being associated with sepsis OR 0.94 (95% CI 0.87 – 1.01, *P*=0.09). Ferritin showed inconclusive results. The sensitivity analyses supported the findings from the IVW analyses.

Using PhenoScanner, we identified the SNP rs2228145, an instrument for serum iron, to be strongly associated with the IL6-receptor, which we considered a potential biasing pathway due to pleiotopy (Supplemental Table 2). In addition, some of the SNPs used were associated with BMI, CRP, coronary artery disease, triglyceride levels, cholesterol levels, blood pressure, diabetes 2, glycosylated hemoglobin and white blood cell counts. The leave-one-out analyses yielded similar results, suggesting that the different potentially pleiotropic pathways did not substantially affect the results (Supplementary Figure S1).

COVID-19

We found indication of a relationship between genetically-predicted higher levels of serum iron and risk of being hospitalized with COVID-19 compared with non-hospitalized COVID cases; OR 1.29 (95% CI 0.97 – 1.72, *P*= 0.08) (Figure 2). Similar associations were observed for TSAT and ferritin, but less pronounced. The sensitivity analyses supported the IVW analyses, and leave-one-out plots suggested no pleiotropic effects (Supplemental Figure S2)

In the sex-stratified analyses, we found tendency among women of a harmful effect of increasing genetically-predicted levels of serum iron; OR 1.63 (95% CI 0.94 – 2.86, *P*= 0.09) and TSAT; OR 1.31 (95% CI 0.99 – 1.75, *P*= 0.06). For TIBC and ferritin the estimates were uncertain (Figure 3). The corresponding results for men were less pronounced, and the wide confidence intervals made

comparison between the sexes inappropriate (Figure 4) The sensitivity analyses supported the main findings (Figures 3 and 4, and Supplementary Figures S3 and S4).

There was no clear evidence that genetically-predicted levels of iron status biomarkers were associated with risk of being hospitalized with COVID-19 compared with the non-hospitalized population including the sex-stratified analyses (Supplemental Figures S5, S6 and S7).

Discussion

In this study we performed two sample MR analyses to estimate the unconfounded effect of iron status on risk of sepsis and severe COVID-19 using data from large GWASs. The MR results provided some evidence that higher genetically-proxied iron load – reflected in higher levels of serum iron and TSAT, and lower levels of TIBC – were associated with increased risk of sepsis. We found a tendency for increased risk of being hospitalized with COVID-19 compared to non-hospitalized COVID-19 cases in subjects with genetically-predicted higher levels of serum iron. We included sex stratified analyses to assess potential sex differences in the effect of iron status on risk of COVID-19 hospitalizations, which provided some indication of a more pronounced harmful effect of high iron status among women compared with men, but with too little precision to strongly support a difference. The sensitivity analyses supported the overall findings.

Our results were consistent with previous observational studies on sepsis, including a prospective study from Turkey found higher serum iron in septic patients compared to healthy volunteers (8). There is a substantial lack of prospective studies investigating the effect of iron status measured before the onset of the infection. In a prospective population-based cohort study from Norway, we found low iron status to be associated with increased risk of future bloodstream infections (6). This is discordant to our MR results where higher genetically-predicted iron status is related to increased risk of sepsis and being hospitalized due to COVID-19, and could be attributed to differences in the epidemiological methods applied, such as residual confounding, but also limitations with the two-sample MR method used that is restricted to assess linear models (37).

Few MR studies have explored iron status and risk of severe infections. An MR-study using iron related SNPs identified in the Genetics of Iron Status-consortia (38) found evidence that higher serum-iron, TSAT and ferritin were related to increased risk of sepsis (21). Using a more updated set of genetic instruments for iron status biomarkers, we replicated these findings for serum iron and TSAT, a tendency for TIBC, but not for ferritin. Another MR study found evidence of increased risk of skin and soft-tissue infections with higher serum iron levels (39).

To the best of our knowledge, no previous study has conducted MR analysis to investigate the effect of iron status on incidence or outcome of COVID-19. Observational studies that have investigated iron status at the time of infection and found evidence of low iron status being a risk factor for a severe course of COVID-19 (12). Another study with COVID-19 patients compared to non-COVID-19 patients showed lower serum iron and TSAT levels in patients with COVID-19 independently of severity. Whereas COVID-19 patients defined as severe and critical had substantially higher ferritin levels (40). Some have linked COVID-19 to the hyperferritinemic syndromes which is associated with hyperinflammation (41). We identified a strong tendency towards an increased risk of being hospitalized with COVID-19 in persons with genetically proxied higher iron status. Differences between our findings and those reported in observational studies could reflect the fact that associations between iron status and COVID-19 may be confounded by factors difficult to adjust for such as poor nutritional status (4) or medical comorbidities associated with functional iron deficiency (42). Despite numerous observational studies in COVID-19 patients, the role of iron status before the time of infection as well as changes in iron status during infection has not been ruled out and the same applies to sepsis. We hypothesize that individuals with higher iron status could be less able to handle the acute iron load seen during severe infections, leaving them more vulnerable to be hospitalized with sepsis and COVID-19.

The role of iron status in the context of infectious diseases has long been noted (1, 2, 10, 11). Both iron deficiency (5, 6), iron overload (8), and iron fortification programs without adequate infection surveillance (43), have been linked to increased risk of infections. To date, treatment with iron chelators in sepsis or COVID-19 have not been studied in any large RCT, although suggested as

potential adjuvant therapy in several reviews (44, 45). Experimental models of sepsis studying different iron chelators, report promising anti-inflammatory and anti-bacterial effects (46). One pilot study in 92 COVID-19 patients using oral and intranasal lactoferrin have shown promising results with faster clinical symptoms recovery and lower serum ferritin levels in patients with mild to moderate COVID-19 compared to controls (47).

The pathway from iron status to risk of severe infections like sepsis and COVID-19 could be multifactorial, including long-term effects of iron status on immune functions and susceptibility to pathogens, but also adaptations in iron status at the time of infection (2, 11, 44). We identified that iron status affects the risk of sepsis and the risk of being hospitalized with COVID-19, indicating that iron status before the time of infection interfere with the response to infection.

It is well established that iron status varies according to sex (3, 4). Observational studies have shown that men are more prone to a severe course of COVID-19 (18, 19), leaving sex-stratified investigations important to reveal potential explanations for the sex differences (23). We assessed sex specific summary-level data on iron status and COVID-19 outcomes and showed that there was some tendency that the effect of serum iron and TSAT was more pronounced among women compared with men. Another study looking at iron status at time of hospitalization for COVID-19 identified sex differences where female patients had significantly lower serum iron, TSAT and ferritin levels and higher TIBC levels compared to men, whereas the association with severity between serum iron and TSAT was observed in both sexes (15).

Major strengths with our study include the use of large GWAS summary data for both iron status, sepsis, and severe COVID-19. Our main MR estimates were similar using IVW, weighted median, weighted mode, and MR Egger methods. As MR studies could carry the risk of pleiotropy, we used various strategies to detect and account for the potential pleiotropy. Taken together, the overall conclusions of our study were less likely to be affected by bias due to pleiotropy. We used GWAS summary data from European decent to reduce confounding due to population stratification. The slight difference in estimation and confidence intervals between the different MR methods were expected and most likely do not represent actual differences (48).

Several limitations should be considered in our study. First, the participants in our study are restricted to European ancestry and as both severe infections and iron deficiency are global concerns, our findings should be examined in other populations. Second, the sepsis phenotype has proven to be heterogeneous depending on how the causal pathogen act on the host immune functions and factors within the host (49). Timing and correct treatment of infections before they evolve to sepsis, further access to organ supportive treatment in intensive care units, and severity of sepsis might also be different. During the COVID-19 pandemic limited hospital resources and capacity might have influenced on hospitalizations. Third, iron status changes substantially during infection and inflammation, further exacerbated by tissue destruction and cell death. Iron status fluctuates during a lifetime, during periods of higher demand and need such as pregnancy and growth, in situations with increased losses (i.e. blood loss or critical illness), and due to chronical medical disorders (50). Genetically-predicted iron status may not perfectly reflect this time-varying exposure (51). The U-shaped risk relationship that has been proposed for the extremes of iron status (10, 15) might cause an attenuated association when evaluated in a linear model as in the two-sample MR methods. Non-linear MR methods could be more suitable to explore this U-shaped relationship but requires large GWAS with both measurements of iron biomarkers as well as the outcomes of interest (37). However, observational studies measuring iron status at time of infection might be biased by the acute phase response leading to iron depletion and hyperferritinemia (i.e. reverse causation) which we avoided using an MR framework. Due to the MR methods' use of genetic instruments, the possibility of confounding was limited. Using sex stratified summary-level data for both exposure and COVID-19 outcomes we were able to investigate potential sex differences in the associations.

In conclusion, our study leveraged large-scale summary data to explore the effects of iron status on risk of sepsis and severe COVID-19. Our findings support a causal association between high iron status and increased risk of sepsis and in verified cases of COVID-19 we identified a tendency of higher risk of being hospitalized in persons with higher iron status. We highlight the importance of sex specified summary-level data to assess potential sex differences in the associations. For being hospitalized with COVID-19 there were indications of a more pronounced effect of higher iron status

in women compared to men. Future studies are needed to explore the exact mechanisms of iron status and severe infections with the potential of prevention management and treatment strategies.

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Consent for publication

No individually identifiable data was included in this manuscript.

Competing interests

The authors declare no competing interests.

Authors' contributions

RMM and TR originated the research hypothesis and conceptual design. RMM and HMF analyzed the data and prepared tables and figures. RMM drafted the manuscript. HMF, KVL, ATD, ES, JKD, BOÅ, LTG and TR revised the manuscript. BOÅ and TR provided critical inputs in study methods and analyses. JKD and TR supervised the study. All authors revised and approved the submission.

Web links

Sepsis GWAS. gwas.mrcieu.ac.uk/

COVID-19 Host Genetics Initiative. covid19hg.org

Sex disaggregated COVID-19 GWAS. NHLBI GRASP catalogue, release date 06.18.21, grasp.nhlbi.nih.gov/covid19GWASResults.aspx

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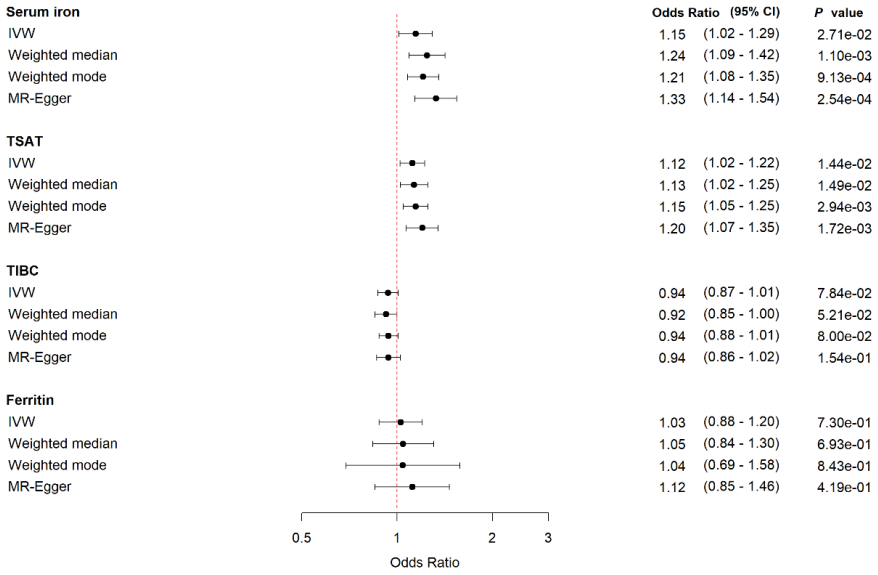
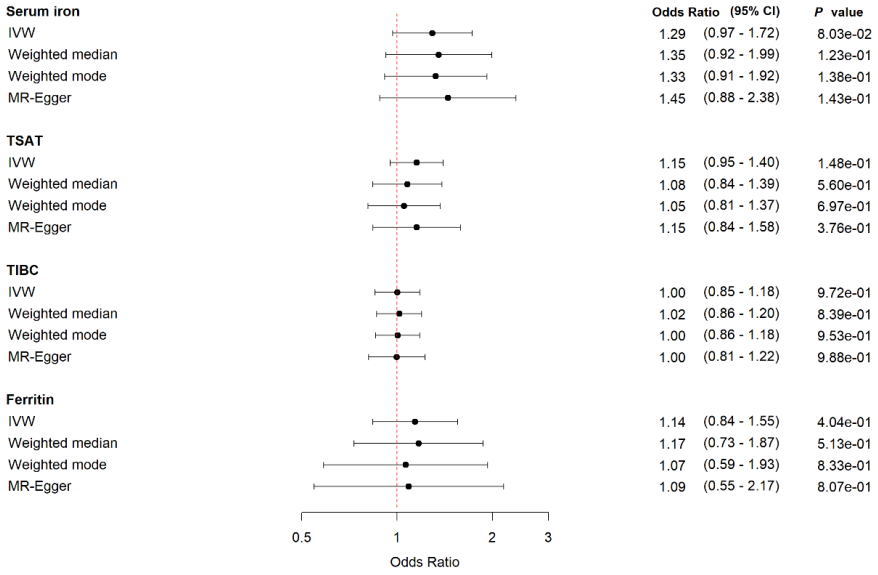
Figure 1: Forest plot with MR estimates for risk of sepsis



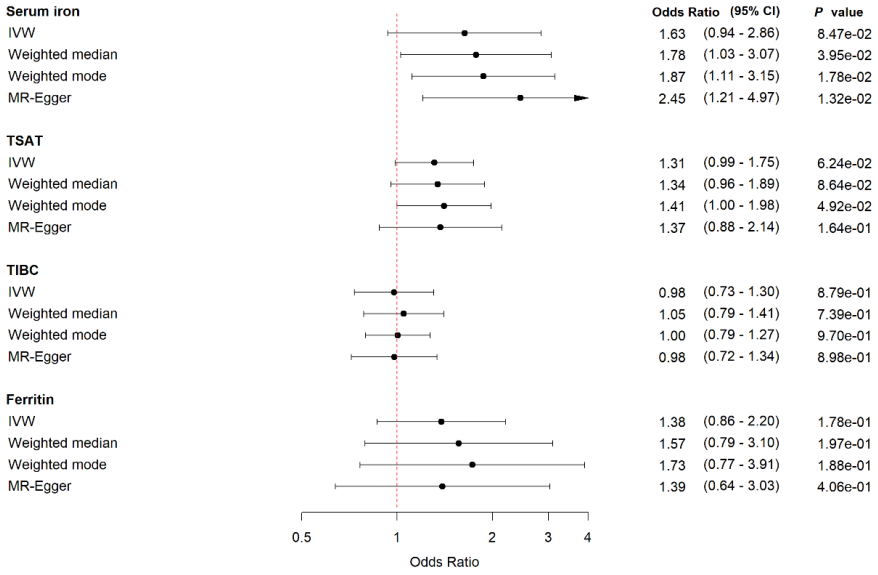
CI: Confidence interval, TSAT: transeferrin saturation, TIBC: total iron binding capacity, IVW: inverse variance weighted

Figure 2: Forest plot with MR estimates for risk of being hospitalized with COVID-19 compared to non-hospitalized COVID-19



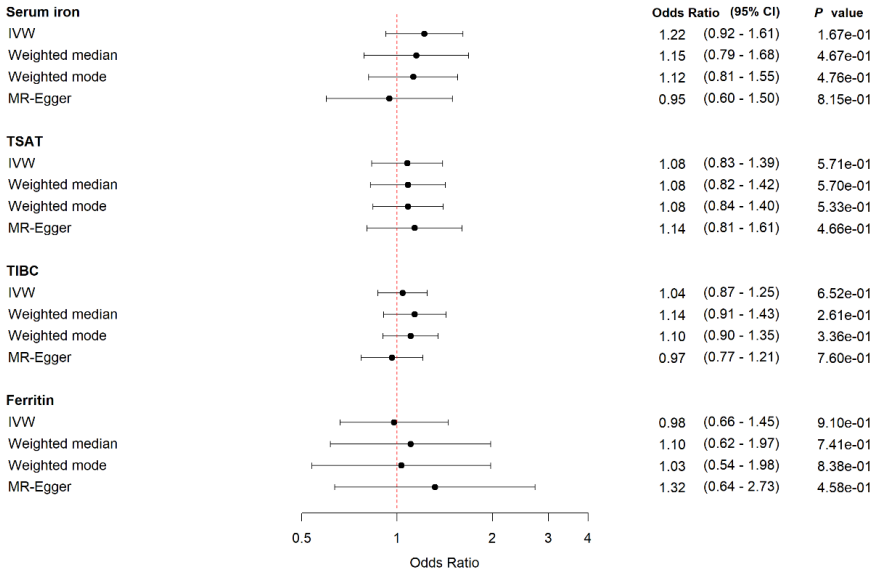
CI: Confidence interval, TSAT: transeferrin saturation, TIBC: total iron binding capacity, IVW: inverse variance weighted

Figure 3: Forest plot for *women* with MR estimates for risk of being hospitalized with COVID-19 compared to non-hospitalized COVID-19



CI: Confidence interval, TSAT: transeferrin saturation, TIBC: total iron binding capacity, IVW: inverse variance weighted

Figure 4: Forest plot for *men* with MR estimates for risk of being hospitalized with COVID-19 compared to non-hospitalized COVID-19



CI: Confidence interval, TSAT: transeferrin saturation, TIBC: total iron binding capacity, IVW: inverse variance weighted

Supplemental material

Iron status and the risk of sepsis and severe COVID-19: A two-sample Mendelian randomization study

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Supplemental Table S1: Included SNPs for all and sex-disaggregated analyses with explained variance and F-statistics for each iron biomarker

		All				Women				Men			
		Serum iron	TSAT	TIBC	Ferritin	Serum iron	TSAT	TIBC	Ferritin	Serum iron	TSAT	TIBC	Ferritin
No of SNPs	Sepsis	14	10	14	33	NA	NA	NA	NA	NA	NA	NA	NA
	Covid-19	11	9	11	27	14	9	15	33	14	9	15	33
Median variance explained (%)		1.8 %	3.4 %	2.2 %	1.5 %	2.0%	3.5%	1.9 %	1.5%	1.9%	3.5%	2.0%	1.4%
Range Variance explained (%)		1.4–7.5 %	1.7–9.7 %	1.4–12.0%	1.1–3.7%	1.1–9.4%	1.4–10.5%	1.3–10.3%	0.6–4.9%	0.9–9.8%	1.3–12.5%	0.5–12.3%	0.7–3.8%
Median F-statistic		291	550	233	158	148	334	138	61	96	232	101	44
Range F-statistic		226–1288	286–1680	147–1413	115–397	79–766	127–1076	90–814	22–205	49–543	82–924	27–702	22–122

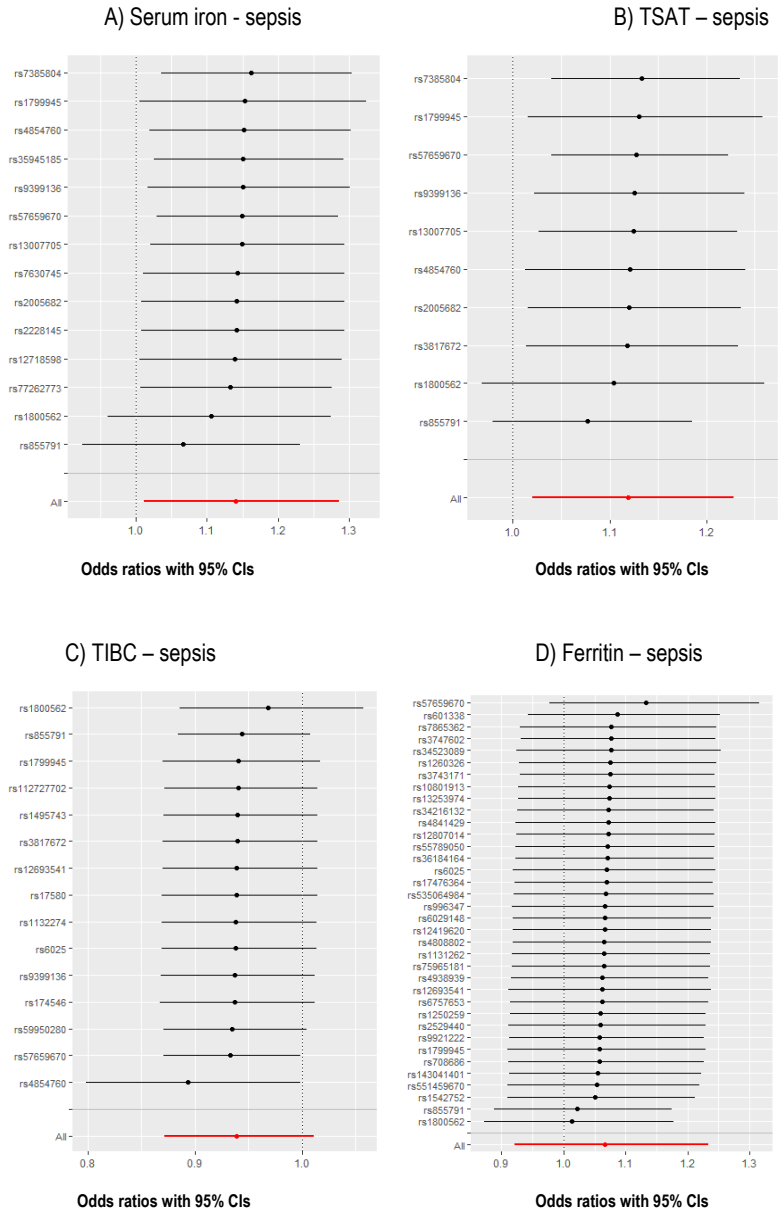
TSAT; transferrin saturation, TIBC; total iron binding capacity, SNP; single nucleotide polymorphism, NA; not available

Supplemental Table S2: Phenoscanner results for included SNPs linked to other biological traits than iron status

SNP	Trait	BETA	*P* value
rs1250259	Total cholesterol	0.03	7.56e-06
rs1250259	LDL cholesterol	0.03	1.46e-06
rs1250259	Blood pressure	-0.02	1.57e-11
rs1260326	Triglyserides	-0.12	4.00e-253
rs1260326	Total cholesterol	-0.05	3.00e-42
rs1260326	Diabetes 2	0.08	3.70e-09
rs1260326	Neutrophil count	-0.03	1.16e-19
rs1260326	Lymphocyte count	0.03	2.00e-12
rs1260326	CRP	-0.07	5.00e-40
rs12807014	BMI	-0.02	1.49e-15
rs2954029	Triglycerides	-0.08	1.00e-107
rs2954029	Total cholesterol	0.06	1.00e-77
rs2954029	Coronary artery disease	0.05	1.68e-22
rs2954029	Granulocyte count	-0.02	5.08e-10
rs2954029	Neutrophil count	-0.03	8.13e-12
rs2954029	Lymphocyte count	0.03	1.17e-13
rs2954029	BMI	NA	NA
rs34523089	Monocyte count	-0.05	1.77e-21
rs34523089	Granulocyte count	0.05	1.71e-26
rs3743171	Monocyte count	0.04	6.31e-14
rs3743171	Granulocyte count	-0.04	7.13e-14
rs3743171	Neutrophile count	-0.02	1.96e-06
rs3743171	BMI	0.01	6.50e-06
rs4808802	Total cholesterol	0.03	3.27e-08
rs4808802	Granulocyte count	0.02	4.72e-06
rs55789050	Diabetes 2	-0.06	7.80e-07
rs601338	Total cholesterol	-0.03	2.41e-10
rs174546	Neutrophile count	0.02	3.11e-10
rs174546	Monocyte count	-0.03	2.71e-14
rs174546	Granulocyte count	0.03	5.03e-16
rs174546	Triglycerides	-3.82	5.00e-24
rs174546	Total cholesterol	0.05	2.67e-37
rs2228145	IL-6	NA	2.00e-57
rs2228145	Coronary artery disease	0.04	4.80e-14
rs2228145	CRP	0.11	1.96e-10
rs2228145	Granulocyte count	0.02	4.23e-07
rs2228145	Monocyte count	-0.02	8.94e-06
rs35945185	Lymphocyte count	-0.02	2.47e-06
rs35945185	Granulocyte count	-0.05	2.74e-36
rs35945185	Neutrophile count	-0.05	6.22e-36
rs1799945	Hypertension	-0.1	2.00e-10
rs1799945	HbA1c	0.02	3.76e-19
rs1800562	HbA1c	-0.04	4.67e-28
rs1800562	Total cholesterol	-0.06	1.91e-12
rs855791	HbA1c	-0.02	3.44e-28
rs17580	Granulocyte count	-0.04	6.30e-06
rs59950280	Triglycerides	0.04	1.00e-10
rs59950280	Coronary artery disease	0.04	1.00e-06
rs59950280	Total cholesterol	0.04	1.00e-10
rs9399136	White blood cell count	-0.05	1.65e-29

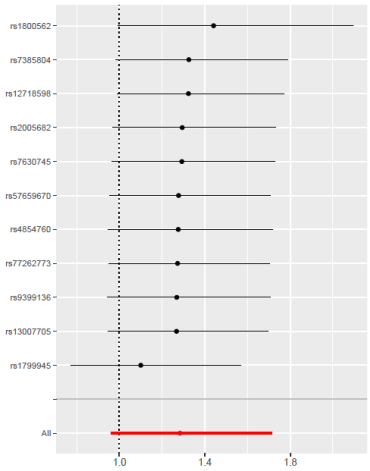
LDL; low density lipoprotein, CRP; C-reactive protein, BMI; body mass index, IL-6; Interleukin-6, HbA1c; glycosylated hemoglobin

Supplemental Figure S1 (A-D): Leave-one-out plots for the association between the iron biomarkers and sepsis

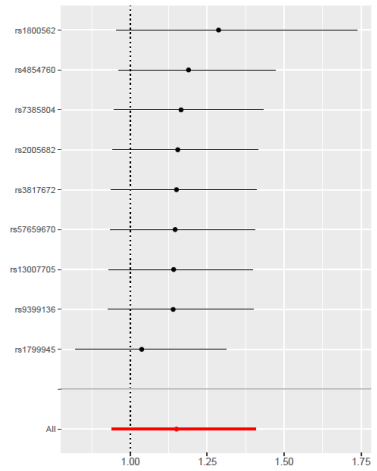


Supplemental Figure S2 (A-D): Leave-one-out plots for the association between the iron biomarkers and hospitalized COVID-19 vs non-hospitalized COVID-19

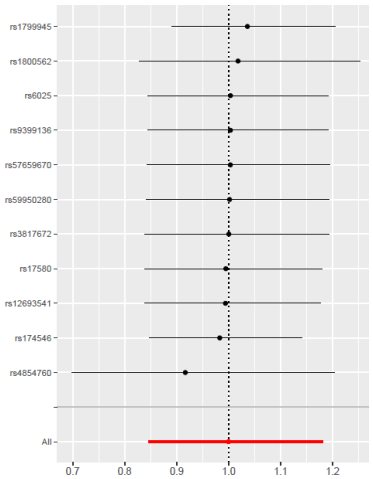
A) Serum iron – hospitalized COVID-19



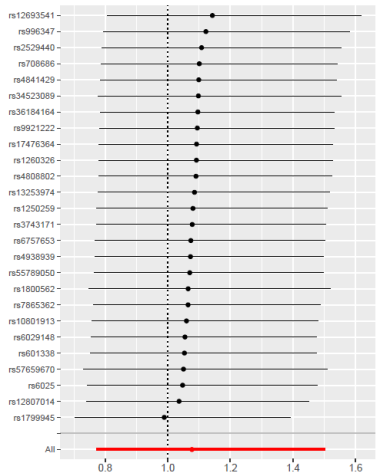
B) TSAT – hospitalized COVID-19



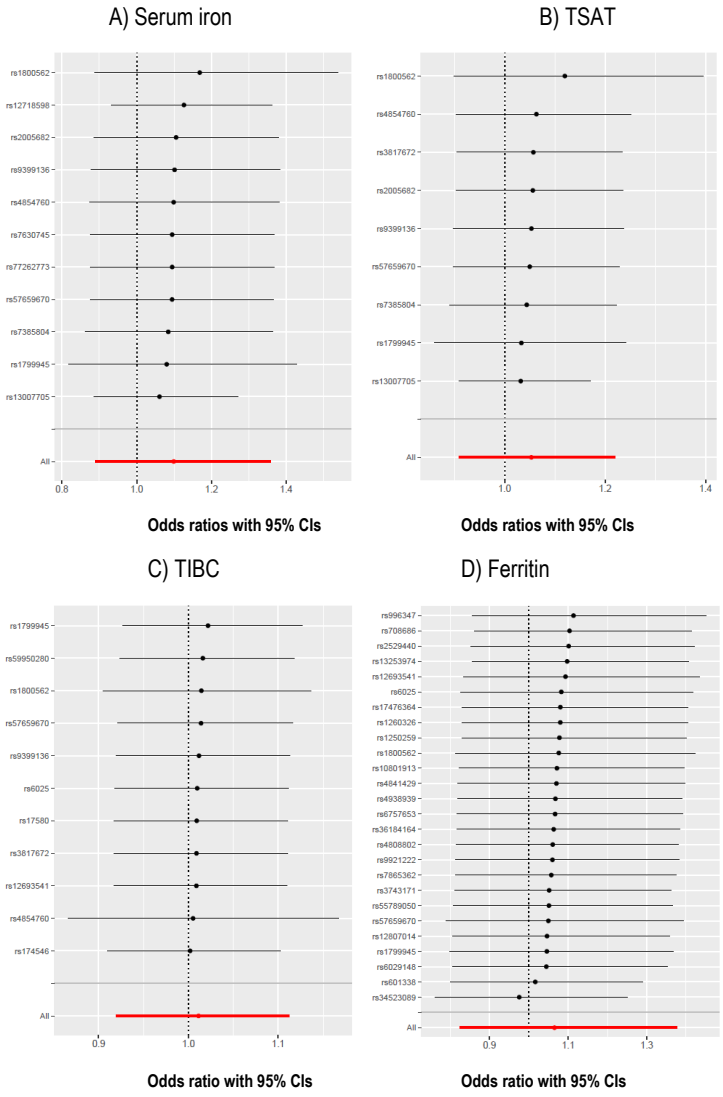
C) TIBC – hospitalized COVID-19



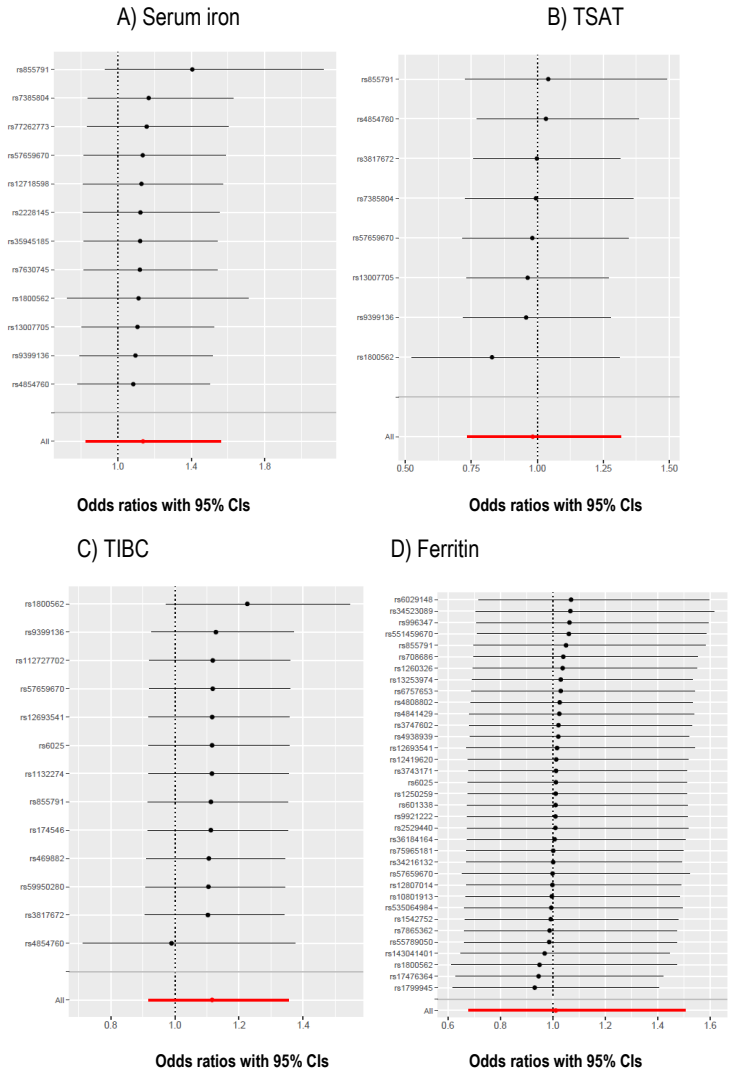
D) Ferritin – hospitalized COVID-19



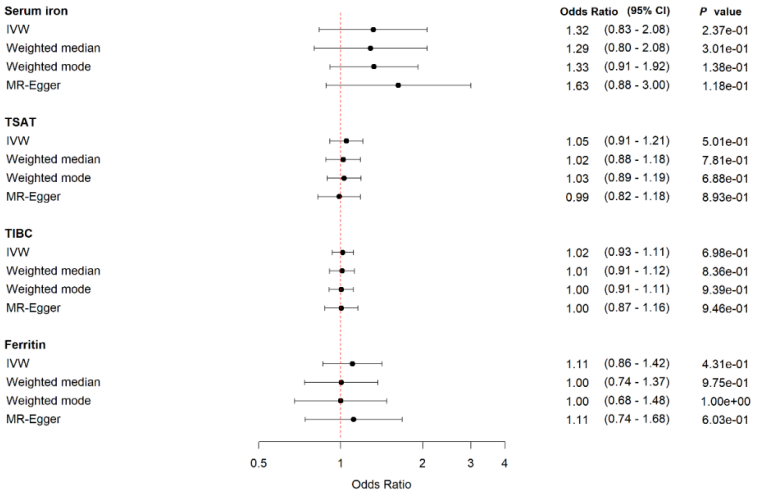
Supplemental Figure S3 (A-D): *Women* - Leave-one-out plots for the association between the iron biomarkers and hospitalized COVID-19 vs non-hospitalized COVID-19



Supplemental Figure S3 (A-D): *Men* - Leave-one-out plots for the association between the iron biomarkers and hospitalized COVID-19 vs non-hospitalized COVID-19

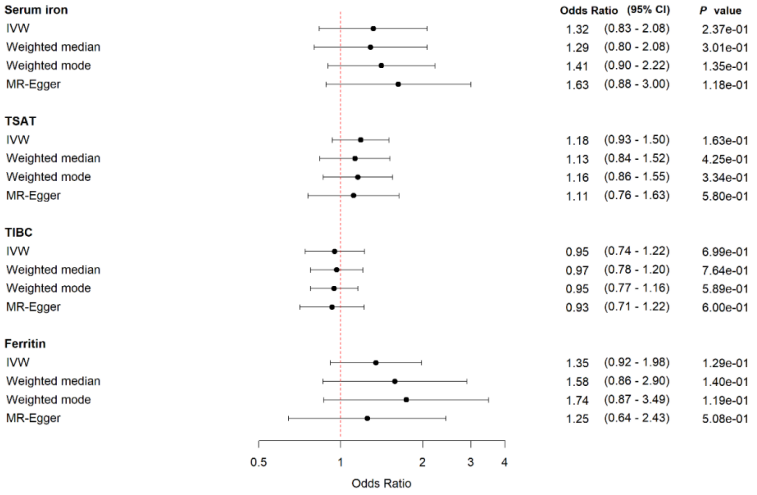


Supplemental Figure S5: Forest plot with MR estimates for risk of being hospitalized with COVID-19 compared with population



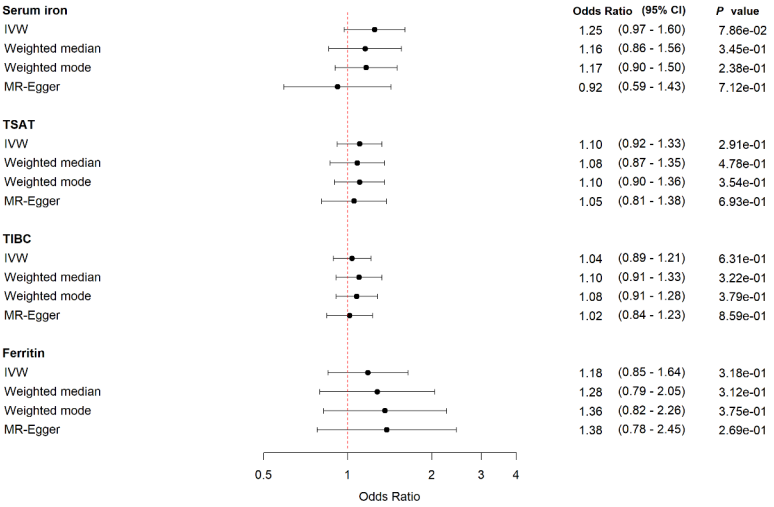
CI: Confidence Interval, TSAT; transferrin saturation, TIBC; total iron binding capacity, IVW: inverse variance weighted

Supplemental Figure S5: Forest plot for *women* with MR estimates for risk of being hospitalized with COVID-19 compared with non-hospitalized population



CI: Confidence Interval, Tsat; transferrin saturation, TIBC; total iron binding capacity

Supplemental Figure S6: Forest plot for *men* with MR estimates for risk of being hospitalized with COVID-19 compared with non-hospitalized population



CI: Confidence Interval, Tsat; transferrin saturation, TIBC; total iron binding capacity

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