Gut microbiota, lipid metabolism and systemic inflammation in common variable immunodeficiency

-A translational research approach

Cand. Med. Magnhild Eide Macpherson

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1. Acknowledgements

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2. Abbreviations

AIHA: autoimmune hemolytic anemia

ALP: alkaline phosphatase

ANCOM: analysis of composition of microbiomes

APC: antigen presenting cells

Apo A-1: apolipoprotein A-1

ASC: antibody secreting cells

ATF3: activating transcription factor 3

BAFF: B cell activating factor

BCR: B cell receptor

CD: Crohn's disease

CETP: cholesteryl ester transfer protein

CID: combined immunodeficiency

CKD: chronic kidney disease

CVD: cardiovascular disease

CVID: common variable immunodeficiency

DAMP: damage-associated molecular patterns

DC: dendritic cells

DMB: 3,3-dimethyl-1-butanol

FFQ: food frequency questionnaire

FMT: fecal microbiota transplantation

GALT: gut-associated lymphoid tissues

GI: gastrointestinal

GLILD: granulomatous lymphocytic interstitial lung disease

HDL: high-density lipoprotein

HFD: high-fat diet

IBD: inflammatory bowel disease

ICOS: inducible T cell costimulator

IDL: intermediate-density lipoprotein

IEC: intestinal epithelial cells

Ig: immunoglobulin

ILC: innate lymphoid cells

ITP: idiopathic thrombocytopenic purpura

IVIG: intravenous immunoglobulins

LCAT: lecithin cholesterol acyl transferase

LDL: low-density lipoprotein

LPL: lipoprotein lipase

LPS: lipopolysaccharide

LRBA: lipopolysaccharide-responsive beige-like anchor protein

LTA: lipoteichoic acid

LXR: liver X-receptor

MAMP: microbe associated molecular patterns

NK: natural killer

NLR: nod-like receptor

NRH: nodular regenerative hyperplasia

OTU: operational taxonomic units

PAMP: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cells

PD: phylogenetic diversity

PID: primary immunodeficiency

P13K: phosphatidyl-inositol-3-kinase

PIK3CD: PI3K catalytic subunit p110δ

PMA: phorbol 12-myristate 13-acetate

PON: paraoxonase

PSC: pluripotent stem cell

QIIME: quantitative insights into microbial ecology

RA: rheumatoid arthritis

RCT: reverse cholesterol transport

ROS: reactive oxygen species

SCFA: short-chain fatty acids

SCIG: subcutaneous immunoglobulin

SFB: segmented filamentous bacteria

SLE: systemic lupus erythematosus

TACI: transmembrane activator and calcium modulator and cyclophilin ligand interactor

TLR: toll-like receptor

TMAO: trimethylamine N-oxide

TREC: T cell receptor excision circles

Treg: regulatory T cell

T2DM: type 2 diabetes mellitus

VLDL: very low-density lipoprotein

XLA: X-linked agammaglobulinemia

3. Articles in the thesis

Paper I

Silje F. Jørgensen, Magnhild E. Macpherson, Tonje Bjørnetrø, Kristian Holm, Martin Kummen, Azita Rashidi, Annika E. Michelsen, Tove Lekva, Bente Halvorsen, Marius Trøseid, Tom E. Mollnes, Rolf K. Berge, Arne Yndestad, Thor Ueland, Tom H. Karlsen, Pål Aukrust, Johannes R. Hov, Børre Fevang.

Rifaximin alters gut microbiota profile, but does not affect systemic inflammation - a randomized controlled trial in common variable immunodeficiency

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Paper II

Magnhild E. Macpherson, Bente Halvorsen, Arne Yndestad, Thor Ueland, Tom E. Mollnes, Rolf K. Berge, Azita Rashidi, Kari Otterdal, Ida Gregersen, Xiang Y. Kong, Kirsten B. Holven, Pål Aukrust, Børre Fevang, Silje F. Jørgensen.

Impaired HDL Function Amplifies Systemic Inflammation in Common Variable Immunodeficiency

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Paper III

Magnhild E. Macpherson, Johannes E. Hov, Thor Ueland, Tuva B. Dahl, Martin Kummen, Kari Otterdal, Kristian Holm, Rolf K. Berge, Bente Halvorsen, Pål Aukrust, Børre Fevang, Silje F. Jørgensen.

Gut Microbiota-Dependent Trimethylamine N-Oxide Linked To Inflammation In Common Variable Immunodeficiency

Manuscript.

4. Introduction

4.1 The immune system- a brief overview

Protecting us from foreign invaders, our immune system combines mechanical and physiological barriers, protein systems and specialized cells to build a multilayered host defense. This immunological fortress is schematically seen as composed by two intertwining systems; the innate and the adaptive immune response. In addition to physical barriers such as skin and mucous membranes, our bodies depend on the innate immune system with its neutrophils, macrophages, monocytes, dendritic cells, complement system and cytokines as a first line of defence (1, 2)(Figure 1). Innate immune recognition of pathogens is facilitated by germ-line-encoded receptors for highly conserved structures present in broad groups of microorganisms; so-called pathogen-associated molecular patterns (PAMPs)(3). This allows for a prompt activation after infection and a swift control of pathogen replication (3), though not a pathogen specific response. The adaptive immune system consists of specialized B- and T-cells. These cells use antigen-specific receptors on their surface to recognize invaders and initiate a targeted effector response (1).

B cells develop from hematopoietic stem cells in the bone marrow before migrating to secondary lymphoid organs such as the spleen and lymph nodes. When B-cells have matured, they can respond to T-cell dependent and T-cell independent antigens to become antibody secreting cells (ASCs), producing immunoglobulins (Ig), neutralizing invading pathogens and facilitating innate immune system functions like phagocytosis. B cells that interact with antigens independently of T cells induce a response which is neither very specific nor long-lived, and no memory B cells are generated (1). In a T cell-dependent response, memory B cell class switching from IgM to another isotype enables the cells to react swiftly to a re-challenge, with IgG production as a secondary response (1). Thus, B-cells contribute to the adaptive immune system both by secreting antibodies and by functioning as memory cells that remember antigens and differentiate into ASCs upon exposure.

T cells are derived from common lymphoid progenitor cells residing in the bone marrow, but migrate to and develop in the thymus (2). The T cell education process results in several types of T-cells leaving the thymus for the periphery: $CD4^+$ Th1 cells that activate macrophages and set into motion a cell-mediated inflammatory response, $CD4^+$ Th2 cells which induce class-switching in B-cells and stimulate antibody production, and $CD8^+$ cells that are cytotoxic and involved in antiviral and potentially antitumor activity (1). T-cells that are activated will also undergo a maturation process that includes formation of memory T-cells, contributing to a central feature of the adaptive immune system: the immunological memory.

In the adaptive immune system, receptors for even the most common pathogen antigens are not inheritable (3). Subsequently, the adaptive immune system has to evolve over time in each individual, and will be determined by the exposure to pathogens to assemble an immunological memory. It usually takes days for the precise adaptive immune response to occur, whilst the innate immune system is able to provide an immediate response to foreign invaders (1)(Figure 1).

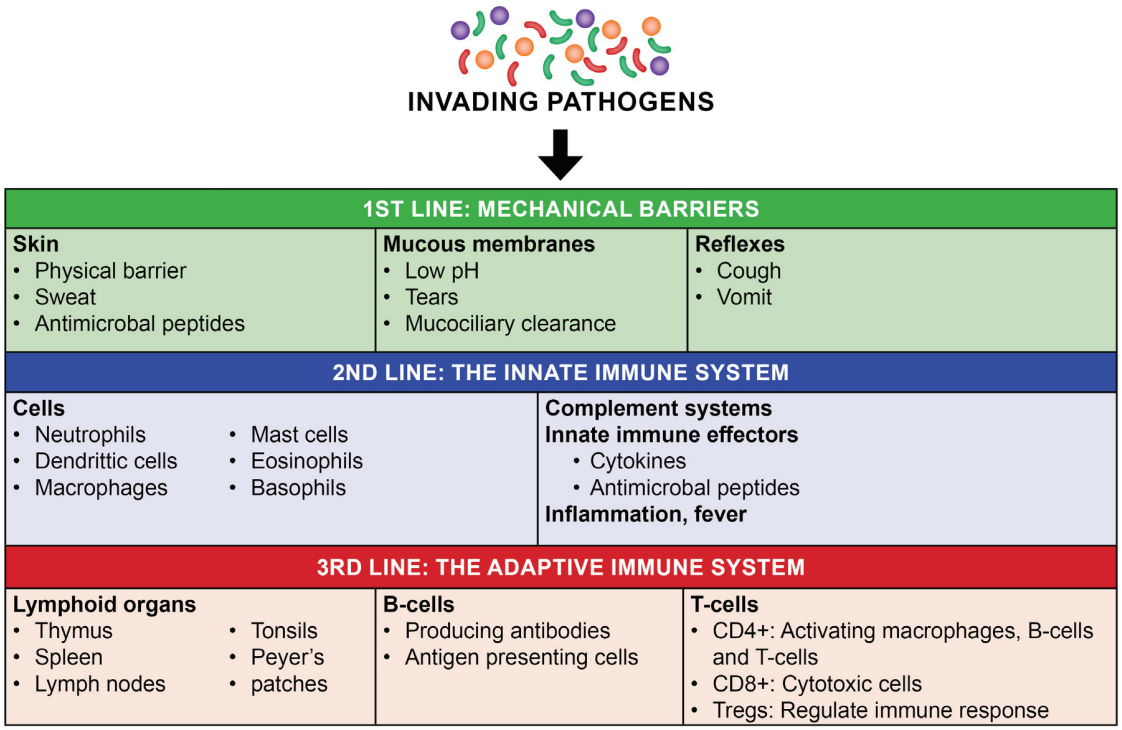

Illustration: Øystein Horgmo, UiO

Figure 1: **Defense lines of the human immune system.** First line of defense is composed of structural barriers that keep invading pathogens out of the body. Second line of defense is nonspecific, consisting of immune cells aided by the complement system and complex biological responses reacting to harmful intruders. Third line of defence is highly specialized, responding to antigens found in foreign microbes.

4.2 Primary immunodeficiency

In 1952, Bruton described an 8-year old boy with recurrent episodes of sepsis and complete absence of the gammaglobulin fraction in serum electrophoresis of his blood (4), a condition later named X-linked agammaglobulinemia (XLA, Bruton-type). One year later, Janeway *et al* characterized a 39-year old woman with recurrent infections in the respiratory tract, bronchiectasis, meningitis and lack of isohemagglutinins in serum (5). This latter case report is considered the first mention of the exceedingly heterogeneous medical condition now referred to as common variable immunodeficiency (CVID). However, the term CVID did not originate until 1971, when a WHO committee used knowledge on Mendelian inheritance and clinical descriptions to separate the hypogammaglobulinemic syndrome CVID from other less well-defined antibody deficiency syndromes (6). Both XLA and CVID are primary immunodeficiency disorders (PIDs), a heterogeneous group of several hundred disorders resulting from abnormal development or function of the adaptive or innate immune system (7). Importantly, PIDs are distinct from secondary immunodeficiencies, which originate from other causes like malnutrition, infections and medical immunosuppressive treatment (7). A review of PIDs in general is beyond the

scope of this thesis, and I will from here on focus on the PID that has been the subject of my thesis and that is the most common symptomatic PID amongst adults; CVID.

4.3 Common variable immunodeficiency

4.3.1 A brief introduction to CVID

CVID affects between 1:25.000 and 1:50.000 individuals in Caucasian cohorts and patients are usually diagnosed between the ages of 20 and 40 years (8). There have been different diagnostic definitions:

- In 1999 an ESID/PAGID committee suggested diagnostic criteria for CVID to include serum levels of immunoglobulin (Ig)G reduced by 2 standard deviations or more from the normal mean as well as low IgA and/or IgM, excluding other causes of hypogammaglobulinemia in patients above the age of two, and additionally absent isohemagglutinins or impaired vaccine responses (9).
- Later, Ameratunga et al proposed new diagnostic criteria where patients had to be older than four years of age, symptomatic with infections, autoimmune or inflammatory complications, and have IgG levels <5 g/l in adults. These criteria also excluded confirmed causative genetic defects from the definition, defining such as CVID-like disorders (10).
- A few years ago, the CVID ICON criteria were published, which were similar to the previous ESID/PAGID criteria using regional laboratory norms for IgG levels and mandatory low IgA and/or IgM levels, also including a demonstrable impairment of vaccine response and allowing for a CVID diagnosis without clinical manifestations being present (11).

We have used a CVID definition based on the first ESID/PAGID criteria in our studies (9, 12).The clinical hallmark of CVID is recurrent infections in the upper and lower respiratory tract, typically caused by encapsulated bacteria (12, 13), but importantly inflammatory and autoimmune manifestations are also common which reflects a profoundly dysregulated immune system. The treatment given to avoid frequent infections consists of regular administrations of Ig, either intravenously (IVIG) or subcutaneously (SCIG). When infections occur, CVID patients often require antibiotic courses that are of broader spectrum and of longer duration than do otherwise healthy individuals. Additional complications are treated on an individual basis but may require the use of immunomodulatory and immunosuppressive drugs.

4.3.2 B-cells in CVID

CVID is primarily characterized by defect B-cell differentiation and function (14, 15), even if T-cell abnormalities (16), macrophage defects (17), malfunctioning dendritic cells (18) and natural killer (NK) cell depletion also occur (19). The central dysregulation can occur at any critical stage of maturation and differentiation of B cells, resulting in reduced production of antibodies. However, impaired T cell function and lack of sufficient help for antibody production can also be underlying the observed B cell dysfunction (11).

Antigen stimulation of immature B cells in the presence of suitable co-stimulation in germinal centers make the B cells develop directly into antibody-secreting plasmablasts or mature into germinal center precursor B cells such as centroblasts and centrocytes (20). Some centrocytes differentiate into plasma cells or memory B cells, which both produce high-affinity antibodies (20). Class-switch

recombination involves a DNA recombination that switches the isotype of antibodies produced by activated B cells.

Whereas most CVID patients have normal total numbers of peripheral B cells in the blood (21), a small subset of patients (5%-10%) have very low peripheral B cell counts indicating early B cell differentiation defects in the bone marrow (14, 22). In 77% of the remaining patients, severely reduced levels of $CD27^+$ memory B cells, and in particular class switched memory B cells ($CD27^+IgM^-IgD^-$), have been found (14). Class switched memory B cells ($CD27^+IgM^-IgD^-$) are viewed as markers for memory B cells that have undergone a germinal center reaction, thus a classification based on B cell phenotyping has been suggested dividing patients into CVID group I (reduced number of peripheral switched memory B cells) and CVID group II (less significant reduction of peripheral switched memory B cells). CVID group I patients have been further divided by proportion of the pro-inflammatory $CD21^{-/low}$ immature B cells, which normally make up <20% of the B cell compartment (14). An increased proportion of $CD21^{-/low}$ B cells associates with more autoimmune cytopenias and cases of splenomegaly, suggesting that these immature cells play a part in non-infectious autoreactive complications of CVID (23). In a large European trial, an enhanced classification of CVID patients combining flowcytometric B cell phenotyping and clinical features was suggested (EUROclass). This separated patients with nearly absent (<1%) B cells, patients with severely reduced (<2%) switched memory B cells and patients with expansion of transitional (>9%) or $CD21^{-/low}$ (>10%) B cells (24). Amongst CVID patients with normal total B cell count, >80% showed reduced switched memory B cells. These patients had lower serum levels of IgA and IgG as well as a higher incidence of splenomegaly and granulomatous disease. Transitional B cells were found to associate with lymphadenopathy, whereas expansion of $CD21^{-/low}$ cells associated with splenomegaly (24). Thus, several distinctive forms of dysregulation in the B cell development can cause CVID, and the levels at which the dysregulation occurs can often be reflected in the clinical phenotype.

Intrinsic B cell activation defects have also been suggested to be involved in the pathophysiology of CVID. Defects leading to reduced B-cell receptor (BCR)-induced calcium signaling, such as reduced BCR dissociation from the B cell surface antigen CD20 upon antigen stimulation, have been identified in subgroups of CVID patients and associated with expansion of $CD21^{-/low}$ cells, lymphadenopathy and autoimmunity (25, 26). Another pivotal intrinsic activation pathway in B cells is conducted by toll-like receptors (TLRs), and TLR9 is an intracellular receptor that detects DNA-containing CpG motifs from bacteria and viruses and activates B-cells. CVID patients have been found to have broad defects of TLR9 activation, which hampers CpG-DNA-initiated innate immune responses, leading to loss of dendritic cell and B cell function (27, 28). TLR7 is another intracellular recognition receptor expressed in B cells, recognizing single-stranded RNA of viruses such as HIV and HCV, which has been showed compromised in CVID (28).

A subset of CVID patients have been found to have reduced expression of co-stimulatory molecules CD86 and CD70 in naïve B cells, molecules relevant in cognate interactions between B cells and T cells, thus important to normal cytokine expression and class switching (29, 30). The absent normalization of CD86 and CD70 B cell expression in CVID upon co-stimulation with autologous CD4+ T cells suggests this represents an intrinsic B cell defect rather than a T cell generated defect of the adaptive immune system (30). However, several surface molecules, including those activating T cells, have emerged as vital to the underlying pathology of CVID as demonstrated by genetic studies.

4.3.3 T-cells in CVID

T cells are imperative to normal B cell activation through providing co-stimulatory signals. Multiple studies support a disturbed T cell immunity in CVID, where reduced numbers of total CD4 T cells (31-34) and reduced regulatory T cells (Tregs) have been identified in CVID patient subgroups (33, 35-37). Recent thymic emigrant levels can also be low in CVID (33), suggesting an overall reduced replenishment of the CD4 T cell pool by newly derived cells from the thymus (16, 33). Supporting this, a reduced number of peripheral lymphocytes carrying T cell receptor excision circles (TRECs) has been identified in CVID patients, thus demonstrating impaired thymic output (38). Phenotyping of T cells in patients with CVID most commonly show reduction of total or naïve CD4 T cells, and this is accompanied by a more severe clinical picture (39, 40). A subgroup of CVID patients have increased numbers of CD8 T cells and elevated plasma levels of IL-7, which affects the homeostasis of lymphocyte proliferation, along with an impaired IL-7 response *in vitro* possibly affecting antigen presenting cell-mediated stimulation of T-cells (41). Some CVID patients show a lack of T cell proliferative responses and decreased cytokine production to recall antigens, an absent response which will remain after immunization (42). Subsequently, defective T and B cell functions can lead to impaired responses to prophylactic vaccinations in CVID (43). Prior to starting immunoglobulin (Ig) replacement therapy, CVID patients often have highly activated T cells, a feature potentially malleable under IVIg treatment (34). IVIg replacement has also been implied in reducing bacterial translocation from the gut, thereby restoring CD4+ T cell functions such as proliferation and production of IL-2 and IFN-γ (44). However, persistently elevated endotoxin levels in CVID patients on regular IVIg suggest other factors are also of importance to gut bacterial translocation (45).

The subgroup of CVID patients that only suffers from recurrent infections and no additional complications does not appear to have significant alterations of their T cell subpopulations, except for lower numbers of early differentiation stage CD4 cells (33). Other T cell abnormalities described in CVID seem to be associated with autoimmune complications (33), moreover the severity of these abnormalities appears reflected in a loss of CD4+ naïve T cells and associated with low levels of class-switched memory B cells (16). The reduced secretion of anti-inflammatory cytokine IL-10 from T cells in CVID patients, unrelated to different proportions of T cell subsets, supports a link between T cell deficiency and an insufficient B cell-function in CVID (46). Hence, T cell regulation of B cells in CVID seems to be important in many patients, especially in those with additional non-infectious complications.

4.3.4 Macrophages, dendritic cells and NK cells in CVID

The strength, direction and efficiency of an adaptive immune response is predominantly determined at the stage of antigen presentation to lymphocytes by antigen presenting cells (APCs) like macrophages and dendritic cells (47), an encounter that could have relevance to CVID pathology. Reports have suggested the interaction between macrophages and T-cells can be defective in CVID patients, leading to inadequate T-cell activation (17, 48).

Dendritic cells (DCs) play an orchestrating role in the immune system, both initiating innate immune responses and inducing adaptive immune responses. DCs of CVID patients have been found functionally impaired with defect antigen presentation and a significantly reduced capacity of secreting IL-12 upon stimulation with LPS, TNFα and CD-40 ligand (18, 49). Additionally, DCs from CVID patients have reduced expression of costimulatory molecules involved in the crucial DC-T-cell cross talk (18).

Furthermore, several studies have discovered a low percentage and absolute number of peripheral blood DCs in CVID patients compared to healthy individuals (50-52). Thus, defective DC function may be contributing to immune dysregulation in a substantial number of CVID patients by reducing functional antigen presentation and thereby reducing T-cell help for B-cell maturation (53).

Natural killer (NK) cells are a critical part of the innate immune defence, providing swift responses to cells infected with viral or bacterial pathogens as well as immune surveillance of tumors. Due to inhibitory surface receptors, they can recognize cells infected with a virus or undergoing tumor transformation by their low expression or loss of MHC I, thus these cells become target cells (54). NK cells also express activating surface receptors, allowing them to recognize viral and bacterial patterns such as TLRs and other surface ligands (54, 55). CVID patients have been found to have significantly reduced numbers of NK cells, which does not appear to bear any pronounced clinical effects but could be of importance to their risk of cancer development (19).

4.3.5 Immune activation in CVID

CVID is characterized by immune activation through recurrent infections, but also by inherent mechanisms of immune dysregulation. Even if inflammation represents a vital and adaptive response to restore homeostasis, it can also be dysfunctional and pathogenic (56). Inflammatory processes can be broadly categorized into acute and chronic inflammation, corresponding to activation of the innate and adaptive immune systems respectively.

Inflammatory inducers initiating the inflammatory pathway are classified as endogenous (releasing signals from stressed, malfunctioning or dead cells or tissues) or exogenous (microbial or non-microbial)(56). Endogenous inducers, characterized by damage-associated molecular patterns (DAMPs), can be released by dying cells to the extracellular environment generating sterile inflammation, while the pathogen-associated molecular patterns (PAMPs) of microbes characterize the exogenous inducers seen in infectious inflammation (57). Both DAMPs and PAMPs are recognized by receptors of the innate immune system, like TLRs and NOD-like receptors (NLRs), promoting production of multiple inflammatory mediators. If the acute neutrophilic inflammatory process is unsuccessful at eliminating invading agents, macrophages and T cells arrive, but if all these efforts fail then chronic inflammation ensues (56).

In CVID, malfunction of these inflammatory responses seems to affect a significant subgroup of patients characterized by chronic non-infectious inflammation and systemic immune activation, however the underlying mechanisms for this are not clear. Granulomatous clustering of immune cells seen in CVID patients are not always associated with specific external agents, but rather of a non-caseating nature (58), suggesting endogenous causes for augmented inflammatory responses need to be further explored in these patients.

4.3.6 Genetics in CVID

In contrast to many other forms of primary immunodeficiency, most cases of CVID appear sporadic and with a considerable genetic heterogeneity (59). Studies have been able to identify a monogenic cause in 5% to 25% of cases (11, 60), and in a recent study in as many as >30% of patients (61), but with a very variable clinical presentation. Genetic variants in modifier genes and environmental factors are believed to play a role in this (60). Also thought to contribute is allelic heterogeneity, where different mutations in one gene lead to varying phenotypic manifestations, as well as locus heterogeneity where mutations

in different genes can give overlapping phenotypes. An autosomal-dominant inheritance pattern is most frequently observed, but families with an autosomal recessive inheritance pattern have also been identified (11). The underlying cause for CVID might in many patients be polygenic or at least influenced by different genetic risk factors (62). The increasing use of comprehensive diagnostic gene panels will improve genetic diagnosis of CVID, but as of today most cases of CVID remain genetically unexplained.

Just under 400 monogenic inborn errors of immunity causing different forms of primary immunodeficiencies are already known, and this number is rapidly increasing (63). CVID is diagnosed based on clinical and immunological findings, but the current widespread use of genetic analyses has triggered a re-evaluation of the diagnostic criteria for CVID. The first disease gene identified for monogenic CVID was inducible T cell costimulator (ICOS) deficiency, which was reported in 2003 but is now considered to cause a combined immunodeficiency (CID)(59). Lipopolysaccharide-responsive beige-like anchor protein (LRBA) and PI3K catalytic subunit p110δ (PIK3CD) mutations have appeared the most frequent monogenic causes of CVID (59), however reclassifications are currently altering these numbers (63). Encoding genes for B cell activating factor belonging to the tumor necrosis factor family (BAFF)-receptors and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) have also been found mutated in CVID patients, and TACI seems to be the most common monogenetic cause of CVID (64, 65). Gene mutations encoding several B cell co-receptor complex deficiencies including CD19, CD81, CD21 and CD20 have been identified in CVID patients, as well as genes encoding the CTLA-4 receptor which prevents excessive T cell activation (59). These patients exhibit a CVID like phenotype, however with the reclassification of recent years CTLA-4 mutations are currently considered a new immune dysregulation syndrome (66).

Other mutations found CVID patients include genes encoding intracellular signaling molecules, such as PKCδ deficiency resulting in B cell deficiency and autoimmunity (67). NF-κB1 and NF-κB2 deficiencies are important to B cell maturation, survival and class-switching and has been observed in CVID (68, 69), as has phosphatidyl-inositol-3-kinase (PI3K) mutations (59) and PIK3CD mutations leading to defects in adaptive immunity but normal IgM levels (70). Several CVID families have a mutation of the transcription factor IKAROS, leading to a progressive loss of B cells and immunoglobulins in serum over time (71). Nevertheless, many of the genetic causes of CVID are estimated to still be unidentified, and some mutations currently known may be reclassified outside CVID.

Several other primary immunodeficiency syndromes can resemble CVID in their initial stages, such as mutations in GATA2, JAK3, RAG1 and the ARTEMIS encoding DCLRE1C gene, where antibody deficiency is detected early in life accompanied by blunted vaccine responses and potentially low lymphocyte counts / cytopenias (59). In these cases, the phenotype will evolve over time to reveal features that are not consistent with CVID and thus lead to reconsideration of the CVID diagnosis.

4.3.7 Clinical presentation of CVID

The most common clinical presentation of CVID is recurrent and frequent infections, occurring in nearly all patients. Respiratory tract infections are most prevalent, followed by gastrointestinal infections, whereas other bacterial infections like meningitis or osteomyelitis are less frequent (8). A large proportion of CVID patients also have non-infectious complications of an inflammatory or autoimmune nature (72)(Figure 2).

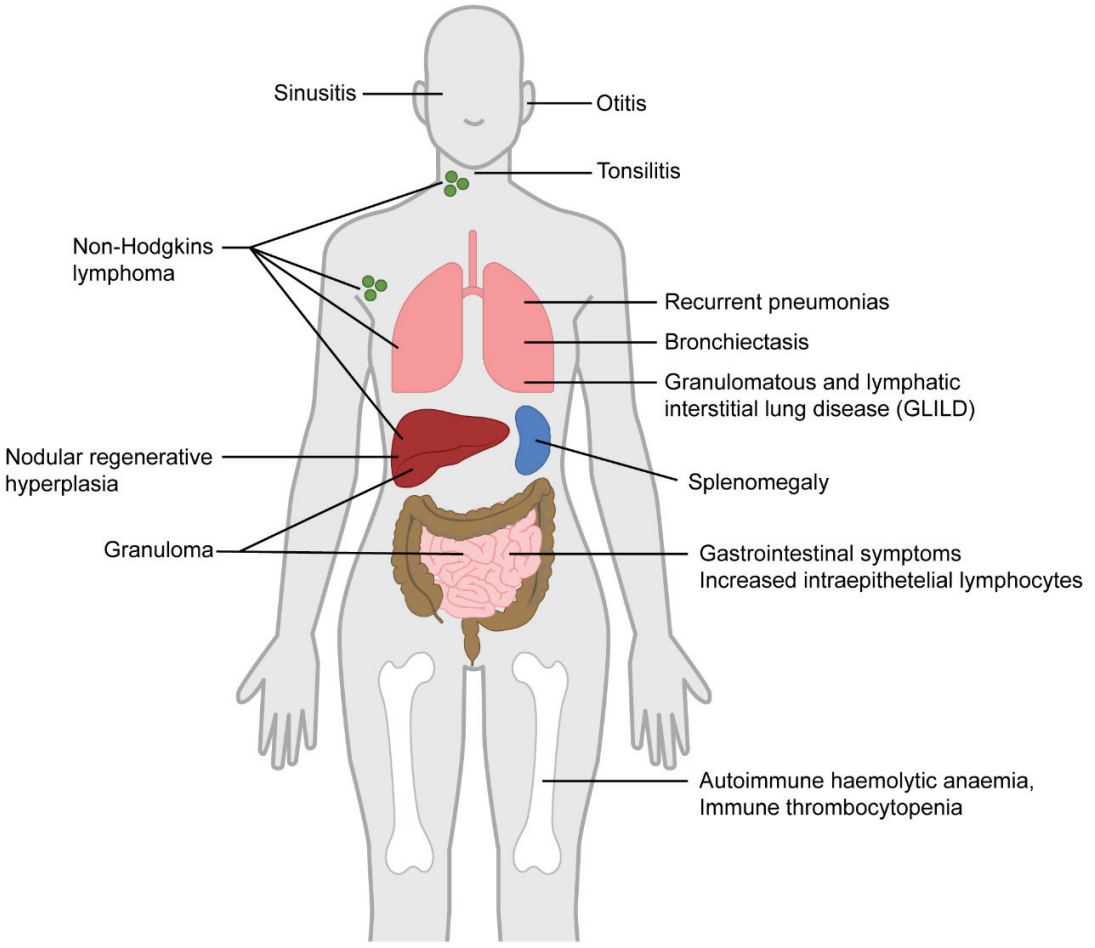

Illustration: Øystein Horgmo, UiO

Figure 2: Non-infectious complications in CVID. Around 70% of patients with CVID have one or more non-infectious complications whilst also suffering from recurrent bacterial infections.

At the time of diagnosis, nearly half of CVID patients have had pneumonia at least once, whereas the prevalence for chronic sinusitis, bronchitis and acute otitis varies between 30-40% (73). Common microbes causing respiratory tract infections in CVID patients are *Streptococcus pneumonia* and *Haemophilus influenzae*, along with several other encapsulated bacteria (74). Some patients endure persistent respiratory disease which develops to bronchiectatic changes despite adequate immunoglobulin treatment (73). When it comes to the incidence of gastrointestinal (GI) infections in CVID patients, reports have been somewhat deviating. *Giardia lamblia* was previously reported to be the most common organism causing GI infection in CVID, however in a more recent cross-sectional study, the only microbial findings on screening of CVID patients prior to endoscopy were one case each of *Clostridium difficile, Cryptosporidium* and *Campylobacter jejuni* (75, 76). Notably, only 3 out of 50 CVID patients in the cross sectional study tested positive for *H. pylori* prior to endoscopy (75). However,

almost a third of CVID patients have chronic gastritis, with gastric mucosal metaplasia the most common finding on gastroscopy (73).

Around 25% of patients suffer from an autoimmune condition, where autoimmune hemolytic anemia (AIHA) and idiopathic thrombocytopenic purpura (ITP) are the most common (72). CVID patients can also develop other autoimmune manifestations like pernicious anemia, autoimmune neutropenia, thyroiditis, rheumatoid arthritis, Sjogren's syndrome and uveitis (8).

Benign lymphoproliferation can be found in up to half of CVID patients, usually presenting as fluctuating lymphadenopathy or splenomegaly (77). Around a third of CVID patients develop splenomegaly during the course of the disease (73, 77). Lungs can be disturbed by lymphoid interstitial pneumonitis, which affects around 3% of patients (77). Between 8% and 22% of CVID patients acquire local or systemic granulomatous changes, sometimes prior to getting their CVID diagnosis (8). Some of the patients who develop granulomas in the lung also get an intense lymphoid infiltration, producing granulomatous lymphocytic interstitial lung disease (GLILD).

Gastrointestinal symptoms are quite common and cause reduced quality of life for many CVID patients, with around 30% of patients reporting diarrhea, pain or bloating (75). Bowel inflammation with increased numbers of intraepithelial lymphocytes in the mucosa resembling celiac disease has been observed in 46% of CVID patients (75). The inflammation disrupts the mucosal surface, thus leading to a leaky gut allowing for endotoxins to enter the blood stream. Clinically proven enteropathy was reported in 9% of a large combined European cohort, however the definition of enteropathy was not entirely unison between these studies (78).

More than 40% of CVID patients have deranged liver function tests, elevated alkaline phosphatase (ALP) levels being the most common (79). On liver biopsy the most frequent finding is nodular regenerative hyperplasia (NRH), and CVID patients with NRH are more likely to have other complications of CVID as well (79). Furthermore, NRH may in some CVID patients lead to hepatitis and portal hypertension, causing ascites and liver failure (80).

The cancer risk in CVID is mainly related to lymphoma (especially non-Hodgkin lymphoma) which is found in 2-8% of patients, but epithelial tumors of the stomach, bladder, breast and cervix also occur with a combined prevalence of around 3% (81). Clinically there is a clear difference in prognosis between CVID patients who suffer from recurrent infections only and those who additionally suffer from one or more autoimmune, inflammatory, granulomatous or malignant complications. Consequently, the distinction into the two phenotypes 'infection only' and additional 'non-infectious complications' is commonly used in scientific studies.

Negative prognostic factors related to the increased mortality have been early age at symptom onset and young age at diagnosis (72), low baseline serum IgG levels, reduced number of peripheral blood B-cells and increased IgM levels at diagnosis (21). Particularly reduced numbers of switched memory B cells seem to be an independent risk factor for autoimmune diseases, splenomegaly and granulomas (82).

4.3.8 CVID treatment

The standard treatment for CVID patients is lifelong replacement Ig-therapy, which reduces the incidence of bacterial infections and possibly enhances survival (77). Additionally, antibiotics have an

indisputable place in the treatment of acute infections. However, good infection control does not resolve the many and problematic non-infectious complications (8), and many patients will also need treatment with anti-inflammatory or immunosuppressive drugs. Corticosteroids and azathioprine have been used for many years, with more recent additions including monoclonal antibodies like rituximab, anti-TNF medications and vedolizumab. The treatment of immunodeficiency with an immunosuppressant comes with a caveat and is not always effective. Despite adequate and aggressive treatment, some patients will develop end-stage organ failure, most notably in liver and lungs. There is therefore a definite need for an improved understanding of the underlying dispositions and triggers causing so many of the CVID patients to develop immune-driven complications. Studies have revealed low-grade chronic immune activation in CVID patients with elevated serum levels of CRP, TNF, soluble (s) CD25 and sCD14 (83, 84), and chronic inflammation is a potential risk factor for developing atherosclerosis, cardiovascular disease, vascular changes in the brain and Alzheimer's disease (85, 86). Elucidating the intrinsic pathology of non-infectious inflammation in CVID patients is therefore a priority, and future CVID treatment will hopefully include modalities that can reduce inflammation and autoimmunity as well as frequency of infections.

4.4 Gut microbiota

4.4.1 An introduction to gut microbiota

The identification of microbial germs as a cause of human disease during the 19th century is one of the most important medical discoveries in history. The success of this understanding in combating communicable disease led to a temporary presumption that all germs were harmful, but we now know that microbes can be both friend and foe. The community of microbes inhabiting a particular individual or organ, the microbiota, is vital not only to good health but also our very survival. Residing in our gut is a microorganism community consisting of 10 trillion to 100 trillion microbes (87). In 1958, Eiseman and colleagues presented the cases of four patients with pseudomembranous enterocolitis treated successfully with fecal microbiota transplantation (FMT)(88). Seven years later, in 1965, Schaedler and colleagues introduced transfer of bacterial cultures to germ-free mice to the research field of gut microbiota (89). This prepared for future studies on the effect of gut microbes on the host. Human gut microbial composition has been discovered to be influenced by mode of delivery, breastfeeding and feeding patterns (90-92). Furthermore, genetics, co-housing with both humans and animals, malnourishment and treatment with antimicrobials in childhood can affect the gut microbial composition (93-98).

The human endogenous flora of microorganisms has yet to be fully mapped, but the larger part of these microbes reside in the gastrointestinal tract (99). To be able to investigate changes in the vast gut microbiota community throughout life, the traditional tools of culture-dependent isolation and growth of bacterial colonies in medium have proven insufficient, as they underestimate bacterial diversity. In 1996, the 16S rRNA technique, identifying specific genetic markers for different bacterial species in a sample, was introduced as a successful alternative to culture-based studies (100). This has become a very useful tool when analyzing human microbial diversity, and has even changed the way we describe these communities, focusing on the collection of microbial genomes in a sample by using the term microbiome. In 1998, the microbial continuum from commensal gut bacteria to becoming a pathogen with an intestinal ecosystem in imbalance was described (101) and in 2002 new insight into

mechanisms by which host-microbial interactions contribute to nutrient metabolism was depicted (102). In 2012, the National Institute of Health (NIH) published the first microorganism reference dataset (103). A growing body of reference databases has since emerged, which combined with ever-evolving bioinformatics technology has aided our ability to analyze gut microbiomes vastly.

4.4.2 Gut microbiota

Establishing what a normal gut microbiota is in humans has proven to be a difficult task. Gradients of pH, oxygen and antimicrobial peptides influence bacterial density and variety throughout the small intestine and colon (104). Moreover, gut bacterial diversity is influenced by host diet, with the diversity increasing from carnivory (meat eaters) to omnivory (eats meat and plants) to herbivory (plant eaters)(105). It has been proposed that an individual's gut microbiome holds a core of permanent commensal bacteria, whilst leaving room for environmental exposures throughout life to alter abundance but not the presence of specific microbial species (106). Such microbial resilience may hold a crucial role in health and disease (107), however the exact mechanisms by which the microbiome protects against or triggers disease are in many cases still unknown. Thus, this is an area that needs further investigation.

Gut microbial pathology can be described by two different axes; one relating to the specific composition of the bacterial community, i.e. the phylum, family and species identified in a sample, and the other axis relating to the diversity of the microbial community, i.e. how many different microbes can be identified. In general, a high degree of diversity is associated with a healthy microbiota whilst low microbial diversity is related to disease. The term dysbiosis is used to describe any alteration of a normal microbiota and will thus typically include both a change in the relative abundance of different microbial species and of the total diversity.

4.4.3 Gut microbiota interacting with other organ systems

Consisting of predominantly non-pathogenic commensal organisms, the gut microbiota functions as a filter for what we eat and influences human health beyond the intestinal tract (108). Obese individuals have an overall reduced bacterial diversity in their gut microbiome compared to lean individuals, along with a reduced relative proportion of the phylum *Bacteroidetes*. However, the relative amount of *Bacteroidetes* can increase with weight loss on fat restricted or carbohydrate restricted diets (109), highlighting a link between metabolic phenotype, diet and gut microbial composition. Whilst germ-free mice are protected from obesity caused by diet, transplanting intestinal microbiota from obese mice to lean germ-free mice leads to more fat deposition than if transplanting microbiota from lean mice (109), demonstrating that metabolic phenotype can be transferred through fecal transplantation in mice.

Gut microbiota in patients with type 2 diabetes mellitus (T2DM) is characterized by a moderate degree of dysbiosis with a decreased abundance of butyrate-producing bacteria (110), while pre-hypertensive and hypertensive individuals display dramatically reduced overall gut microbial richness compared to healthy controls (111). Fecal transplantation from hypertensive human donors to germ-free mice shows that high blood pressure can be transferred by fecal transplantation (111), whilst blood pressure also seems malleable by diet through alterations of the gut microbiota as demonstrated in studies on mice fed a high-fiber diet or given direct acetate supplementation (112). Microbial dysbiosis has also been linked to asthma (113), and inflammatory bowel disease (IBD) is thought to be related to an abnormal immune response to gut bacteria provoked by environmental factors in hosts with genetic

susceptibility to atypical recognition and processing of bacteria (114). The abundance of phyla *Bacteriodetes, Firmicutes* and *Proteobacteria* appear to be closely linked to a range of medical conditions (114, 115), however the association between gut microbiota and different diseases is accompanied by a striking lack of mechanistic and pathogenic understanding of its relationship. One concept does seem clear though; the interaction is bidirectional, with the host influencing the microbiota and the microbiota influencing the host.

4.4.4 Gut bacteria and the immune system

The gut microbiota has substantial effect on the function of the human immune system and vice versa, and a stable and diverse gut microbial community is regarded as essential for host physiological processes and immune functions of the mucosa (116).

As part of its surveillance duty the immune system keeps sampling the gut microbiome, adapting to the exposures it detects in the microbial community. Pattern-recognition receptors of the innate immune system can recognize conserved molecular products of microbes like lipoteichoic acid (LTA) recognized by TLR2 and lipopolysaccharide (LPS) recognized by TLR4 (117). Commensal bacteria will thus normally be recognized as such by TLRs and this is an essential interaction in the maintenance of intestinal epithelial homeostasis (118). TLRs also operate as sensors of microbial infections in the body and are integral players in the initiation of immune responses and inflammation, thereby contributing to both activation of innate immunity and the development of antigen-specific adaptive immunity (117). Defects in the signaling of other innate receptors like NLRs can affect the composition of gut microbiota through altering the expression of antimicrobial peptides (116).

There is only a single layer of epithelium separating the gut-associated immune system and the millions of bacteria in the lumen, and some of the gut microbes will attempt to cross the intestinal mucosal barrier. They do however have several functional parts to overcome; firstly a gel layer of intestinal mucus which transports luminal components and protects the epithelium from gut contents that could be damaging. Secondly, tight junctions seal the paracellular space between epithelial cells of the intestinal epithelium, thereby maintaining the barrier against free diffusion. Large components like bacteria can normally not pass through the gut mucosal epithelium (119). However, certain bacterial products from the gastrointestinal tract have been discovered getting through to lamina propria, a mechanism termed bacterial translocation (120). One such bacterial product is lipopolysaccharide (LPS) found in the outer membrane of *Gram negative* bacteria, which after the bacterial translocation activates the innate immune system. Below the intestinal epithelium, the mucosal immune system consists of various immune cells acting to maintain a balance between protecting against microbial invasion whilst avoiding unrestrained inflammation (121). In healthy conditions, bacterial translocation is a physiological process and an important part of host immunity. As a reaction to bacterial translocation, epithelial cells in the gut release chemokines generating recruitment of dendritic cells (DC) to the mucosa. Intestinal DCs sample commensal bacteria at the mucosal surface, prompting IgA-production from B-cells and ensuring the intestinal IgA repertoire represents the dominant bacterial species in the intestine at any given time (122). If the rate and degree of bacterial translocation from the gastrointestinal lumen increases and becomes continuous, it turns into a pathological condition (123)(Figure 3).

Several diseases have been associated with increased intestinal permeability, including inflammatory bowel disease, coeliac disease, irritable bowel syndrome, metabolic diseases, obesity and critical illness (124). In treated HIV patients, an abundance of *Gram negative* bacteria in the gut was found to correlate with the endotoxin pathway and chronic immune activation, implying inflammation and bacterial translocation as contributing factors (125). A similar mechanism has been suggested in CVID, where elevated levels of immune activation markers and endotoxins were observed in the setting of gut microbial dysbiosis (45); an association warranting further proof-of-concept investigations.

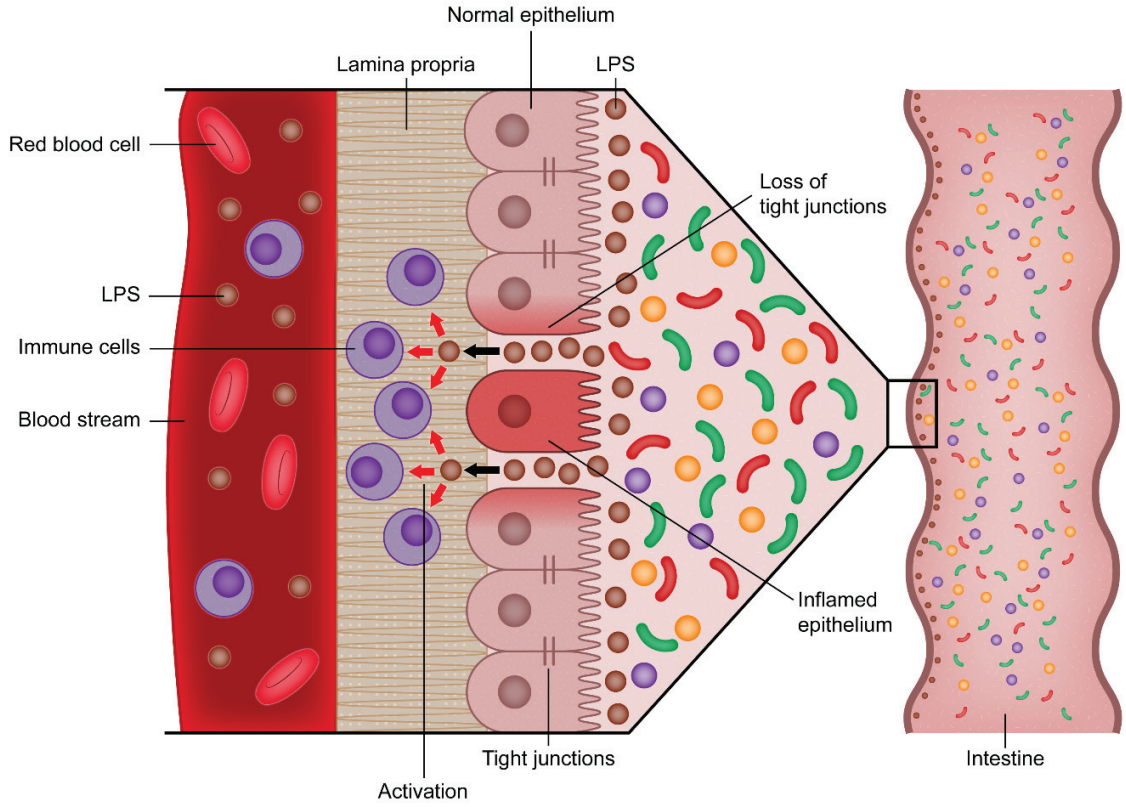

Illustration: Øystein Horgmo, UiO

Figure 3: Leaky gut. Inflamed epithelial cells in the gastric mucosa lose their tight junctions and start allowing small particles like lipopolysaccharide (LPS) through. These particles can then translocate to the underlying lamina propria and bloodstream, activating immune cells.

Various components of the adaptive immune system also influence the gut microbiome. B-cell deficient mice have reduced bacterial diversity in their gut; moreover, their concentration of LPS is higher than in controls, indicating a break in the gut barrier integrity related to lack of B-cells (116-118, 126). However, overall gut bacterial composition in B-cell knockout mice appears similar to controls except for some small changes within the microbial community, thus implying that a relatively low level of dysbiosis can cause substantial alterations in intestinal function (126). Intestinal epithelial cells (IECs)

have mechanisms for adapted responsiveness to microbial signals, making them crucial to intestinal homeostasis by enabling tolerance for the relentless exposure to commensal bacteria (127). IECs reveal microbial signals to immune cells in the mucosa, eliciting appropriate immune responses to pathogens and commensal bacteria by interacting with APCs. Subsequently, IECs can modify B- and T cell responses (127). However, various factors can hinder the gut bacteria in accessing the host cells, such as secreted bacteria-specific IgA reducing intestinal proinflammatory signaling (128) and the organization of mucous layers including mucosal biofilm formation (87, 104). Thus, IECs may not encounter all bacteria in the intestinal lumen and fecal microbiota could represent a combination of shed mucosal bacteria and nonadherent luminal bacteria (129), worthwhile keeping in mind when studying the cross-talk between gut microbes and the immune system. The immune system utilizes multiple mechanisms to neutralize and kill harmful viruses and bacteria. As a natural consequence, a parallel development of immune evasive mechanisms within pathogens occurs, making human endogenous intestinal microflora an important factor instructing innate immunity (129). Microbe-derived immunomodulins, vitamins and short-chain fatty acids (SCFAs) can modulate signaling within the host, thereby altering immune cell activity and cytokine production (130). Some bacteria use specific secretion systems with effector molecules to attack pivotal signal transduction mediators in the host immune cells, and thereby inducing host cell apoptosis (131) or decreased transcription of important pro-inflammatory genes within the innate cells (132). This can affect the host immunity to such a degree that it generates long-lasting effects (133). Pathogens can also cleave and bind antibodies using a variety of proteases and proteins, as well as dampen or alter T cell responses (133). Several bacteria manage to interfere with the integral MHC-TCR interaction, where innate cells present antigens to the adaptive immune system (133).

Dietary fiber has emerged as a central modulator of gut microbial function, and has been proposed to reduce colon cancer risk based on enhancing SCFA activity which subsequently inhibits cancer cell migration and invasion, augmenting cancer cell cycle arrest and apoptosis as well as reducing carcinogen contact time within the intestinal lumen (134). SCFAs, and in particular butyrate, influence the regulatory T cell (Treg) network through enhancing induction and fitness of Tregs (135). Butyrate has epigenetic properties by inhibiting histone deacetylases, which has been suggested an underlying mechanism for increased generation of Tregs in the gut (136). Thus, bacterial metabolites can modulate communication between commensal microbes and the immune system, bringing about changes in pro- and anti-inflammatory cells (135). The gut microbiota is also emerging as an important mediator of tumor responses to chemotherapy and immunotherapy (137). This suggests that bacterial composition in the gut can be targeted to enhance the effects of specific treatments, a fascinating and promising field to explore further.

4.4.5 Gut microbiota in CVID

The combination of a hampered microbial defense and immune-driven enteropathy is commonly seen in different forms of primary immunodeficiency, suggesting there are alterations of the gut microbial flora. This is indeed seen in CVID, where a large shift in gut microbial composition with reduced intra-individual bacterial diversity has been shown (45). This is supported by experimental models that have demonstrated a lack of B- or T cells and IgA in mice leading to reduced bacterial diversity in the gut (138).

A CVID specific dysbiosis index has been calculated based on relative abundance of the 10 taxa mostly differentiating the gut microbiota in CVID patients from healthy controls (45). This index is based on a model previously used in Crohns' disease (CD), where scientists had observed taxa within certain

families to be regularly found together and correlating with clinical disease severity (139). The taxa found upregulated in CVID include *Bacilli, Dorea, Roseburia* and *Gammaproteobacteria*. The taxa found reduced in CVID include *Bifidobacterium, Odoribacteracea, Christensenellaceae, Blautia, Sutterella* and *Desulfovibrionacea* (45).

Commensal bacteria at the intestinal mucosal surface generate IgA responses. Studies on germ-free mice have demonstrated stepwise and highly specific IgA responses to bacterial exposure, establishing an antibody repertoire matching the current commensal bacterial content (122). However, in mice lacking B-cells, the intestinal epithelium responds to gut microbes by activating innate immune genes as a defense mechanism, much to the detriment of metabolic genes thus affecting fat absorption (126). This indicates a dysfunctional immune system with little IgA-mediated regulation of gut microbes will stimulate intestinal epithelial cells to acquire immune functions whilst concurrently reducing their metabolic activity. Gene expression profile studies of duodenal biopsies from CVID patients with gastrointestinal pathology have indeed shown genes involving immune components to be upregulated and genes involving metabolic functions to be downregulated compared to controls, launching a possible association between immunodeficiency and defective absorption of lipids, though lacking a clearly detectable link to the human gut microbiome (126).

B-cell effects on intestinal metabolism thus seem to depend on the ability of plasma cells to secrete IgA antibodies specific for intestinal flora, as induced by antigen-presenting dendritic cells (140). IgA can limit bacterial access to the epithelium and thereby penetration to intestinal immune cells (140, 141), but also facilitate survival of certain bacteria through a symbiotic relationship (142). Gut bacteria extensively coated with IgA make up a subgroup of bacteria that will selectively stimulate intestinal immunity, and has been associated with susceptibility to inflammatory bowel disease (143). Hence, gut bacteria evoking vast amounts of IgA may have a competitive disadvantage to those evoking less IgA, highlighting that the principal role for antibodies in the gut appears to be mediating tolerance (128).

A normally functioning adaptive immune system strives to maintain a link with the gut microbiome by selectively inducing immune responses to bacteria stimulating the innate immune system, thus allowing greater microbial diversity without abandoning the vital protective role the innate immune system has in maintenance of the mucosal barrier (128). If the adaptive immune system is deficient, upholding this balanced interaction between gut bacteria and the immune system through coordination of gut microbial homeostasis becomes challenging. In successfully treated HIV patients, links have been found between gut bacterial community, modified metabolic pathways and markers of systemic immune dysfunction, suggesting chronic immune activation could in fact be sustained by host-microbiota interactions (125). Thus, the relationship between gut microbes, metabolic function and systemic immune activation in CVID needs more investigation.

4.4.6 Modulating gut microbiota

The effect of microbial pathogens on our immune system has already been an area of much research, whilst a possible modulation of the immune system by our commensal microbial flora is a new and promising field to be explored. Highlighting this, an adverse alteration of the intestinal immune-microbial homeostasis can lead to development of inflammation (144).

Without causing major changes to the gut microbial composition, rifaximin (an antibiotic acting locally in the gut) has been shown to reduce endotoxemia, increase serum fatty acids and alter urine

metabolites in patients with liver cirrhosis (145). It has been suggested that alteration of virulence and biological properties of gut microbes, achieved through the use of locally acting antibiotics at subinhibitory concentrations, could be underlying such changes (146). At the same time, the use of probiotics in HIV-infected individuals on stable antiretroviral therapy has shown a tendency to reduce inflammatory markers CRP and IL-6 and a significant reduction of D-dimer levels without inducing major changes to microbial translocation from the gut (147). The crosstalk between our commensal gut microbiota and immune cells could thus lead to modulation of the immune system, dependent on shifts in microbiota composition or microbial properties.

If the symbiosis between the immune system and the microbes is somehow disturbed, we may end up with a microbiome not fit to establish immune responses or maintain immune homeostasis. As a result, autoimmune and inflammatory disorders may arise (148, 149). Despite this knowledge, little is known about how the immune system, use of antibiotics, gut microbial dysbiosis and systemic inflammation connect. There is a lack of knowledge on how extensively and for how long the gut microbial composition is affected by antibiotic disturbance (Figure 4). Moreover, it is unclear which factors most significantly affect this connection in patients with primary immunodeficiency; the dysfunctional immune system influencing gut microbial diversity, or the gut microbiota dysbiosis seen in CVID augmenting immune dysregulation? Thus, it is essential to further investigate whether altering the bacterial composition in the gut of CVID patients can contribute to shaping their adaptive immune system.

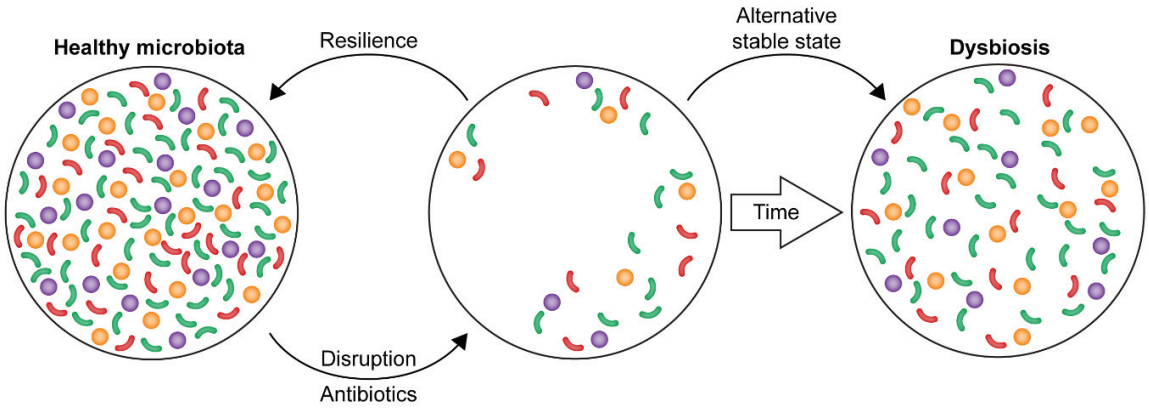

Illustration: Øystein Horgmo, UiO

Figure 4: The effects of antibiotics on gut microbial composition. The gut microbial diversity is disrupted by antibiotics, but depending on the length and type of antibiotic treatment the microbiota it could either stabilize as an alternative state with some degree of dysbiosis or, in hosts with a resilient gut microbiota, return to its origin. Figure is inspired by Lange *et al*. (264) and reused with permission from Karger Material.

Modulating gut microbiota to treat extra-intestinal or even systemic human disease is an exciting field where little has been done so far. With its combination of dysregulated gut microbiota and

dysregulated immune system, CVID is an attractive model to use for exploring this concept. Importantly, such studies could prove the validity of the concept as well as give insight into the mechanistic interactions between gut microbiota and the host immune system.

4.5 Trimethylamine N-oxide (TMAO)

Intestinal microbial flora has an essential role in generating the organic compound TMAO from dietary phosphatidylcholine (lecithin) and free choline (150). This obligatory function of the gut microbes in converting dietary nutrients into TMA, leading to the oxygenated product TMAO, has been confirmed in multiple human studies (151-154). TMAO levels in plasma seem to be a robust prognostic marker for cardiovascular disease (CVD) risk, including in individuals with low overall traditional risk factors for major adverse cardiovascular events (152, 154). The underlying cause of this could be connected to the observed increase in atherosclerosis with higher TMAO levels, potentially related to the attenuating effect of TMAO on reverse cholesterol transport (RCT)(151, 153, 155).

Plasma concentration of TMAO in healthy subjects correlates positively with inflammatory marker TNF and soluble TNF receptors, but does not reach significant correlation with CRP or IL-6 levels (156). It is not clear exactly how the increased levels of TMAO could be causally linked to inflammation, but several mechanisms have been suggested. Studies investigating the effects of dietary TMAO and high-fat diet (HFD) on glucose tolerance in mice concluded that TMAO added to the diet augmented impaired glucose tolerance, probably by blunting insulin signal transduction and causing adipose tissue inflammation (157). Mice receiving dietary supplementation with TMAO precursor choline have demonstrated an upregulation of scavenger receptors on macrophages, leading to foam cell development (150). This will ultimately lead to activation of necrotic and apoptopic pathways resulting in cell death and release of cholesterol and inflammatory cytokines (158). Thus, TMAO is dependent on the gut microbiota for its production and has been linked to several inflammatory conditions potentially through interfering with RCT, making it an interesting organic compound to investigate further in CVID patients.

4.6 Lipid metabolism

The importance of gut microbiota in modulating nutrients also extends into the realm of lipids where it has been shown that the function of gut microbes can have an impact on host cholesterol levels. Lipids exist in humans in the form of fatty acids, glycerophospholipids and non-glyceride lipid components like sterols including cholesterol, lipoproteins and sphingomyelin. The many functions of lipids include energy storage and transportation, structural components of cells and cellular signaling. Cholesterol is considered the most immunologically active form of lipids, with particular effects on the innate immune system.

4.6.1 Cholesterol uptake and production

Cholesterol is an essential molecule to form and maintain cell membranes, but also crucial for the synthesis of hormones, fat-soluble vitamins and production of bile acids. Humans obtain cholesterol from endogenous production in the liver and from the diet through intestinal absorption. Cholesterol is also excreted from the liver through bile, thus cholesterol in the gut lumen originates both from bile (approx. 1 g/day) and from the diet (approx. 400 mg/day)(159). Unlike previous misconceptions of intestinal cholesterol uptake as merely a passive diffusion process, research of recent years have

identified three cellular transmembrane transporters pivotal in cholesterol absorption: NPC1L1, ABCG5 and ABCG8 (160, 161). Cholesterol absorption starts with micellar solubilization of cholesterol in the intestinal lumen, before transportation to the enterocyte brush border membrane where it interacts with the enterocyte and gains entry (161). This process is dependent on NPC1L1 activity, a sterol transporter localized in the proximal jejunum (160). ABCG5 and ABCG8 are transporters expressed in both the liver and the intestine; they act as a heterodimer and perform to prevent sterol accumulation in humans through sterol efflux from hepatocytes and enterocytes (160).

4.6.2 Cholesterol metabolism

Once cholesterol has been absorbed, it undergoes intracellular esterification with fatty acids before it is packaged into chylomicron particles for secretion at the basolateral surface of enterocytes (160). Chylomicrons circulate to transport fatty acids and cholesterol to skeletal, muscle, cardiac and adipose tissue, and are subsequently hydrolyzed by lipoprotein lipase (LPL) to form a chylomicron remnant which is taken up by the LDL-like receptor in the liver. From the liver, cholesterol can enter the metabolic pathway as very low-density lipoprotein (VLDL), of which the triglyceride core is hydrolyzed to produce intermediate-density lipoprotein (IDL)(162). The IDL particles are either taken up by the liver or further hydrolyzed to produce LDL. If LDL becomes oxidized, it can enter macrophages via scavenger receptors CD36 and SR-A and join the intracellular cholesterol pool. Macrophages loaded with excess cholesterol become foam cells, important in the pathogenesis of inflammatory conditions like atherosclerosis, but intracellular overload of cholesterol is also toxic and will eventually lead to apoptosis (163).

4.6.3 HDL formation

Nascent high-density lipoprotein (HDL) particles are made in both the liver and intestine. These particles mainly contain apolipoprotein A-1 (apo A-1) and phospholipid, and lack the spherical form of more mature HDL particles (162). Their principal function is to remove unesterified cholesterol from peripheral cells such as macrophages with the help of membrane associated ABCA1 transporter proteins. The cholesterol in nascent HDL particles is subsequently esterified to fatty acids under the influence of lecithin cholesterol acyl transferase (LCAT), producing mature and spherical HDL particles that can further accept cholesterol from macrophages via the ABCG1 transporter (164, 165). Finally, cholesteryl ester in the HDL core is transferred to apo B-containing lipoproteins by cholesteryl ester transfer protein (CETP), or returned to the liver via the SR-B1 receptor (162).

4.6.4 Reverse Cholesterol Transport

Increased levels of low-density lipoprotein (LDL) in the blood have been shown to induce cholesterol accumulation and arterial wall inflammation, whereas HDL appears to oppose this process (166). Over the recent years, studies have suggested that HDL function rather than the mere levels of HDL is of importance to cardioprotection. HDL improves endothelial dysfunction and exerts antioxidative and antiapoptopic effects, but one of the most important antiatherogenic functions of HDL is the capacity to promote cholesterol efflux from peripheral cells via the circulation to the liver, thereby reducing inflammation (167). This process of transferring cholesterol from extrahepatic tissues to the liver is called reverse cholesterol transport (RCT). The concept was first introduced by Glomset in 1968 (168), and has been extensively studied ever since. Preserving cholesterol homeostasis in macrophages is vital to prevent detrimental formation of foam cells (169)(Figure 5), thus RCT is a key mechanism to avoid atherosclerotic disease and cell death.

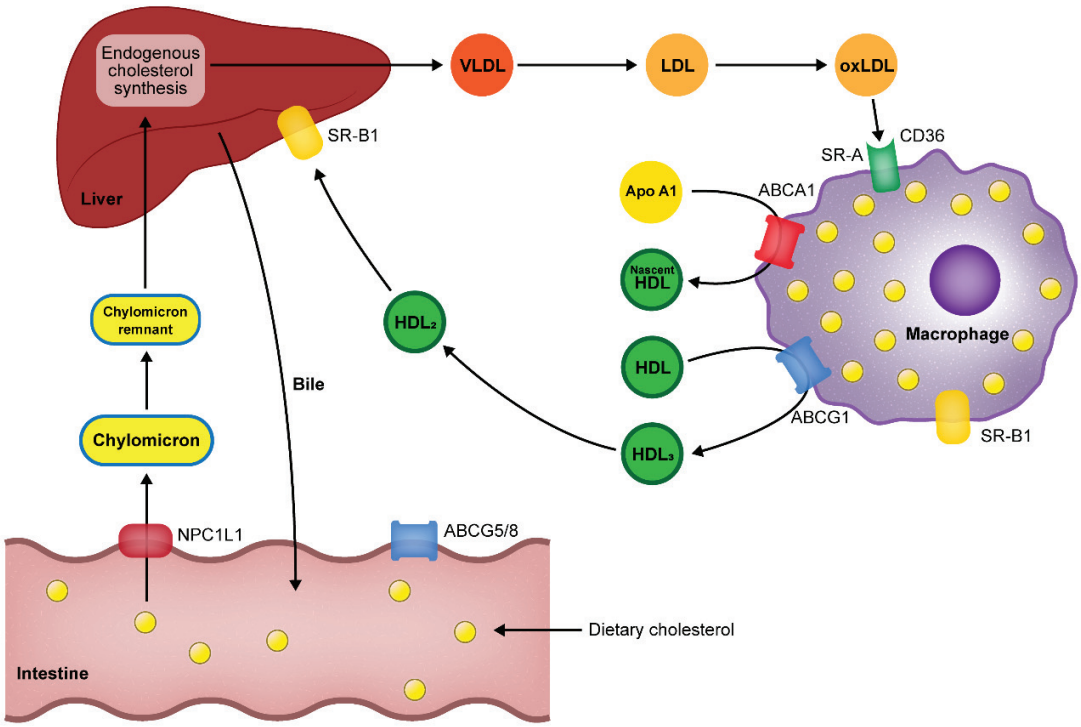

Illustration: Øystein Horgmo, UiO

Figure 5: Schematic overview of reverse cholesterol transport. Dietary cholesterol is absorbed from the intestine via Niemann-Pick C1-like 1 (NPC1L1) proteins in the brush border membrane of enterocytes and transported in the blood as large chylomicrons, where they exchange components with HDL and become chylomicron remnants that can be taken up by the liver. Cholesterol is also endogenously synthesized in the liver, and along with deposited cholesterol, it can be assembled into very-low density lipoproteins (VLDLs) or excreted into the bile via ATP-binding cassette (ABC)G 5/8 transporters. The ABCG5/8 transporters efflux cholesterol from enterocytes to the intestine as well. In the bloodstream, VLDL is converted to low-density lipoprotein (LDL), which can be further oxidized to oxLDL. The oxidized LDL is engulfed by macrophages via scavenger receptors CD36 and SR-A to form foam cells. Apo A-1, secreted by the liver and intestine, accepts cholesterol from peripheral cells via ABCA1 transporters, forming nascent HDL particles. The nascent HDL particles pick up phospholipids and more cholesterol from ABCA1 transporters before becoming more mature HDL particles, which subsequently accept cholesterol from ABCG1 and possibly SR-B1 in macrophages. After enzymatic modifications, the HDL molecule is converted to a mature HDL_2 particle which can return to the liver and transfer its cholesterol to the liver through the SR-B1 transporter.

Membrane transporters ABCA1, ABCG1 and the SR-B1 receptor pathway all contribute to exporting intracellular cholesterol to HDL (169). Apo A-1 binding to ABCA1 increases the amount of ABCA1 transporters in the plasma membrane, which subsequently regulates the rate of free cholesterol and phospholipid efflux as well as formation of nascent HDL particles (167). Less than 5% of the HDL cholesterol content comes from RCT, meaning HDL cholesterol levels alone are not appropriate as a surrogate marker for this imperative HDL function (165, 167). Rather, studies assessing the functional aspects of HDL need to apply models reflecting the specific functionality they want to explore. To assess RCT, which will normally result in reduced amounts of intracellular cholesterol in peripheral cells, measuring lipid loading in macrophages can be done before and after cholesterol efflux activity.

However, many of the studies on these mechanisms have been conducted on mice, and the need for studies on human cells has emerged.

4.6.5 HDL and inflammation

High LDL cholesterol levels in the blood can be modified by free radicals, resulting in LDL oxidation, whereupon intracellular cholesterol accumulation occurs and inflammatory responses are triggered via TLR signaling and NLRP3 activation (166). HDL opposes this by promoting RCT from peripheral cells, but also by exerting anti-oxidative functions through associated proteins (PON1, PON3, apo A-1, transferrin and ceruloplasmin), thus reducing levels of oxidized LDL (170). Furthermore, HDL reduces expression of endothelial cell adhesion molecules, thereby interfering with recruitment of monocytes from the blood into arterial walls, as well as inhibit platelet aggregation in the face of inflammation (171). Perhaps most strikingly, HDLs attenuating effect on inflammatory responses lies in its ability to modulate the inflammatory response in different cell types. HDL can bind to LPS and block its ability to stimulate inflammation in monocytes and macrophages (172). However, acute inflammation induced by endotoxemia can also lead to a reduction in selective HDL efflux functions (173), and our research group has previously found elevated plasma levels of lipopolysaccharide in CVID patients associating with systemic immune activation (45). HDL further induces transcription factor ATF3, a target gene in macrophages which is expressed during TLR-dependent immune responses, to limit the inflammatory response by modulating the magnitude of innate immune receptor activated IFN-beta production (174). An in vivo mouse model has in fact demonstrated that the protective effects of HDL against TLR-induced inflammation is fully dependent on ATF3 (175), leaving the question whether this transcription factor could also affect anti-inflammatory functions of HDL in CVID.

During infections and other acute medical conditions, the levels of HDL decrease and HDL functions can be modified (176). Moreover, during systemic inflammation, HDL functions can become attenuated with reduced cholesterol efflux from macrophages and subsequently increased proinflammatory signaling (177). Autoimmune diseases like Crohn's disease, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) all have altered levels and functions of HDL (178). Mouse studies have shown that cholesterol accumulation in dendritic cells with induced deficiency of cholesterol transporters ABCA1 / ABCG1 generates intracellular activation of the NLRP3 inflammasome, leading to more autoimmunity (179). The majority of patients with CVID have low grade systemic inflammation as well as autoimmune and/or inflammatory manifestations (72). CVID patients also have lower levels of apo A-1 and HDL than healthy controls (180). However, the question has remained whether inflammation can be linked to demonstrable changes of HDL function in CVID, which could open up for modulation of HDL as a therapeutic target to reduce inflammation and autoimmunity in these patients.

5. Aims

The overall aim of this project was to explore and define novel molecular mechanisms involved in the pathogenesis of chronic non-infectious inflammation in CVID. Furthermore, immunoglobulin replacement therapy reduces the number of infections in CVID patients but does not alleviate non-infectious complications, thus there is a definite need for new therapeutic targets. In the present project we wanted to investigate whether gut microbiota and lipid metabolism could be potential targets for therapy to reduce autoimmune and inflammatory manifestations of CVID. To elucidate this we applied three different approaches:

I. A proof of concept study to explore if altering the dysbiotic gut microbiota in CVID by the use of the non-absorbable antibiotic rifaximin could reduce systemic inflammation in CVID.

II. Exploring the anti-inflammatory effects of high-density lipoprotein (HDL) and functional lipid metabolism in CVID patients

III. Examining the amine oxide trimethylamine N-oxide (TMAO), which has previously been linked to inflammation, gut disease and lipid metabolism, in CVID

6. Summary of the papers

Paper I

Rifaximin alters gut microbiota profile, but does not affect systemic inflammation - a randomized controlled trial in common variable immunodeficiency

We have previously shown that reduced gut microbial diversity is associated with systemic inflammation and gut leakage in CVID patients. In paper I, we wanted to explore if modulation of the gut microbiota by an antibiotic could reduce systemic inflammation and gut leakage in CVID. We included 40 CVID patients in a randomized open prospective clinical trial where 20 patients received the oral antibiotic rifaximin 550 mg every 12 hours for 2 weeks and the other 20 patients received no study intervention. Rifaximin was chosen as intervention drug as it is non-absorbable, has bactericidal activity against a broad range of enteric pathogens and has previously been shown to reduce serum levels of LPS in chronic liver disease. All patients delivered stool samples and had blood samples taken at baseline, at the end of the intervention period (2 weeks) and at follow-up 8 weeks after inclusion. We found rifaximin to have no significant effect on markers of systemic inflammation (soluble [s] CD14, $p=0.56$; sCD25, $p=0.64$; sCD163, $p=0.32$ and neopterin, $p=0.06$) in CVID, nor on levels of LPS as a marker of gut leakage. Moreover, rifaximin treatment had no significant effect on mRNA levels of pro- and anti-inflammatory cytokines and chemokines in peripheral blood mononuclear cells (PBMC) from patients, nor did it affect the distribution of B- and T cell subpopulations. However, intra-individual microbial diversity (alpha diversity) in the gut was reduced after the rifaximin intervention ($p<0.001$), as was the relative abundance of multiple gut bacteria. The gut microbial diversity returned to baseline in the intervention group by follow-up visit at week 8, demonstrating that rifaximin imposes a transient effect on the gut microbiome. Furthermore, rifaximin did not affect the CVID-specific dysbiosis index, consisting of ten gut bacteria at different taxonomic levels that captured a difference in alpha diversity between CVID patients and healthy controls in a previous study. Taken together, this study confirmed a significant effect of rifaximin in temporarily altering the gut microbiome, but showed no concurrent significant effect on markers of systemic inflammation or gut leakage. This result implies utilizing a short course of rifaximin to alter the gut microbiota is ineffective with regards to affecting systemic inflammation through the gut-immune system axis in CVID patients.

Paper II

Impaired HDL function amplifies systemic inflammation in common variable immunodeficiency

High-density lipoprotein (HDL) is an established endogenous anti-inflammatory mediator. In paper II, we hypothesized that levels and functional activity of HDL were altered in CVID patients, affecting systemic inflammation in these patients. We first measured the plasma lipid profile in 102 CVID patients and 28 healthy controls, discovering that CVID patients had significantly lower levels of HDL ($p<0.0001$) whilst levels of LDL and total cholesterol were similar to controls. The CVID patients with non-infectious complications had the lowest levels of HDL ($p<0.01$ compared to patients with infections only), and low HDL was correlated with elevated levels of inflammatory markers CRP ($p=0.02$) and sCD25 ($p<0.001$). Furthermore, levels of the main protein in HDL; Apo A-1 were lower in CVID patients than in controls ($p<0.01$), as were levels of cholesterol transporter ABCA1 mRNA from patient PBMC ($p<0.0001$). Hence,

two important factors in the reverse cholesterol transport (RCT) mechanism from peripheral cells to the circulation appeared affected in CVID patients. In order to assess HDL functional activity we exposed THP-1 macrophages to ^{14}C-cholesterol for 48 hours before inducing cholesterol efflux from the macrophages by adding serum from either CVID patients or controls. The results showed that serum from CVID patients had decreased cholesterol acceptor capacity compared to controls ($p<0.05$), in line with the reduced Apo A-1 levels. Moreover, monocyte derived macrophages from CVID patients had reduced cholesterol efflux capacity to serum when exposed to ^{14}C-cholesterol for 48 hours followed by overnight stabilization ($p=0.04$), consistent with their low ABCA1 expression in PBMC. Impaired cholesterol efflux capacity has been associated with inflammation and autoimmunity via intracellular inflammasome activity in murine models, and the anti-inflammatory role of HDL has been linked to the ability to reprogram macrophages from TLR-induced inflammation via the transcriptional regulator ATF3. We therefore measured ATF3 mRNA levels in PBMC from CVID patients and controls, and found decreased levels in patients ($p<0.0001$). To explore this further, we incubated freshly isolated PBMC exposed to different concentrations of human HDL for 6 hours before stimulating the cells with TLR4 ligand (LPS) and TLR2 ligand (Pam3Cys) for 12 hours. We subsequently measured inflammatory cytokines TNF and IL-6 in the cell supernatants and found supplemental HDL to suppress TNF release from Pam3Cys stimulated cells of healthy controls, whereas supplemental HDL made no difference to the TNF release from the corresponding patient cells ($p<0.05$). A similar effect was not observed after LPS stimulation or for IL-6 release. In conclusion, we found reduced levels and impaired anti-inflammatory activity of HDL in CVID patients, particularly in those with non-infectious complications. Low HDL levels were associated with systemic inflammation and a more severe clinical phenotype. Reduced HDL-driven reverse cholesterol transport activity seems to be an important factor contributing to systemic inflammation and non-infectious manifestations in CVID patients, thus representing a potential therapeutic target to reduce inflammatory and autoimmune complications.

Paper III

Gut microbiota-dependent trimethylamine N-oxide linked to inflammation in common variable immunodeficiency

Production of the organic compound trimethylamine N-oxide (TMAO) is dependent on gut bacteria converting carnitine and choline from meat, fish and egg in the diet to its precursor trimethylamine (TMA), which is then oxidized to TMAO in the liver. TMAO has previously been shown to be a biomarker for systemic inflammation in cardiovascular disease, but has also been suggested to be a mediator of inflammation. In paper III, we explored whether TMAO could be a missing link between altered gut microbiota and systemic inflammation in CVID. To investigate this, we measured plasma levels of TMAO and inflammatory markers in 104 CVID patients and 30 healthy controls. TMAO levels were significantly higher in CVID patients ($p=0.02$) and associated positively with markers of systemic inflammation; TNF ($p=0.01$) and IL-12 ($p=0.01$) but not IL-6 ($p=0.26$) or IL-8 ($p=0.21$). Furthermore, plasma levels of LPS correlated significantly with TMAO ($p=0.03$), indicating a connection between gut leakage and TMAO. Examining the gut microbiota, we found an abundance of *Gammaproteobacteria* in CVID patients, which correlated positively with plasma levels of TMAO ($p=0.02$). Moreover, on genus level, an abundance of *Escherichia-Shigella* associated positively with TMAO ($p=0.03$). Thus, the data suggested that a gut microbiome abundant in *Gram negative* bacteria was linked to elevated plasma levels of TMAO in CVID patients. To investigate the role of diet on levels of carnitine, choline and TMAO, we had patients filling in a food frequency questionnaire. This revealed no greater intake of meat, fish or eggs among CVID patients compared to controls that could explain the increased TMAO levels. Overall, diet did not appear to particularly affect TMAO levels in CVID patients. However, dietary intake of table sugar correlated

with elevated TMAO levels ($p<0.01$). Furthermore, consumption of red wine seemed to attenuate levels of TMAO ($p=0.02$), possibly related to effects of the choline analogue 3,3-dimethyl-1-butanol (DMB) which can be found in red wine. In summary, CVID patients have elevated levels of TMAO in plasma which is associated with increased abundance of *Gammaproteobacteria* in their gut microbiota and elevated markers of systemic inflammation. This suggests the gut microbiota could be a therapeutic target to reduce TMAO formation and thereby systemic inflammation in CVID.

7. Methodological considerations

7.1 Clinical trial

7.1.1 Planning of a randomized controlled trial

Clinical trials are set up to observe the outcomes of specifically assigned exposures and can provide strong evidence to prove causality. Randomization of patients into groups is imperative to avoid selection bias and confounding factors, and blinding of both study participants and observers is the ideal set-up yet not always feasible. Choosing study intervention brings about numerous considerations, such as type of drug, dose and length of active treatment, where the underlying evidence to guide these decisions may not be strong.

In order to explore our hypothesis for paper I that the gut microbial composition in CVID patients contributes to low-grade systemic inflammation through interaction with the intestinal innate immune system, we wanted to implement a short-term antibiotic intervention to modulate the gut microbiota and subsequently immune activation in these patients. Several different antibiotics were initially considered, including vancomycin which had been demonstrated to reverse inflammatory processes in patients with primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD) (181). Azithromycin was discussed, as it had been reported to improve clinical outcomes in patients with cystic fibrosis and inflammatory diseases of the respiratory tract, believed secondary to its anti-inflammatory effects on neutrophils and macrophages, potentially involving alteration of gut microbiota (182). However, to ensure any effects seen on immune activation in our study were related to modulation of the gut microbiota, rather than a direct effect on the immune system by the study drug, rifaximin emerged as a good option. Rifaximin is a locally acting antibiotic in the gastrointestinal tract with negligible intestinal absorption after oral administration, which also has the benefit of a great safety profile (183). Moreover, rifaximin has low potential of inducing resistance to the study drug outside the intestine, and the use of rifaximin in prevention and treatment of travelers' diarrhea had previously shown a limited burden of side-effects (184). Recent studies had demonstrated promising results using rifaximin to alter the gut microbiota and reduce LPS levels in the treatment of acute and chronic hepatic encephalopathy (145, 185). Rifaximin had also been demonstrated to have beneficial effects in patients with irritable bowel syndrome (186), Crohn's disease and ulcerative colitis (187), suggesting it could have an impact on intestinal inflammation. Based on this knowledge, rifaximin was thought to be a safe and suitable antibiotic to use in order to study effects on systemic inflammation by modulation of gut microbiota in CVID.

In the planning of an intervention study, getting the sample sizes right is also important. To avoid incorrectly rejecting the null hypothesis (type I error) when evaluating the effect of an intervention, but also to avoid accepting the null hypothesis incorrectly (type II error), power calculations during the planning phase are essential. However, sample size calculations may be of little value in the initial phases of explorative studies, where there is little data to base such calculations on (188). In paper I, we did a proof-of-concept intervention study, implicating there were no prior studies to build power calculations on, and thus strict sample size calculations were not found appropriate. However, as an estimate, we wanted to achieve a clinically important difference of 50% change in inflammatory markers between groups with a p-value of 0.05 and desired power of 95%. This meant we would need 25 patients in the intervention group and 25 patients in the control group. We pre-screened all CVID patients between 18 and 74 years of age registered at our clinic, and had defined exclusion

criteria (Table 1). Patients remaining after pre-screening were invited to participate in the study, and eligibility to participate was evaluated again at the first study visit. Partly because CVID is a rare disease, and partly due to 'use of antibiotics in the last 12 weeks' being an exclusion criterion, we were eventually able to recruit 20 patients in each group. Subsequently, we may have missed some patients that could have had a more significant effect of the study intervention, and statistically the smaller *n* demands slightly greater differences between groups to reach statistical significance. Furthermore, although we have extensive and longitudinal clinical and laboratory data on the CVID cohort at our clinic, some medical aspects of the patients were difficult to determine with certainty at the time of inclusion. These include prevalence of granulomatous changes amongst the CVID patients, as we do not routinely biopsy livers, lungs or other organs unless a definite clinical indication is present, nor can we be categorical about the possibility of ongoing low-grade infections but merely base this on clinical assessments, laboratory markers such as CRP and neutrophil counts and radiological modalities where indicated. We do however have good knowledge of the prevalence of GI-symptoms and enteropathy in our CVID cohort, as many of them have undergone endoscopies as part of previous studies. The rarity of the disease makes recruitment of patients to studies challenging in general, and these challenges also relate to paper II and III. Especially for the *in vitro* studies in paper II, numbers are low due to difficulties recruiting during a given timeframe.

Exclusion criteria for The Rifaximin study
Antibiotics in the last 12 weeks
History of allergic reaction to rifaximin
Malignancy
Impaired kidney function
Pregnancy or lactation
On-going infection
Use of probiotics in the last 6 months
Immunosuppressive drugs
Comorbidity that may influence with the patient's safety or compromise the study results (e.g., cardiovascular disorders, alcoholism, psychiatric disease, HIV infection)
Polypharmacy (patients with an extensive medication list i.e. ten drugs or more)

Table 1: Exclusion criteria used prior to inclusion into the rifaximin study.

7.1.2 Paper I, study design

The Rifaximin study was a randomized, open, single-center clinical trial. Using computer-generated randomization (1:1), half of the CVID patients enrolled received the study drug rifaximin and the other half no intervention. In an ideal setting, it would have been a blinded trial with placebo tablets for the no-intervention group to avoid any bias in the reporting of outcomes based on knowledge of who got the study drug and not. However, budget limitations prevented this, as this trial was fully financed through independent research funding and not through funding from pharmaceutical companies. On a positive note, the physicians reporting outcomes had no ties to any third party as a consequence of this. The random allocation sequence was generated through a randomization list with a combination of permuted blocks of four (n=10) and blocks of two (n=5) to avoid assignment to intervention / no intervention for unequal numbers of patients. The possibility of creating groups of patients unbalanced for prognostic features is a known limitation to simple randomization (189). However, no phenotype

based stratification of the patients between the two study arms was performed here because of the low number of study participants. As the randomization unfolded, it appeared the patients who had been randomized to rifaximin treatment had less inflammatory and autoimmune complications than the no intervention group, which may have interfered with the results.

Including a gastrointestinal symptom score questionnaire was considered, but as this was an open-label study where both patients and physicians knew who were receiving the study drug, the value of such a questionnaire was considered too low to include. The study being a single-center clinical trial meant a limited number of physicians were involved in the inclusion and follow-up of patients, reducing the risk of inter-individual differences in handling of the ongoing follow-up, and any potential influence this would have on the reporting. Simultaneously, multi-center studies are in general more robust when it comes to reproducibility, and the limited number of patients enrolled could also have been increased had more centers been involved in the study.

The study protocol included three visits to the outpatient clinic at Oslo University Hospital Rikshospitalet; one at inclusion, one after 2 weeks (end of intervention) and a follow up visit 8 weeks after inclusion (Figure 6). Each visit included physical examination, fecal sample and blood tests. The patients also sent a fecal sample by post 14 days prior to their planned inclusion into the study, to have two sets of baseline samples in order to take into account possible individual variances between these baseline samples. The patients who were randomized to intervention with rifaximin received 28 tablets of rifaximin 550 mg at the first visit, and were to take the drug every 12 hours for the next 2 weeks. Previous rifaximin studies had daily doses varying from 400mg to 1800mg divided on 1-3 doses (185-187, 190). We wanted to administer a dose sufficient to achieve an effect on the gut microbiota, but at the same time not exceeding the highest approved treatment dose. We thought a twice daily regime would ensure better compliance than taking the study drug three times a day. Long term use of rifaximin 550 mg x2 had been used in chronic hepatic encephalopathy for years. We considered this dose to be sufficient to test our hypothesis, and a 2 week intervention length to be adequate in order to modulate the gut microbiota. The patients got information on potential side effects of the study drug as well as contact details to investigators in case of adverse events. At the *end of intervention* visit, compliance was assessed and any suggestion of side effects was evaluated and logged in the patient file to allow for premature cessation of the study if needed, as well as to report side-effects of rifaximin at the end of the study which could aid future trials where rifaximin is considered as a study drug.

Primary endpoints in the rifaximin study were changes in inflammatory and anti-inflammatory mediators and gut leakage markers over time as a response to the drug intervention. Secondary endpoint was changes in the gut microbiota after drug intervention based on 16S rRNA sequencing, both expressed by diversity measures and abundance of specific bacteria. These endpoints were as per standard protocol defined in the study protocol and registered in ClinicalTrials.gov prior to commencement of the study, and thus not possible to change as the clinical trial progressed. As the secondary endpoint was reached, but the primary endpoint was not, swapping them around after completion of the trial would have made this a positive study rather than a negative one. This highlights why it is so important to comply with international guidelines for registering and publishing clinical research, but perhaps also why publishing negative studies is important too.

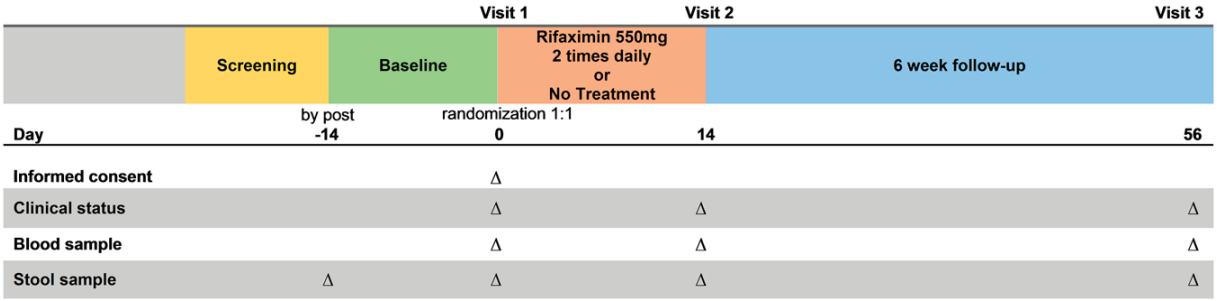

Figure 6: The Rifaximin study protocol. CVID patients assessed eligible for participation in the study sent a stool sample by post 14 days prior to their inclusion visit. Another stool sample, along with blood samples and clinical status, was obtained at visit 1. After informed consent the patients were randomized to intervention with rifaximin for two weeks or follow-up as normal. New stools samples, blood samples and a repeated clinical status were performed at visit 2 (end of intervention) and visit 3 (end of study). Figure reused from paper I.

7.2 Blood sampling and cytokine analysis

The pre-analytical phase of blood sampling, including patient- and lab variables, appears responsible for most laboratory errors and can potentially influence results (191). An inherent problem when measuring cytokine levels is that the primary source of cytokines, white blood cells, are in the blood sample and thus available for stimulation through for instance bacterial contamination (192, 193). Cytokine measurements will reflect in vivo status best if blood samples are collected into tubes with an endotoxin-free anticoagulant such as EDTA, inhibiting ex vivo cytokine production in the tube and maintained at 4°C until rapid processing (194, 195). Serum levels of soluble (s)CD25 and sCD14 have been shown consistently increased in CVID patients with or without systemic endotoxinaemia, thus acting as relatively stable markers of immune activation in CVID (45, 83). Peripheral venous blood from patients and controls was drawn into sterile collection tubes without any additives for serum and with EDTA as anticoagulant for plasma samples. The collection tubes were immediately immersed in melting ice and within 15 minutes of sampling, they were centrifuged at 2000*g* for 20 minutes to obtain platelet-poor plasma, or allowed to clot at room temperature prior to centrifugation at 1000*g* for 10 minutes for serum samples.

When measuring inflammation markers, the use of previously unthawed specimens is recommended, though similar measurements have been found between paired samples that have one and two freeze-thaw cycles at the time of assay (196). Both our plasma and serum samples were stored at -80°C in aliquots, and for analyses thawed only once. When available sample volumes are small, multiplex-based immunoassays can allow for simultaneous measurement of several cytokines. Cytokines that are present in quite large concentrations will give the most accurate estimates when using such multiplex-based assays (197). Circulating levels of certain cytokines studied by multiplex immunoassays in paper II and III do however exist at rather low concentrations, raising an issue of accuracy. Up to 30% of CVID patients in our studies received regular intravenous immunoglobulin (IVIG), which has been shown to increase serum levels of several cytokines after infusion (198), and blood samples from patients were therefore collected prior to IVIG infusion. Various antibiotics appear to have an effect on monocyte generation of certain acute-phase inflammatory cytokines. CVID patients are treated with antibiotics more frequently than the general population, which could potentially affect circulating cytokine levels, but patients on antibiotics were not included in any of the studies.

7.3 LPS measurements

Lipopolysaccharide (LPS) levels in paper I, II and III were measured by Limulus Amebocyte Lysate (LAL) chromogenic assay. LAL contains an aqueous extract of blood cells (hemolymph) from the horseshoe crab, which agglutinates upon addition of endotoxin (199). The colour produced after cleavage of the synthetic peptide-chromogen complex, measured by a spectrophotometer, represents the specific endotoxin concentration in the sample. The LAL assay is the gold standard for detection of lipid A, which is the endotoxic component of LPS (200). Lipid A is conserved among several bacterial species and serogroups, thus it can be debated whether identifying the more specific O-polysaccharide antigenic part of LPS, which gives a more accurate measure of serogroup specificity, is also important (200). However, the O-polysaccharide tests are less sensitive. For the purpose of measuring the overall endotoxin load as a consequence of gut inflammation and leakage, we considered the LAL assay to be most useful in our papers.

7.4 PBMC isolation

Peripheral blood mononuclear cells (PBMC) can be extracted from whole blood by using ficoll, a hydrophilic polysaccharide that separates layers of blood. Gradient centrifugation separates the blood into a bottom layer of erythrocytes and a plasma layer on top, with a layer of PBMC and a fraction of polymorphnuclear cells in between (201). The step of removing the PBMC layer can be somewhat operator-dependent, and may affect the yield of cells from each isolation. To negate this effect, we used an automated cell counter (Countess™) to assess cell viability and the yield from each isolation, before calculating the sample volume needed to achieve the same amount of cells making up each aliquot of pellet prior to freezing or further functional studies.

In human serum, there are numerous cytokine inhibitors and the cytokines have a short half-life, thus measuring cytokine levels in serum may not precisely reflect the production potential of immune cells (202). PBMC model systems can be used instead for measuring cytokine production in supernatants or mRNA cytokine gene expression. These mononuclear cells are however very sensitive and can respond to various factors inferring activation or inflammatory stimulation (203), which must be considered when interpreting the results. We isolated PBMC from heparinized venous blood adding ficoll and starting centrifugation within 1 hour of collection to minimize additional cell stimulation. PBMC pellets were immediately stored at -80°C until mRNA analyses for paper I and II. Freshly isolated PBMC was used for two of the functional studies in paper II.

7.5 PCR

Total RNA was isolated from thawed PBMCs using RNeasy spin columns as described by the manufacturer (Qiagen, Hilden, Germany). The isolated RNA was treated with DNase (Qiagen) and stored at −80 °C for later analysis. RNA concentrations and purity were assessed by spectrophotometer absorbance (NanoDrop ND-1000 Thermo Scientific, Wilmington, DE). 500 ng of RNA was loaded into the cDNA synthesis using q-Script cDNA Synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantification of mRNA was performed using Perfecta SYBR Green qPCR Fast Mastermix (Quanta Bioscience) on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the accompanying software SDS 2.4. We were handling small volumes of human samples, so to increase accuracy and reduce the risk of human error, we used robot pipetting for the quantitative reverse transcription PCR (RT-qPCR) reactions. To avoid missing data due to failed reactions, we conducted RT-qPCR in duplicate for each transcript. Target transcript levels were quantified by the comparative Ct method using the

average Ct-median value from reference genes β-actin and GAPDH as endogenous control. The purpose of normalization to endogenous reference genes is to reduce any confounding noise, however this of course presupposes that any noise introduced affects the reference genes and the genes of interest in the same way (204). We therefore decided to use GAPDH which is commonly used as a housekeeping gene due to its often stable and constitutive expression at high levels in many cells, and the β-actin gene which is also generally expressed across eukaryotic cell types with little variation to its expression.

7.6 Microbiota analyses

7.6.1 Sampling for microbiota analyses

Patient stool samples for paper I and III were collected by the participants at home with a standardized collection device to facilitate stool sampling and minimize introduction of biochemical artefacts (205). The samples were immediately transferred to Stool collection tubes with Stool DNA stabilizer (Stratec Biomedical, Birkenfeld, Germany) (206). Participants delivered two baseline samples in the clinical trial, the first was collected 14 days prior to the day of inclusion and returned to the hospital by post. The second baseline sample was collected at home within the last 24 hours of their hospital visit for inclusion into the study, and stool samples collected at visit 2 and 3 in paper I were collected the same way. Baseline samples from paper I were also used for the gut microbiota analyses in paper III. Once received at the hospital, the stool samples were immediately stored in a -20°C freezer until DNA extraction. The gold standard is using fresh stool for immediate DNA extraction at a study centre, or at least freezing of samples as soon as possible after collection to avoid post-sampling changes to bacteria in the samples, but this is difficult to implement when participants are from geographically spread-out sites. Delivery of stool samples by mail may thus have impacted the results. However, directly transferring the faecal samples to a spin stool DNA sampling tube (the PSP® method) appears to generate both bacterial recovery and DNA yield on par with more standard methods in comparative studies, despite allowing stool samples to be in room temperature for up to 48 hours (206). Obtaining mucosal samples by gastrointestinal endoscopy could perhaps have been an alternative to stool samples, however this would have been an invasive procedure with risk of complications, making it more difficult to recruit patients, and more challenging to use for longitudinal follow-up as well.

7.6.2 DNA extraction

The process of DNA extraction aims at exposing genomic DNA from the nucleus without degrading too much DNA whilst doing so. Samples are cleaned to achieve representative DNA with sufficient purity and yield for further processing. It is absolutely critical to avoid contamination of samples, and to handle DNA carefully to avoid damage and enzymatic degradation. DNA extraction method has been shown to have great impact on the resulting observed bacterial diversity (207). In paper I and III, bacterial DNA was extracted using the PSP® *Spin Stool DNA Plus Kit* (Stratec). This is a combined kit for collection, transportation and storage of stool samples, as well as subsequent DNA purification. The kit permits rapid and efficient isolation of DNA from fresh or frozen stool samples. Because of the many compounds in stool samples that can degrade DNA and inhibit downstream enzymatic reactions, optimized prelysis buffer under high temperature and subsequent pre-incubation of the sample was used to remove PCR inhibitors. PCR inhibitors and particles that were not dissolved were subsequently removed by a centrifugation step.

Another constant challenge to pure DNA extraction lies in the nature of stool; it does not only contain DNA from gastrointestinal commensal bacteria and pathogens, but also parasite DNA, food DNA

and host DNA from colon epithelial cells. Nearly all DNA extraction protocols use a combination of chemical, thermic and mechanical methods for cell lysis and cleaning. Various lysis conditions permit enrichment or reduction of bacterial DNA content in the total DNA. Mechanical cell disruption by bead beating seems vital to obtain adequate lysis, especially of *Gram negative* bacteria (208). The DNA then binds to a spin column membrane, subsequent washing steps remove contaminants and ethanol, before purified DNA is extracted directly in a low-salt buffer. The method used for DNA isolation is thus of importance to successful recovery of gut bacteria. The PSP® *Spin Stool DNA Plus Kit* we used applies a combination of extensive homogenisation, thermic and mechanical bead beating lysis prior to DNA extraction, which has performed well in comparative evaluations with recovery of a greater proportion of bacteria in the *Firmicutes* phylum (206).

7.6.3 16S ribosomal RNA gene sequencing

When selecting which genetic target to amplify in order to best compare the microorganism communities present, an advantage of 16S ribosomal RNA (rRNA) genes is that they are essential and thus occur in at least one copy in prokaryotic genomes. Sequencing of the 16S rRNA gene can be performed using several platforms. Multiple factors need to be considered when selecting which platform to use, including sequence quality, number of reads obtained per run, sequence length (to improve taxonomy assignment) and costs (209). 16S rRNA gene sequencing has been extensively studied and allows sequence comparison and taxonomic assignment at genus level using well established databases, thereby providing a good resolution of the microbial community (210). There are however several pitfalls, for instance that species-level resolution is sometimes not achieved. Moreover, some organisms have multiple copies of the 16S rRNA gene, which can result in an overestimation of the relative abundance of certain taxa (211). In paper I and III, high-throughput sequencing of the 16S rRNA gene was performed with dual indexed barcodes as per an established protocol (209), followed by sequencing using Illumina MiSeq. The dual indexed barcode, i.e. barcodes in both the forward and reverse primers, has the benefit of allowing multiplexing of many samples with a limited number of primers and coverage of important variable regions of the 16S rRNA gene (209). The platform used here allows for up to 300 paired-end reads.

7.6.4 Choosing 16S rRNA hypervariable region

There are several 16S rRNA gene hypervariable regions (V1 through V9), thus amplifying a specific region by PCR introduces a general but unavoidable bias. The different regions have a distinct taxonomic coverage (212), hence the choice of which hypervariable region to amplify will have a definite impact on the overall bacterial community results generated. The hypervariable region V3-V4 has previously yielded good results and provided good taxonomic coverage for gut microbiomes in studies on humans and mice (209, 213). Therefore, we chose to amplify the hypervariable V3-V4 region with barcodes for both the forward and reverse primers.

7.6.5 Library preparations and operational taxonomic units (OTUs)

During quality filtering for the microbiota data, the cut-off for number of reads per sample was set at 9000, where samples with less reads were excluded from further analysis. Sequences were then clustered into operational taxonomic units (OTUs). There are several picking algorithms for OTUs, which can have an impact on downstream findings and data interpretation. The three main categories of OTU algorithm clustering are *de novo*, *open reference* and *closed reference*. De novo picking includes sequences being compared internally and clustered together depending on a similarity threshold, prior to matching of a representative sequence from each OTU cluster to a reference database. Open-

reference picking includes two steps: first clustering of sequences to a reference database, then de novo clustering of sequences that do not match the reference database. Closed-reference OTU picking consists of matching sequences to a reference database and subsequently discarding of the sequences that do not match the database (214). In our studies, merged reads were quality filtered and mapped at 97% sequence similarity using a closed-reference clustering, applying the software pipeline QIIME 1.9.1 (215) against the Silva reference database. The 97% threshold was based on convention, thus could have been set otherwise. Choosing closed-reference OTU picking has a definite limitation in not allowing the detection of novel diversity with reference to the existing database. However, for comparing abundance of different bacteria in stool samples from different cohorts, we considered its advantages of efficiency, inter-analysis comparability and predefined OTUs in the reference sequence collection to be more important. Quantitative insights into microbial ecology (QIIME) is a community developed, open source bioinformatics pipeline which transforms data from raw sequences through de-multiplexing, quality filtering, picking of OTUs, taxonomic assignment and phylogenetic reconstruction to diversity analyses. This bioinformatics pipeline was chosen to achieve a post-sequencing workflow that was standardised through incorporating software from several developers as well as obtaining a certain level of quality control. OTUs containing less than two reads were filtered out prior to generation of a rarefied OTU table with assigned taxonomies, as is recommended in the literature (216)(Figure 7), however by doing this we were also running the risk of missing less prevalent but potentially important OTUs.

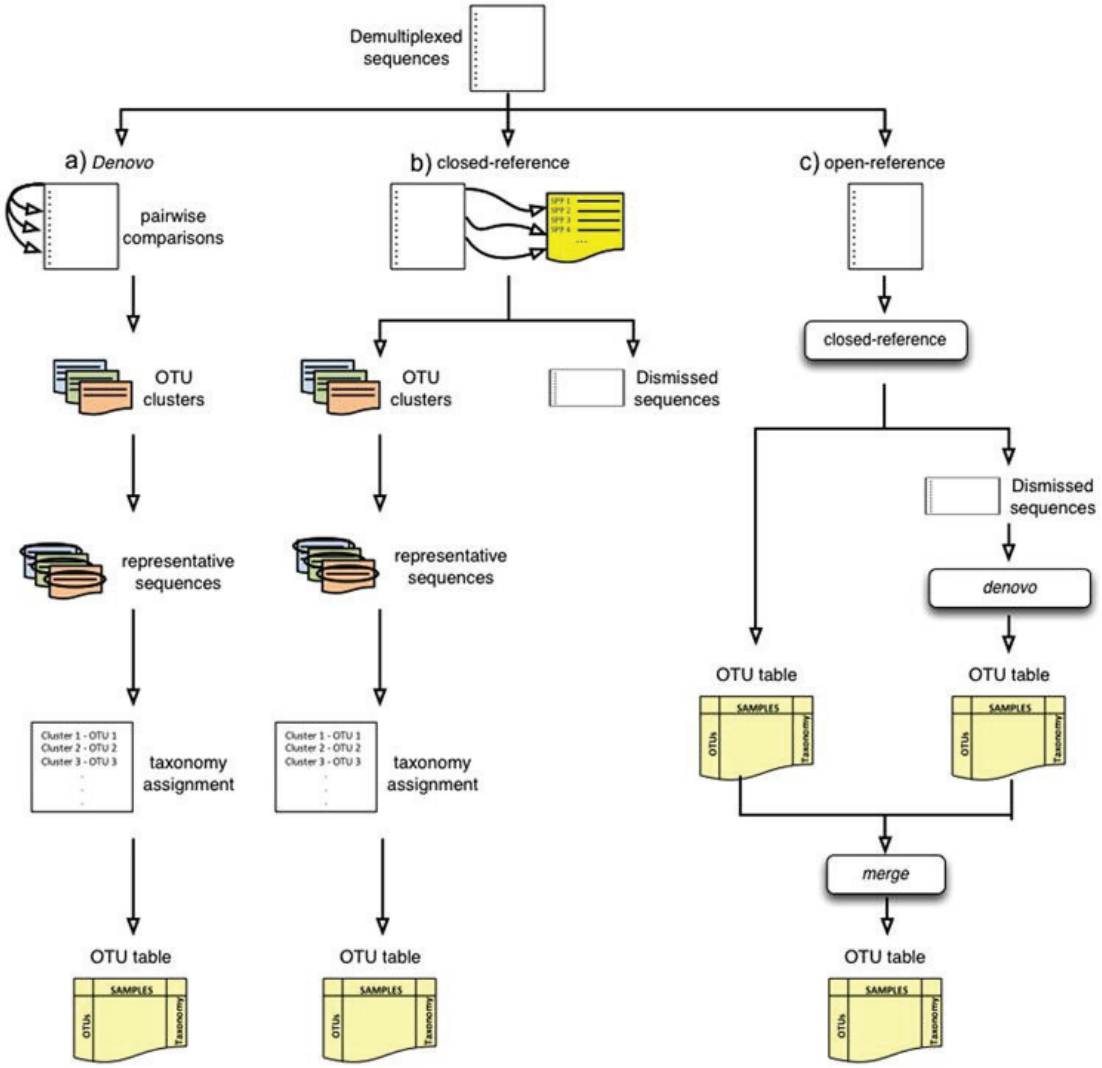

Figure 7: OTU picking approaches. a) The *de novo* method compares sequences to each other, forming OTU clusters, from which representative sequences are selected. Taxonomy is assigned to the selected sequence, and applied to the other sequences making up that OTU, before OTU tables are made. b) The closed-reference method compares sequences directly to a reference dataset, and sequences that match a reference sequence are clustered. The remaining sequences are dismissed. Once a cluster is formed, a representative sequence is selected and taxonomy is assigned to that sequence before OTU tables are made. c) The open-reference method combines closed-reference and *de novo*. The first step is the same as with closed-reference, however sequences dismissed in in the first step are here clustered into OTUs by the *de novo* method. Both OTU tables are merged into a single final OTU table. Figure by Navas-Molina *et al.* (216) reused with permission from Elsevier Books.

7.6.6 Alpha and beta diversity

The diversity of a microbiome can be measured in several ways.

Alpha diversity describes the diversity of species within a community or site (e.g. an individual or disease phenotype). The total number of species (species richness), relative abundances of the species (species evenness) or a combination of these two commonly characterizes the alpha diversity (217).

Chao1 estimates alpha diversity based on species abundance (richness) through estimating the total OTU count to be expected in a sample with infinite sampling. Shannon index characterises species diversity taking both abundance and evenness of the species present into account, but is more sensitive to species richness. Simpson index is also a measure of diversity considering both number of species present and the abundance of each species, though it is most sensitive to species evenness.

Phylogeny is the history of a species' evolution, and a diagram of such an evolution will often be shown in the shape of a tree, visualizing how evolutionary relationships have changed over time. Phylogenetic diversity (PD) is a measure of biodiversity which incorporates phylogenetic difference between species. Faith's PD defines diversity as the sum of lengths of all the tree branches found in a sample, thus incorporating the relative number of new features arising along that specific part of the tree.

Beta diversity describes the difference in biological diversity between communities or sites (e.g. between individuals or disease phenotypes). In its simplest form, beta diversity is a measure of the degree to which samples differ from one another (214). Beta diversity is usually characterized using the number of species shared between two communities (217), and these metrics can be quantitative or qualitative. Quantitative metrics (like Bray-Curtis and weighted UniFrac) use sequence abundance, whereas qualitative metrics (such as unweighted UniFrac and binary Jaccard) use presence-absence of sequences. They can also be phylogeny-based (like UniFrac metrics) or not (like Bray-Curtis) (214). In paper I, we used Bray-Curtis to quantify compositional dissimilarity between participants at four different time points, as it is an established way of quantifying the difference between samples of ecological abundance data when the samples are taken from the same physical size. Using Bray-Curtis comparison, 0 equals the samples being compared are exactly the same and 100 signifies the maximum difference that can exist between two samples, producing a scale which is easy to understand.

7.7 Lipid analyses and HDL subfractions

In paper II, levels of total cholesterol, Apo-A1, HDL- and LDL cholesterol were measured using the immunoassay system Roche Hitachi 917. This is a well-established method (218, 219), however static measures for dynamic processes always has inherent limitations. Methods used to classify HDL subfractions include ultracentrifugation, either by density gradient which divides HDL into subclasses by falling peak diameter (220), or by vertical auto profile (VAP) which measures cholesterol content of the two major HDL subfractions (221), however ultracentrifugation is time-consuming and reproducibility can be an issue. HDL subfractions can also be separated based on size and charge using 2-D gel electrophoresis (222), or based on apolipoprotein composition or ion mobility (220). Nuclear magnetic resonance (NMR) spectroscopy is another method, which unlike other methods for analyzing HDL particles does not require a physical separation step. The protons within the lipoproteins have a natural magnetic distinctness, allowing for division into subfractions (220). In paper II, a high-throughput proton NMR metabolomics platform (Nightingale Health Ltd, Helsinki, Finland) was used to quantify plasma samples, where HDL subclass diameters were weighted with their corresponding particle concentrations generating four subclasses: XL (14.3 nm), L (12.1 nm), M (10.9 nm) and S (8.7 nm). The sample recovery and reproducibility of results is very good with NMR spectroscopy. Sensitivity and selectivity are however lower for NMR than for mass spectrometry (MS)(223), thus for targeted analyses on selected metabolites, MS-based analyses would perform best. However, for more untargeted metabolomics analyses, NMR is an appropriate choice that is often used (223). Additionally, the minimal sample

preparation required, combined with cost-effectiveness (224), makes NMR spectroscopy a reasonable choice for metabolomics analyses, and a practical method to use for HDL subfraction analyses.

7.8 Functional studies of reverse cholesterol transport

7.8.1Serum as cholesterol acceptor

We used the human cell line THP-1 monocytes (ATCC, Chicago, IL) for studies of reverse cholesterol transport. The THP-1 monocytes were differentiated to THP-1 macrophages using 100nM phorbol 12-myristate 13-acetate (PMA, Sigma) over 24 hours. They were subsequently loaded with ^{14}C-cholesterol (0.5 µCi/ml[18.5mBq/l] American Radiolabel Chemicals, Saint Louis, MO) in regular growth medium added oxidized LDL (10µg/ml) and then further grown for 48 hours. The cells were incubated overnight in sterile 0.2% (wt/v) human serum albumin in RPMI-1640. A study comparing phenotypes of the differentiated promonocytic THP-1 cell line with primary human monocytes or monocyte-derived macrophages found that differentiating THP-1 cells with 200nM PMA for 3 days before removing the PMA-containing media for further cell incubation in fresh RPMI for an additional 5 days enhanced the differentiation (225). Thus, a modified PMA differentiation protocol might have enhanced macrophage differentiation in our study too, however for the cholesterol acceptor function of serum that we wanted to investigate, these factors were less likely to be central. After cell incubation, we added 2.5% (v/v) heat-inactivated serum in RPMI-1640 medium from CVID patients and healthy controls respectively to the lipid-laden THP-1 macrophages. The medium was collected after 3 hours and ^{14}C-cholesterol measured by liquid scintillation counter (TRI-CARB 2300 TR Scintillation Counter [Packard , Waltham, MA]). Total cell-associated ^{14}C-cholesterol loading of THP-1 macrophages was assessed in a parallel set up. Percentage efflux was calculated using the equation: $[DPM_{medium}/(DPM_{(cell+medium)})] \times 100$. Serum was employed on the lipid-laden cells as its cholesterol acceptance is mainly driven by its concentration of HDL and Apo-A1 as well as HDL function. To achieve an even more accurate set up for exploring CVID patients' HDL function as cholesterol acceptor, we would have had to freshly isolate HDL from the patients and dissolve this in a medium employed on lipid-laden THP-1 macrophages.

7.8.2 Cholesterol efflux capacity from macrophages

We used freshly isolated PBMC from CVID patients and controls to investigate macrophage cholesterol efflux capacity. For optimal comparison, we sampled blood from patients and age- and sex-matched controls at the same time, ensured PBMC isolation was performed by one operator, as well as counted number of cells in the suspension (using an automated cell counter) to seed equal numbers of cells onto a 24-well plate (Nunclon™ Delta Surface, Thermo Fisher scientific). After one hour incubation, cells were washed twice and plastic adhered monocytes were stimulated with TNF 10 ng/ml for the mononuclear cells to differentiate into a macrophage-like phenotype. After two days of incubation, the macrophage-like cells were loaded with ^{14}C-cholesterol (0.5 µCi/ml [18.5 mBq/l]) in regular growth medium added oxidized LDL (10 µg/ml). As an alternative to the carbon-14 (^{14}C) cholesterol, tritium-3 (^{3}H) cholesterol could have been used, which has a higher specific activity and shorter half-life. However, ^{14}C cholesterol labelling is a well-established method with less potential for label loss. The cells had 48 hours of lipid loading before overnight incubation with 0.2% (wt/v) human serum albumin in RPMI-1640. To assess cell cholesterol efflux capacity, we used 2.5% heat inactivated serum from one healthy individual to cells from both CVID patients and controls, as detailed above. It is not given that a healthy control harbours normal reverse cholesterol transport conditions, however the cholesterol acceptor capacity in this serum was first assessed using lipid-laden THP-1 macrophages and found within normal range prior to being utilized for further studies.

7.9 Functional studies of HDL anti-inflammatory effect (ATF3)

To examine the effect of HDL concentration on the inflammatory response to cell stimulation with TLR2 ligand (Pam3Cys) and TLR4 ligand (LPS), we set up a study using freshly isolated PBMC from patients and age- and sex matched controls. Using a 96-well plate (Nunclon™ Delta Surface sterile, Thermo Fisher scientific), we seeded out duplicate wells for each HDL concentration and each stimuli for each participant, as well as duplicate wells for unstimulated cells. The cells were pre-treated for 6 hours with HDL (0.5 mg/ml, 2 mg/ml and no HDL, respectively [purified human HDL, Kalen Biomedical]). They were then stimulated overnight with the TLR4 ligand LPS from Escherichia coli 026:B6 (2.5 ng/ml) and TLR2 ligand Pam3Cys (0.8 µg/ml), in addition to the duplicate set of cells remaining unstimulated for control. The supernatant was collected after 12 hours and stored until all samples had been collected, prior to analysis in the same V-plex run (Proinflammatory Panel 1 kit, Meso Scale Diagnostics) for levels of TNF and IL-6 (QuickPlex SQ120). We wanted to analyse all samples in the same V plex run to avoid potentially interfering confounding factors between runs. Although a similar experiment has previously been conducted on human PBMCs (175), there is always the possibility that our choice of HDL concentrations added to the cell suspensions, or our ligand stimulation with Pam3Cys and LPS, was not sufficient to provoke a greater difference between patients and controls in cytokine production.

7.10 Trimethylamine oxide (TMAO) measurements

7.10.1 TMAO precursor measurements

In paper III, free carnitine and the carnitine precursors trimethyl lysine (TML) and γ-butyrobetaine (γBB) were analyzed in plasma using a high-performance liquid chromatography (HPLC)/mass spectrometry (MS) method. Some modifications were made to the HPLC conditions; the LC system was an Agilent 1200 series with binary pump (Waldbronn, Germany), variable volume injector and a thermostat autosampler. HPLC separation was conducted at 30°C using a gradient solvent mixture. One of the main advantages of MS is the small amount of sample needed for analysis, and for paper III only two µl of the samples were injected. Detection was achieved using ion-trap mass spectrometer run in tandem mass spectrometry (MS/MS) mode. Accuracy for carnitine determination lies >95% with this method (226). As opposed to other methods, no derivatization step is necessary. The presence of endogenous carnitine and acyl-carnitine makes it difficult to use plasma as a matrix for calibrators, because carnitine-free plasma is difficult to obtain, thus a 4% bovine serum albumin (BSA) solution in water was utilized as calibration matrix, validated for standards and plasma quality controls (226).

7.10.2 TMAO measurements

Plasma levels of TMAO, choline and betaine were measured applying the same assays used to determine carnitine concentrations. Stable isotope dilution liquid chromatography-tandem mass spectrometry in positive multiple reaction monitoring (MRM) mode was used for quantification of the plasma analytes. This is a highly sensitive and accurate platform for targeted metabolic measurements used in previous studies (227), where compounds within a complex mixture (such as plasma) can be selectively quantified. Prior to protein precipitation, the internal standards TMAO-trimethyl-d9 (d9-TMAO), choline-trimethyl-d9 (d9-choline) and betaine-trimethyl-d9-methylene-d2 (d11-betaine) were added to plasma samples and monitored in MRM mode. Various concentrations of TMAO, choline and betaine standards and a fixed amount of stable, isotope-labelled internal standards (Cambridge Isotope Laboratories, Inc.) were spiked into 4% BSA to prepare the calibration curves for the quantification of plasma analytes. TMAO measurements have previously shown great intra-individual variations, where factors such as age, renal function and diet could affect the results (228). Hence, this represents an

inherent pitfall in TMAO studies. To assess whether these factors influenced our data, we adjusted our analyses to take into account age and sex, in addition to running correlation analyses between TMAO and BMI, renal- and liver function parameters.

7.11 Food and dietary supplements questionnaire

Patients contributing fecal samples to our randomized controlled trial were asked to complete a self-administrated and validated Norwegian food frequency questionnaire (FFQ) as well. This was a multiple choice questionnaire designed to reflect dietary habits over the past year (229). Participants could add supplementary information by hand if they had any specific dietary restrictions or habits. The questionnaire was developed by the Department of Nutrition at Institute for Basic Medical Sciences, University of Oslo. It included questions about 180 food items grouped together according to a Norwegian meal pattern (229). Frequency alternatives varied from once per month to several times per day, whereas portion sizes were given as recognizable household units. These sizes were then converted into grams based on standard Norwegian portion sizes. The FFQ also included use of dietary supplements. For paper III, rather than using all the available data from the 180 item FFQ in a non-selective way, and to obviate correcting for multiple testing, we looked at selected food items previously described to be associated with metabolites in the carnitine-TMAO pathway; meat, fish, egg and dairy products (230). We also looked at selected food items relevant to gut microbiota and inflammation; fiber, protein, wine and sugar (231-234). By not including all 180 items in our analyses, we may have missed some impact on TMAO levels by different food groups. However, with more variables introduced, the pretest probability of potential positive findings being of actual clinical importance to TMAO levels would be reduced.

7.12 Statistics

7.12.1 Basics

In paper II, the datasets for HDL analyses and HDL subclass analyses were log-transformed to achieve normal distribution. Two-tailed multivariate testing or non-parametric testing was subsequently applied to compare groups. Related samples Wilcoxon signed rank test was used to analyze longitudinal data, whereas matched pair Wilcoxon signed rank test was applied to the functional studies on patients and age- and sex matched controls in paper II. Functional studies with repeated measurements under different conditions had repeated measures ANOVA analysis applied.

In paper III, datasets for TMAO pathway metabolites were log transformed to achieve normal distribution and then analysed using multivariate testing. Where normal distribution was not achieved, non-parametric testing (Mann Whitney) was applied.

7.12.2 Multivariate distribution analyses

In paper I, linear regression analysis of significant taxa was performed along a timeline, where baseline taxa and treatment groups were independent variables whilst taxa at 2 or 8 weeks were dependent variables in a forced model.

To investigate the connection between lipid levels and inflammatory markers in paper II, we applied bivariate correlation analyses as well as forced and stepwise linear regression analyses.

In paper III, correlation analyses between TMAO and inflammatory markers, gut microbes and dietary factors were performed using Spearman's rank or Pearson's correlation test as appropriate. To adjust

for variables that could influence our data, we ran stepwise linear regression analysis. Friedman test was used to compare longitudinal data collected at three different time points.

SPSS version 24 (IBM, NY) was used to perform statistical calculations.

7.12.3 Comparison of α- and β-diversity

When analyzing alpha diversity, dysbiosis index and the different taxa in paper I, we used UNIANOVA *a priori*. Calculation of rarefied alpha- and beta diversity was performed in QIIME, which can scale to large datasets and visualize trees and taxonomy. Rarefied tables allow for standardizing of data obtained from samples with different sequencing depths, where the sample with the lowest read-count decides the rarefication level. This is a main reason for discarding samples with low read-counts, as described above. Chao1 bacterial richness is a measure of alpha diversity which estimates the total OTU-count one could expect in a sample with infinite sampling. It is however important to look at both community richness *and* evenness too, like the Shannon index does. Additionally, it is useful to examine the phylogenetic diversity (Faith's PD) to consider all aspects of the alpha diversity. The use of antibiotics is a known confounder to measures of alpha diversity, conceivably avoided here using 'no antibiotics last 12 weeks' as an exclusion criterion.

Beta diversity represents the comparison of differences between microbial communities, visualised in a square, hollow matrix, where a distance is calculated between every pair of microbial samples reflecting the dissimilarity between them. Caution is required as low specificity due to assumptions that each group of samples has equal variance can occur. Also, with increasing sample size the likelihood of finding significant p-values increases, thus looking at the test effect size is important. In general, beta diversity metrics are robust to noise and low sequence counts (214).

8. Ethical considerations

International ethical guidelines for medical research are stated in the Helsinki Declaration, where the health of the patient always has to be the first consideration and participation in medical research should be voluntary, under informed consent and after potential risks have been calculated and assessed to be satisfactorily managed (235). Prior to starting the randomized controlled trial in paper I, there were discussions in the research group regarding subjecting patients to a course of antibiotics which was not given as a direct result of a detected infection, but rather to prove a pathophysiological concept. On the grounds of Rifaximin being minimally absorbed systemically, and the intervention only being given for two weeks, it was considered unlikely that this intervention would harm the patients. Any signs of side effects or adverse reactions were followed closely. One of the study participants developed what we interpreted as an allergic reaction to the drug shortly after inclusion, and the drug was immediately stopped. Any researchers undertaking an interventional study would be careful not to expose the patients to potential harm. However, it can be difficult ensuring no allergic or negative reactions to a chosen intervention occur, hence the close follow-up regarding adverse reactions during the intervention study. We sent an application to the Regional committee for Medical and Health Research Ethics of South-Eastern Norway and the Norwegian Medicines Agency for permission to go ahead with the Rifaximin study, and our applications were approved prior to commencement. The trial was registered with clinicaltrials.gov, number NCT01946906, and conducted in accordance with the Helsinki Declaration. All participants provided written, informed consent and we allocated study ID-numbers which were used for handling samples and analyses throughout to maintain anonymity.

Hospitals and medical centers treating patients with rare and orphan diseases have an obligation to pursue research, as these diseases rarely get the same attention as more commonly occurring diseases, potentially reducing their chances of effective treatment. In paper II and III, such research could be conducted on CVID patient samples without exposing the patients to any substantial risks, apart from the very unlikely event that blood sampling should somehow harm them. When reporting the research we had completed, we applied the Vancouver recommendations from the International committee of medical journals editors (ICMJE) to ensure accurate, reproducible and equitable publishing of new medical knowledge.

9. Discussion

9.1 Inflammation in CVID.

9.1.1 The inflammatory phenotype of CVID

Patients with CVID are subject to recurrent acute infections with bacteria and viruses that trigger the innate and adaptive immune systems. Albeit hampered by the immunodeficiency, these infections are thought to follow an established pattern of activation and resolution of the inflammatory process. The observation of persistently elevated markers of inflammation like TNF and IL-12 in patients with CVID, apparently independent of acute infections, has led to a search for other causes of these findings. It has been shown that CVID patients with an inflammatory and autoimmune phenotype have significantly increased levels of TNF, as well as reduced levels of regulatory T-cells and increased numbers of CD4+INFg+TNF+ T-cells all pointing towards an inflammatory T-cell phenotype in CVID (84). Furthermore, CVID patients display reduced overall numbers of CD4+ and increased numbers of CD8+ cells (236). Granulomas are a common histopathological finding in CVID specimens, consistent with the findings of elevated TNF and altered T-cell phenotype. To better understand this pathophysiology, there has been a search for triggers of chronic T cell activation in CVID patients.

9.1.2 Microbes as a cause for chronic inflammation in CVID

Chronic infection would be an obvious cause of persistent inflammation in patients with immunodeficiency, but despite extensive search this is rarely seen in CVID. Chronic norovirus enteritis is perhaps the predominant cause of infectious enteropathy in CVID (237), but seems to affect only a minority of patients (75). One of the research hypotheses has been that microbial translocation from the gut leads to low-grade endotoxemia which stimulates systemic immune activation. However, in a study of 35 CVID patients, 53 sIgAD patients and 63 controls, elevated levels of T cell activation marker sCD25 were uncovered, but no increase in serum lipopolysaccharide (LPS) concentration was observed (83). Some years later, our research group conducted a study of 44 CVID patients, 45 IBD patients and 263 controls, examining their gut microbiota, LPS levels and systemic inflammation. Contrarily, this study showed elevated plasma levels of both LPS and sCD25 in CVID patients, correlating negatively with their gut microbial alpha diversity (45). Hence, one could propose that microbes in the gut are somehow linked to systemic inflammation.

9.1.3 Sterile triggers of inflammation in CVID

Foreign bodies, trauma, radiation, toxins, damaged cells, and cholesterol crystals can all trigger an inflammatory response, resulting in sterile inflammation. Comparable to the inflammatory response triggered by an invading microbial pathogen, sterile inflammation induces recruitment of macrophages and neutrophils, production of pro-inflammatory cytokines and chemokines as well as stimulating T cell-mediated adaptive immune responses. CVID is in itself not characterized by tissue damage, but recurrent infections repeatedly trigger their innate immune system, stimulating an inflammatory process. However, the chronic low-grade inflammation seen in CVID has some resemblance to the phenomenon of inflammageing

Inflammageing is a term that has been used to describe raised inflammatory markers in blood samples from older individuals predicting the risk of chronic morbidity including cardiovascular diseases, disability, frailty, reduced cognitive function and premature death (238). Several potential mechanisms of inflammageing have been suggested, such as genetic susceptibility, increased gut permeability,

immune cell dysregulation and chronic infection (238). Furthermore, trials have suggested there is a causal relationship between inflammageing and cardiovascular disease that can be targeted therapeutically (239-241). In line with this, the mortality risk in CVID patients with inflammatory and/or autoimmune complications has been shown to be 11 times higher than for patients who have infections only over a time span of 40 years (72). Hence, identifying factors that trigger or sustain inflammation could be of great importance to reduce morbidity and improve mortality rates in CVID patients.

9.2 Could gut microbiota contribute to inflammation in CVID?

Exposure to environmental factors and resident microbes influence the development of the mucosal immune system both in the gut and lungs, where complex molecular and cellular regulatory pathways prevent inappropriate stimulation of the immune system and shape adaptive immune responses (242). Where tolerance to dietary nutrients and environmental antigens is not acquired, or if cellular composition is disturbed or the epithelial barrier does not form properly during maturation of mucosal immunity, it could result in inflammation (242). One of the most important contributions gut microbes have to human hosts is normal evolution of gut-associated lymphoid tissues (GALT)(243). Crypt patches, Peyer's patches and isolated lymphoid follicles make up GALTs, where antigens can be presented by antigen presenting cells (APCs) and influence lymphocyte functions resulting in inflammation or tolerance (243). Furthermore, regulation by commensal bacteria of gut-specific immune-cells, such as IL-22 producing innate lymphoid cells (ILCs), can lead to overgrowth of potentially pathogenic bacteria and increase the risk of intestinal damage and systemic inflammation (244).

Mice studies have demonstrated an accumulation of T_H17 cells as a result of colonization with segmented filamentous bacteria (SFB; a novel genus in the order *Clostridiales*)(245, 246), increased numbers of Tregs in gnotobiotic (i.e. with defined bacterial communities) mice colonized with *Clostridial* strains (247) and systemic T-cell responses affected by polysaccharide A from *Bacteroides fragilis* (248). These commensals are influencing the inflammatory properties of the innate immune system beyond the effect on local mucosal tissues (249). The term microbe associated molecular patterns (MAMPs) has been used to describe the molecular signature of these commensal bacteria, as opposed to pathogen associated molecular patterns, PAMPS, for pathogenic microbes (250). On an intracellular level, multiprotein complexes of the nod-like receptor (NLR) family called inflammasomes monitor our cellular health by detecting pathogenic substances that emerge in the course of infection, metabolic disturbances and tissue damage (251). Inflammasomes trigger programmed cell death in the form of pyroptosis when a host cell has been compromised by microbial pathogens, a process that is inherently inflammatory (252). Demonstrating this, the NLRP6 inflammasome plays a crucial role in recruitment of inflammatory cells as a response to endogenous or exogenous damage-associated molecular patterns (DAMPs), and a disturbance of this pathway could trigger inflammatory bowel disease in some humans (253). Toll like receptors (TLRs) also mediate inflammation induced by infectious triggers, as well as triggers such as saturated fatty acids, via endogenous molecules (254). A dysregulated interaction between TLRs and commensal bacteria can induce both chronic inflammation and tissue damage (118).

Gut microbial composition which has been altered sufficiently to be considered dysbiotic has been demonstrated to drive inflammation in colitis, insulin-resistance and hyperlipidemia (255). In patients with severe sepsis, plasma levels of CRP and IL-6 have been shown to be significantly higher in patients with *Gram negative* bacteremia than with *Gram positive* bacteremia, indicating that different PAMPs elicit varying intensity of the immune response (256). In paper III, we found CVID patients to

have increased abundance of the *Gram negative Gammaproteobacteria* in their gut microbiota compared to controls, linked to elevated levels of inflammatory markers in plasma. Thus, not only the reduced microbial diversity, but also the distribution of species in the gut microbiota could be contributing to systemic inflammation in CVID.

9.3 Rifaximin does not alter systemic inflammation in CVID

Previous studies by our research group had linked gut microbiota to elevated circulatory levels of LPS and systemic inflammation in CVID (45), but paper I is to our knowledge the first intervention study targeting the dysbiotic gut microbiota in CVID. LPS is shed from *Gram negative* bacteria in the gut and through microbial translocation/leaky gut mechanisms (see 4.4.4) it activates the innate immune system (117). Based on this, targeting the gut microbiota using a drug known to reduce LPS and ameliorate symptoms in liver disease (145) was appealing.

Rifaximin is a broad-spectrum antibiotic from the rifamycin family, which inhibits bacterial RNA synthesis and has *in vitro* action against *Gram positive, Gram negative*, aerobic and anaerobic bacteria. In addition, rifaximin has a very advantageous side-effect profile and low risk of interactions with other drugs (257). It appears to alter stool concentrations of certain bacteria without affecting the overall composition of the gut microbiota (145, 258), and has been shown to reduce the length of gastrointestinal infections without wiping out enteropathogens (146, 257). In rodent studies, rifaximin has reduced ileal bacterial load and changed the bacterial composition to a relative abundance of *Lactobacillus* species, by which it prevents mucosal inflammation and impairment of the gut barrier (259). It also decreases colon bacterial load, thereby diminishing bacterial translocation and subsequent inflammation (260).

There is negligible systemic absorption of rifaximin after oral administration (<0.4% of administered dose) and the drug is essentially excreted in feces unchanged (261). Due to its localized activity in the GI-tract, any potential effect on systemic inflammation would be trough gut microbial mechanisms. Therefore, irrespective of the disease studied, this was an important “proof of concept” study exploring whether alteration of gut microbial composition could alter systemic inflammation. We therefore explored if rifaximin for 2 weeks could alter gut microbial composition and thereby modulate the systemic inflammation in CVID (Figure 8).

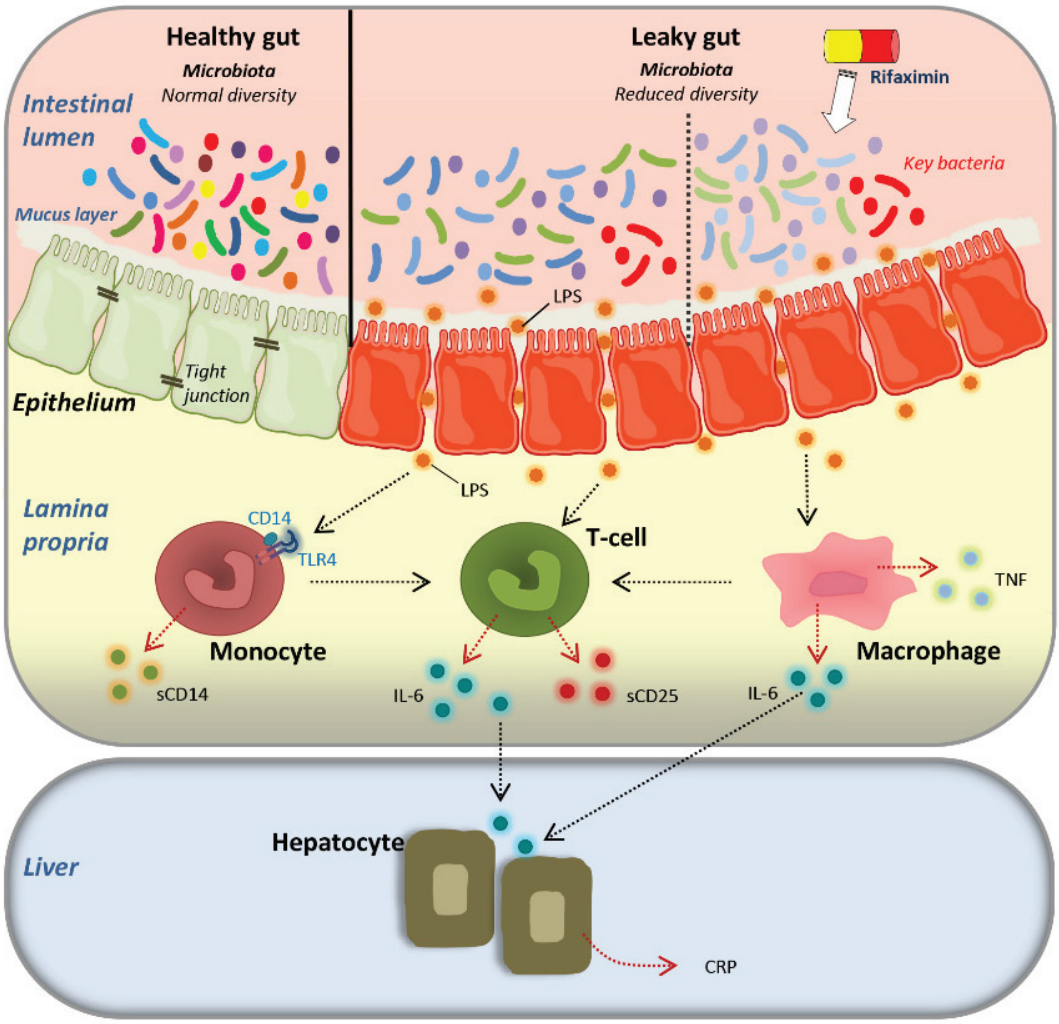

Illustration: Kari Otterdal, RIIM, OUS Rikshospitalet

Figure 8: Graphical illustration of the hypothesis tested in the rifaximin study. CVID patients have dysbiosis of their gut microbiota with reduced bacterial diversity compared to healthy controls and altered abundance of certain key bacteria (red). Many CVID patients also have enteropathy with inflamed epithelial cells in the mucosa, causing a loss of tight junctions and possibility for gut leakage. LPS translocating to lamina propria activates immune cells and generates secretion of inflammatory cytokines.

In our study, whilst rifaximin had indeed altered the gut microbiome significantly by the end of the intervention period, we failed to prove that this change in bacterial diversity was sufficient to modulate immune parameters. In addition, as expected, rifaximin only caused a temporary shift in gut microbial composition of the patients randomized to this intervention. Six weeks after the end of intervention, gut microbial alpha diversity of the group as a whole had returned to its pre-interventional state. One could argue that exposing the study patients to rifaximin for 2 weeks was too short a period to be able to conclude that altering the gut microbiota does not lead to significant modulation of the

immune system. However, a previous clinical trial focusing on reducing symptoms of irritable bowel syndrome showed significant relief of gastrointestinal symptoms after only 2 weeks treatment with rifaximin (186), though this of course focused on symptoms and not objective laboratory parameters. Also, a study on the effect of repeated antibiotic disturbance by ciprofloxacin on the gut microbiota showed altered bacterial community composition starting 3-4 days after drug initiation, with the microbial communities starting to return to their original state one week after cessation of antibiotics (98). Still, in hindsight, it is possible that a longer duration of the rifaximin intervention could have led to a greater impact on the immune response in our study, brought about by sustained alterations of the gut microbiota over some time. Supporting this, a study on patients with moderately active Crohn's disease (CD), where a 12-week intervention with extended intestinal release rifaximin was given to achieve remission, demonstrated significant effect on the patients' symptoms (262). Furthermore, a study on patients with moderately active CD given rifaximin treatment for up to 24 weeks showed remission and a sustained effect on symptoms up to 48 weeks follow-up (263). It is likely that long-term antimicrobial therapy modulates a variety of diseases through altering the gut microbiota (264), however the underlying effects are largely still not understood, and the length of antibiotic intervention required to achieve significant modulation is also not known.

In paper I, we used alpha diversity to measure microbiome change, a well-established group variable for gut microbial composition, but it does not necessarily reflect all important changes in abundance of critical bacterial species that could influence the immune cells. We found that rifaximin had not altered the previously defined CVID dysbiosis index significantly, suggesting that an important feature of the dysbiosis of CVID was not targeted with rifaximin (Figure 8). Rifaximin treatment has previously led to a relative abundance of *Lactobacillus* species and a reduction in the number of segmented filamentous bacteria in the gut microbiota of rodents, linked to reduced gut inflammation (265). A study conducted on patients with colonic active CD showed daily administration of rifaximin causing increased concentrations of *Bifidobacterium, Atopobium* and *Faecalibacterium prausnitzii*, without altering the overall composition of their gut microbiota (258). Hence, rifaximin failing to alter key bacteria in the gut microbial dysbiosis found in CVID patients could have contributed to us not finding any significant effect on levels of LPS or inflammatory markers.

Overall, based on the negative results from this study, there is currently no obvious role for the use of rifaximin in CVID. Moreover, short-term use of rifaximin is not likely to achieve short or lasting immune modulation in chronic diseases characterized by systemic inflammation and gut leakage.

9.4 Carnitine metabolism and the microbiota to disease axis

Alterations of the gut microbiota has been found in nearly every disease where this has been studied, but we are still far from understanding the mechanisms central to this association. The well-established principles for interactions between pathogens and the immune system have served as a template for the interaction between our commensals and the immune system as well, but there might be altogether different mechanisms at work. The pivotal importance of gut microbiota in digesting nutrients to absorbable metabolites, and the strong association between gut dysbiosis and metabolic disease, have therefore highlighted metabolites as an important area of future research.

Trimethylamine N-oxide (TMAO) is an organic compound generated from carnitine, choline and betaine from the diet via metabolism by gut microbes. Carnitine is a nonessential nutrient in most

people, as the body can synthesize carnitine from lysine side chains. It can however also be acquired through food such as red meat, chicken, dairy products and wheat. Choline is a near essential nutrient that exists either bound within another compound (like phosphatidylcholine) or free. It is obtainable from dietary sources like red meat, chicken, fish, eggs, cruciferous vegetables and whole grains. It is a component of lecithin and a precursor for acetylcholine. Betaine is a nonessential nutrient that we can get through foods such as red meat, turkey, seafood, sugar beets, spinach, wheat and grains.

When carnitine, choline and betaine are ingested through diet, the gut microbes metabolize these nutrients to the intermediate compound trimethylamine (TMA) (Figure 9). In addition, TMAO can also be consumed directly through certain fish types, and this absorption appears largely complete without the processing of gut microbes (266). Plasma levels of TMAO can also be influenced by factors other than diet and gut microbes; renal function has been suggested to play a role as TMAO is mainly excreted through the urine (267, 268), additionally, age can have an impact on plasma TMAO levels (269) and hormones such as estrogen and testosterone on FMO3 activity (270, 271).

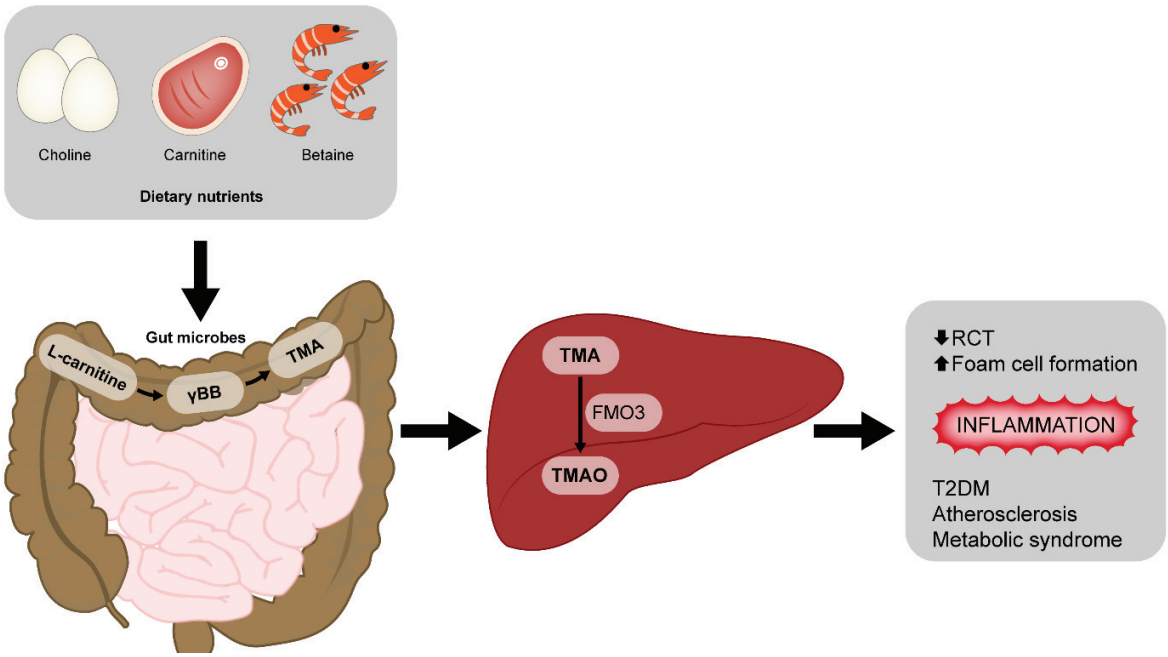

Illustration: Øystein Horgmo, UiO

Figure 9: Dietary nutrients converted to trimethylamine N-oxide (TMAO). Red meat, eggs and seafood are important dietary sources of carnitine, choline and betaine. Microbes in the gut metabolize these dietary nutrients to γ-butyrobetaine (γBB) and further to trimethylamine (TMA). The majority of TMA in the gut is passively absorbed into the portal circulation by diffusion and subsequently transported to the liver. In the liver, the enzymes flavin monooxygenase FMO1 and FMO3 oxidize TMA to TMAO. FMO3 has higher specific activity in the liver than FMO1, thus constituting the main enzyme in TMA conversion to TMAO (272). Increased concentrations of TMAO are linked to reduced reverse cholesterol transport (RCT), increased foam cell formation, inflammation and associated medical conditions.

9.5 TMAO: a marker or mediator of gut microbiota mediated inflammation in CVID?

In paper III, we focused on identifying a link between gut microbiota, metabolites (mainly TMAO) and the systemic inflammation seen in patients with CVID. Studies on human gut microbiota rarely contain data on diet and one of the strengths of paper III was that it also included a standardized assessment of the participants' diet. We found increased TMAO levels in CVID compared to controls and TMAO correlated positively with markers of systemic inflammation and abundance of *Gammaproteobacteria* in stool samples from these patients.

9.5.1 TMAO and systemic disease

Increased levels of TMAO have so far been linked to several medical conditions such as cardiovascular disease (CVD)(150, 152), renal dysfunction (273), diabetes mellitus (157) and colorectal cancer (274), but the evidence is somewhat equivocal (272). However, the TMAO concentration in plasma seems to be a robust prognostic marker for CVD risk, including in individuals with low overall traditional risk factors for major adverse cardiovascular events (152, 154). The underlying cause of this could be connected to the observed increase in atherosclerosis with higher TMAO levels (153, 155). Thus, the composition of gut bacteria plays a role in progression of CVD, however it does not conclude whether TMAO is a biomarker or a contributor to such a development. In our study, the CVID patients did not have an increased incidence of CVD, despite their increased TMAO levels.

The kidneys excrete TMAO, and several studies have reported increased TMAO levels in patients with impaired renal function. Hence, TMAO could be a mechanism-based marker of CVD risk in the setting of reduced kidney function (273). TMAO levels have also been identified as an independent predictor of mortality in patients with chronic kidney disease (CKD), correlating with systemic inflammation in these patients (275). However, the significant correlations between TMAO and inflammatory markers in CKD patients disappeared when renal function was accounted for, thus implying TMAO could act as a surrogate marker for GFR in these patients (275). In our CVID cohort, eGFR did not correlate significantly with TMAO levels. Furthermore, eGFR values of our study patients did not significantly differ from the eGFR values of the control cohort, showing CVID patients have elevated levels of TMAO in the setting of normal renal function.

Plasma TMAO levels are also increased in patients with type 2 diabetes (276) and there are associations between TMAO levels and metabolic syndrome (277). Circulating levels of TMAO have been observed to increase with increasing BMI in adults (277), potentially related to specific taxa in the gut microbiome associating with both diet and plasma TMAO levels (151). Despite all this accumulating knowledge, it is still not clear whether TMAO is merely a marker or indeed a mediator of inflammatory disease processes that can be therapeutically targeted (228). Our data links TMAO to inflammation and an altered gut microbiota in patients with CVID, even in the absence of renal dysfunction.

9.5.2 TMAO, gut microbes and diet

In paper III, we focused on the ten gut microbial taxa identified in the CVID dysbiosis index, and additionally five taxa identified as increased or decreased in CVID using a statistical framework called Analysis of composition of microbiomes (ANCOM)(278) when exploring a possible link between key gut microbes and the carnitine-TMAO-pathway. This helped us identify a pattern where abundance of key *Gram negative* bacteria in the gut associated with elevated plasma levels of TMAO and its precursors. Since nutrients converted into TMA come from our diet, the diet represents a potential therapeutic

target to affect TMAO levels. Hence, we also wanted to illuminate the impact of diet on TMAO levels in CVID patients, representing a patient population with known low-grade systemic inflammation.

L-carnitine, a TMA precursor abundant in red meat, is used as dietary supplement by some athletes (279) and vegetarians (280, 281), but has also been tried out as a supplement to patients with renal disease on hemodialysis (282). Vegetarians, and especially vegans, have a lower incidence of atherosclerotic cardiovascular disease than omnivores, proposed to be linked to dissimilar TMAO formation (283). Studies on vegans/vegetarians and omnivores/carnivores have shown dietary habits to significantly associate with alterations in the composition of intestinal microbes, and elevated TMAO levels have been found to attenuate reverse cholesterol transport (RCT)(151). Geographical differences in TMAO levels among patients and controls from different continents also point towards unique dietary habitats and gut microbial communities indeed affecting TMAO levels (284). Thus, diet influences levels of TMAO and its precursors, however the composition of gut microbiota appears the most important contributing factor regulating plasma TMAO levels (151). Our findings support this observation, where certain key bacteria in the gut microbiota were demonstrated to associate with increased levels of TMAO. The jury is still out as to how big an impact diet has on circulating levels of TMAO in humans. However, based on the results in paper III, it is likely that the impact is closely related to the dietary induced modulation of the gut microbiota.

9.5.3 TMAO and dietary considerations in CVID

Many studies of the gut microbiome have so far been performed in animals, particularly mice, which makes it difficult to assess the impact of the human complex diet on intestinal bacteria. Laboratory mice are usually fed specific diets, such as a high-fat diet made to resemble a Western diet. But as humans often introduce a variation of factors into their diets at irregular intervals, a strict study dietary pattern may not necessarily adequately translate to real life. In paper III, information on the patients' diet was particularly important since the metabolites measured could be affected by dietary intake.

To examine the impact of diet on TMAO levels and inflammation we used self-reporting food frequency questionnaires (FFQs). These have an inherent bias in the human ability to recollect exact amounts of food compounds making up their diet, and the potential inclinations to report a more optimal dietary intake than is perhaps the case. We also had a relatively low number of patients completing the questionnaire, which must be taken into consideration when interpreting the FFQ results. What we did find was that the diet of CVID patients did not differ significantly from the Norwegian reference population, particularly when it came to foods high in carnitine and other TMA precursors such as red meat, milk and dairy products. Importantly, we did not find any significant associations between TMAO levels and intake of meat, fish, dairy products or fiber in our patients. We did however find intake of red wine to negatively correlate with TMAO levels, and there was a positive correlation between TMAO levels and intake of table sugar but not total sugar consumption.

Western diets high in sugars and saturated fat have previously been found to associate positively with levels of TMAO (228, 285), thus it is tempting to suggest that our table sugar finding is proof of this concept in CVID patients too. Nevertheless, we choose to interpret the finding with some caution, as the estimated total intake of sugar through the diet did not associate significantly with TMAO in our patients. Consumption of red wine has previously been linked to reduced inflammation and a reduction in cardiovascular disease (233, 286, 287), but until recently the exact mechanisms underlying

this association have remained elusive. In 2015, a group of researchers explored how they could “drug the microbiome” to inhibit meta-organismal pathways responsible for TMAO generation, with the aim to prevent atherosclerosis (288). They surveyed multiple natural and synthetic choline analogs and found 3,3-dimethyl-1butanol (DMB) to have cutC/D choline TMA lyase inhibitory activity, a compound that can be found in some red wines. DMB was further investigated through mice studies and revealed to inhibit TMA formation in the gut, but also to reduce choline diet-enhanced endogenous macrophage foam cell formation and atherosclerotic lesion development (288). Hence, our results in paper III, showing attenuating effect of red wine on TMAO levels, substantiate a potential role of DMB in preventing inflammation.

To mechanistically prove the associations we found in this study describing a role of TMAO in generating systemic immune activation in primary immunodeficiency (PID), mice studies would have to be considered. The lack of a CVID mouse model however makes this approach difficult. If available, study mice could have their gut microbiome modified to represent the dysbiosis typically seen in CVID patients, whilst another group could be kept as normal gut microbiota controls. Both groups could be fed a diet rich in TMAO precursors, allowing for subsequent measurements of TMAO levels in plasma and examination of immune cell activation and inflammatory markers. However, as conventional laboratory mice may be too distant from natural environmental conditions to represent a true mirror of the physiology of free-living humans (289), natural microbiota based mouse models may be needed to improve translatability of immunological results to humans, potentially making the outlined study setup difficult to implement.

We suggest that other mechanisms than diet are mainly responsible for the increased levels of TMAO in CVID patients, but certain dietary factors could have an impact on TMAO and are thus worthwhile exploring as therapeutic targets. The gut microbiota composition seems to be more influential for TMA and subsequent TMAO formation than diet alone, thus likely representing a more potent therapeutic target to reduce inflammation.

9.5.4 TMAO and inflammation

In paper III, we hypothesized that TMAO could act as a missing link between the gut microbiome and systemic inflammation. We found increased TMAO levels in CVID compared to healthy controls, correlating positively with markers of systemic inflammation and abundance of *Gammaproteobacteria* in stool samples from these patients.

In healthy subjects, plasma concentration of TMAO correlates positively with inflammatory marker TNF and soluble TNF receptors, but does not reach significant correlation with CRP or IL-6 levels (156). It is not clear exactly how the increased levels of TMAO could be causally linked to inflammation, but several mechanisms have been suggested. In mouse studies, TMAO added to diet augmented impaired glucose tolerance, causing adipose tissue inflammation (157). TMAO has also been shown to promote vascular inflammation by activation of the NLRP3 inflammasome through reactive oxygen species (ROS) signaling pathways (290, 291), as well as increasing activation of caspase-1 along with the NLRP3 inflammasome causing increased IL-1β production (292). However, more mechanistic studies are needed on human cells to explore whether and how raised levels of TMAO affect pathways leading to inflammasome activation and further triggering of systemic inflammation. Based on our results in paper III, TMAO presents itself as a link between bacterial composition in the gut and systemic inflammation,

suggesting that modulation of the gut microbiota could be a target in order to affect the level of systemic inflammation in CVID through TMAO (Figure 10).

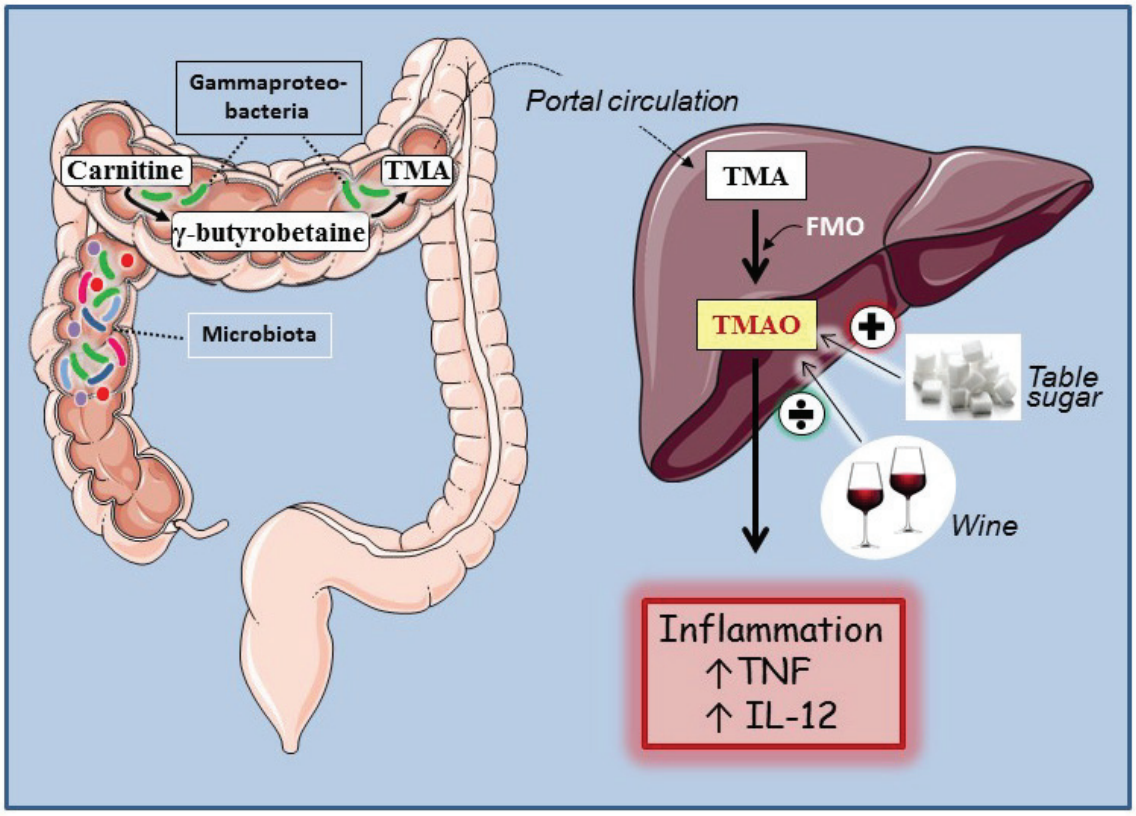

Illustration: Kari Otterdal, RIIM, OUS Rikshospitalet

Figure 10: The impact of gut microbiota and diet on TMAO concentration and inflammation in CVID. We found increased abundance of *Gammaproteobacteria* in the gut microbiota of CVID patients, linked to increased concentrations of TMAO and elevated inflammatory markers TNF and IL-12 in plasma. Intake of table sugar correlated with increased TMAO concentrations whereas intake of red wine was associated with lower concentrations of TMAO. Figure reused from paper III.

9.6 Metabolic disturbances in CVID

In paper II, we wanted to explore the significance of lipid metabolism, and in particular HDL levels and function, in relation to inflammation and autoimmunity in CVID. We considered several hypotheses; do reduced levels and altered function of HDL in CVID patients cause systemic inflammation as well as autoimmune and inflammatory complications? Are recurrent infections and chronic low-grade inflammation in CVID patients the cause of their impaired HDL function? If so; could this be sustaining the problem through inferring further inflammatory conditions by failing to perform RCT in a satisfactory way? We started by analyzing plasma levels of central lipoproteins in CVID patients and controls, which revealed reduced levels of HDL in CVID compared to controls. The analysis of HDL subclasses showed reduced levels of M-HDL, L-HDL and XL-HDL in CVID. S-HDL levels were also lower in CVID, though not

reaching statistical significance. Moreover, HDL levels were consistently lower in CVID than in healthy controls, reaffirmed by temporal testing of a smaller group of the CVID patients. Furthermore, Apo A-1 levels were reduced in CVID patients, of importance when investigating RCT in these patients. There was no significant difference in plasma levels of LDL or total cholesterol between the patients and the controls.

When dividing CVID patients into phenotypes (infections only versus complications), the difference in HDL levels between patients and healthy controls turned out to be driven by the group that has non-infectious complications. This could fit with previous knowledge about the connection between HDL and inflammation. We also found that low HDL levels correlated with high levels of inflammatory markers CRP and sCD25 (indicating activated T-cells) in CVID patients, further supporting this. We did however not see significant associations between HDL levels and LPS or sCD14.

The strongest predictor of HDL levels in CVID patients was sCD25, suggesting a link between T-cell activation and lipoprotein metabolism. However, as previous studies had proposed HDL function to be of more importance than HDL levels in terms of affecting inflammation, we set up some functional studies to look further look into this. Macrophage reverse cholesterol transport is a function of intracellular cholesterol concentration, cell cholesterol efflux capacity and serum HDL cholesterol acceptor function. In a subgroup of CVID patients, including patients with infections only and patients with non-infectious complications, we measured the HDL cholesterol acceptor function in serum and compared it to healthy controls. THP-1 macrophages were exposed to ^{14}C-cholesterol for 48 hours prior to cholesterol efflux being induced by serum from either CVID patients or controls. This experiment showed a significantly decreased cholesterol acceptor function in serum from CVID patients compared to healthy controls, and in particular amongst the patients with non-infectious complications.

Pursuing this, the cholesterol efflux capacity in macrophages of CVID patients with non-infectious complications and controls was tested by exposing monocyte derived macrophages to ^{14}C-cholesterol for 48 hours to allow for cholesterol loading, followed by overnight stabilization, exposure to a uniform control serum and subsequent analysis of cholesterol efflux. Macrophages from patients and controls showed no difference in cholesterol loading, but cholesterol efflux capacity to serum was significantly decreased in CVID patient macrophages. This efflux of intracellular cholesterol is largely reliant on functioning ABCA1 and ABCG1 transporters, so we isolated PBMC from CVID patients and controls to measure mRNA expression of these coding genes by qPCR. We found significantly reduced mRNA expression of ABCA1 in CVID patients, but no significant difference in mRNA expression of ABCG1. The reduced expression of ABCA1 in PBMC could definitely contribute to the overall reduced RCT in CVID patients, and protein data would have been useful to substantiate this interpretation.

Finally, we looked into the importance of the modulating transcription factor ATF3 to HDL's anti-inflammatory function on macrophages in CVID patients, and we found that CVID patients had reduced mRNA expression of ATF3 in their PBMCs. To further explore this, freshly isolated PBMC from a smaller group of CVID patients with the inflammatory phenotype, and the same number of healthy controls, were incubated in the presence of different concentrations of human HDL (HDL 2.0 mg/ml, 0.5 mg/ml and no extra HDL added) for 6 hours. We then stimulated the cells with TLR4 (LPS) and TLR2 (Pam3Cys) ligands for 12 hours, before analyzing the cell supernatant for TNF and IL-6 levels to assess the inflammatory state. Not surprisingly, we found a suppressive effect of HDL on TNF release in Pam3Cys exposed cells from healthy controls, but this anti-inflammatory effect of HDL was significantly reduced in

cells from CVID patients. Cell stimulation with TLR4 ligand LPS did not show the same differences in TNF release, nor did the cells show a similar pattern for IL-6 release when stimulated with Pam3Cys or LPS. This suggests that the anti-inflammatory effects of HDL on PBMC is to some degree attenuated in CVID, which could reflect their reduced expression of ATF3. Experimental studies including even more patients and controls, as well as protein data on ATF3, should be endeavored to consolidate these findings.

9.7 Regulation of HDL in CVID

The immunomodulatory effects of lipid metabolism have been studied in several medical conditions and it is clear that lipids have important both pro- and anti-inflammatory effects (166, 293-295). HDL and Apo A-1 are modulators of T cell activity, whilst oxidized low-density lipoproteins (ox-LDL) promote foam cell formation and inflammation, linking lipid metabolism to immune system regulation.

Based on our findings in paper II, CVID patients have impaired serum cholesterol acceptance capacity in the setting of decreased HDL and apo A-1 levels, as well as reduced expression of ABCA1 in macrophages resulting in reduced RCT, linked to elevated markers of systemic inflammation (Figure 11). Intracellular accumulation of cholesterol in dendritic cells can activate the NLRP3 inflammasome and result in cytokine release triggering autoimmunity and inflammation, and it is conceivable that cholesterol accumulation in macrophages of CVID patients could generate a similar effect. To avert or counteract this, therapeutical interventions could be directed at improving RCT either by boosting Apo A-1 / HDL function as cholesterol acceptors, or by enhancing the macrophage cholesterol efflux capacity via the ABCA1 transporter. Interventional studies with in vitro components would be necessary to prove this concept.

Statins have a modest effect on HDL cholesterol levels (up to 16%), as do fibrates (up to 20%), but the limited clinical benefit of raising low HDL cholesterol levels has brought about a search to identify new agents affecting HDL function (296). Several polypeptides have been created to advance the anti-inflammatory function of HDL, including apolipoprotein mimetics. Administering the peptide D-4F to mice through their drinking water has generated significantly improved anti-inflammatory properties of HDL without altering HDL plasma levels (297, 298). Moreover, tandem 4F-based peptides (mimicking the punctuated α-helical repeats of full-length apo A-1) have been shown to further increase cellular cholesterol efflux via the ABCA1 and ABCG1 pathway in mice (299). In humans, patients with type 2 diabetes mellitus (T2DM) who were given a recombinant HDL infusion developed improved plasma HDL ability to receive cholesterol from THP-1 macrophages, thereby enhancing its anti-inflammatory properties (300). Thus, more studies on the use of recombinant HDL to aid the immune system in combating endotoxemia and general inflammation could lie in the future. Additionally, cholesteryl ester transfer protein (CETP) inhibition, administered to reduce cardiovascular disease, has been demonstrated to increase HDL and cholesterol efflux capacity (301, 302), suggesting this could be an interesting therapy to investigate further for CVID patients as well.

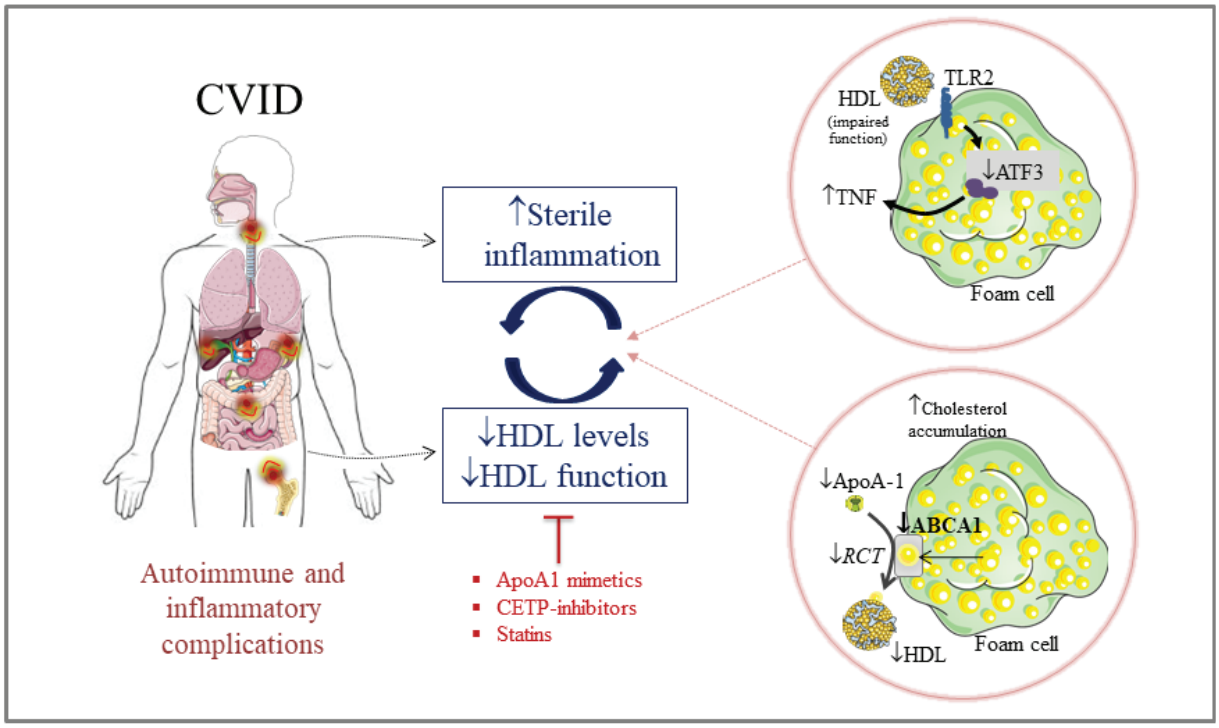

Illustration: Kari Otterdal, RIIM, OUS Rikshospitalet

Figure 11. The vicious cycle of low HDL, impaired HDL function and inflammation in CVID. Patients with CVID have lower levels of HDL and reduced HDL function via impaired reverse cholesterol transport compared to healthy controls, correlating with elevated inflammatory markers and a more severe phenotype with autoimmune and/or inflammatory complications. Loss of HDL's anti-inflammatory effects leading to increased levels of sterile inflammation in these patients could be related to their reduced expression of transcription factor ATF3, which is vital in the protective effect HDL has against inflammation induced via toll-like receptors. However, the inflammatory state in CVID patients could also contribute to reduced function of HDL in itself, thus sustaining a vicious cycle.

9.8 Could HDL have anti-inflammatory effects in CVID?

With many components of the lipid metabolism linked to inflammation and inflammatory diseases, the lowering of LDL levels has in multiple studies been shown to reduce the risk of CVD, while it has been more challenging to prove that increasing HDL levels has a cardioprotective effect (303, 304). Functional aspects of HDL appear more potent in modulating inflammation than HDL levels alone (305). Simultaneously, systemic inflammation has been proposed to modify HDL to a dysfunctional form with reduced antiatherogenic capacity (177).

Our research in these three papers has focused on gaining knowledge of the impact gut microbial composition has on immune activation, as well as trying to identify the link between gut microbes, altered lipid metabolism and systemic inflammation seen in CVID. A previous study applying subacute endotoxemia in a rodent macrophage-to-feces RCT model demonstrated that inflammation *in vivo* impairs RCT and HDL efflux function independent of HDL levels (306). Murine monocytes that were exposed to LPS showed a decrease in both ABCA1 and SR-B1 gene expression (307). However, in

addition to LPS-induced inflammation downregulating ABCA1 expression, it seems ABCA1 can also act as an anti-inflammatory receptor contributing to reduce the expression of inflammatory factors (308). Furthermore, *in vitro* studies have detected that TNF, which is typically elevated in CVID patients, can down-regulate ABCA1 expression in THP-1 macrophage-derived foam cells, thereby increasing intracellular cholesterol content. IL-10 can upregulate ABCA1 expression and thereby decrease cholesterol accumulation (309), whilst pro-inflammatory cytokines IFNγ and IL-1β have been implicated in downregulating ABCA1 expression.

A dysregulation of intracellular lipid metabolism has been shown in HIV-infected macrophages, where HIV-1 impairs ABCA1 dependent cholesterol efflux due to post-transcriptional down-regulation of ABCA1 and inhibited internalization of apo A-1 (310). Monocytes are thus primed for foam cell formation in HIV positive individuals, a mechanism further enhanced by elevated TNF levels, likely contributing to increased atherosclerosis in these patients (311). But despite existing knowledge about lipid metabolism and inflammation in patients with secondary immunodeficiency, little has been known about how these mechanisms are regulated in primary immunodeficiency. Our studies have confirmed that elevated levels of pro-inflammatory cytokines are correlated with low HDL levels as well as reduced HDL function, low ABCA1 expression and impaired RCT in CVID.

9.9 How to modulate inflammation in CVID

9.9.1 The gut microbiome, metabolites and inflammation

Several diseases characterized by systemic inflammation display a pattern of diet and gut microbial composition connecting with metabolites or low-grade endotoxemia, seemingly contributing to trigger inflammation. In a large clinical cohort, the three metabolites choline, TMAO and betaine were identified to predict increased risk of CVD (150). In chronic kidney disease, altered gut microbial flora and the related endotoxemia appears to contribute to systemic inflammation (312, 313). Obesity and diabetes are also characterized by low-grade inflammation, where changes in the gut microbiota controls metabolic endotoxemia, which has been demonstrated to control the inflammatory tone (314). CVID patients with enteropathy have been found to have exceedingly low levels of gastrointestinal mucosal IgA, which appears to allow for specific pathobionts to induce inflammation, thereby diverting molecular pathways from lipid metabolism towards processes inducing enteropathy (315). These findings illustrate a complex interaction between microbes in the gut, lipid metabolism, metabolites and inflammation which needs further investigation.

9.9.2 Modulating via the gut microbiota

In paper I, we tried out whether a short term antibiotic course inducing temporary alterations of the gut microbiota in CVID patients would be sufficient to modulate their immune response. We found that, despite achieving a significant reduction in gut bacterial diversity after the two-week long intervention with rifaximin, we were not able to identify significant changes in immune response parameters. This is not to say our hypothesis would not have been confirmed had we chosen a different study drug or extended the intervention period, but it taught us that an intervention drug must likely target some central 'key bacteria' to significantly and permanently modulate the immune system. With the limitation that rifaximin is a locally acting antibiotic in the gut, these results also suggest that short courses of oral antibiotics may not cause major long term overall disturbances to our gut microbiota.

9.9.3 Modulating via metabolic functions

In paper II, we explored the role of HDL on systemic inflammation as well as inflammatory and autoimmune complications in CVID. We discovered that apo A-1 and HDL levels were significantly lower in CVID patients than in healthy controls, a difference driven by the subgroup of patients that had non-infectious complications. Moreover, HDL levels correlated negatively with plasma levels of inflammatory markers CRP and sCD25. This suggested that low HDL was associated with systemic inflammation, which was in line with findings from other studies of diseases characterized by sterile inflammation. Through functional studies, we found reduced HDL cholesterol acceptor function in serum and decreased macrophage cholesterol efflux capacity to serum in CVID patients compared to healthy controls. CVID patients also had low mRNA expression of ABCA1 in PBMC. Overall, paper II identified both reduced HDL levels and impaired HDL function in CVID patients with inflammatory and/or autoimmune complications, suggesting that HDL dysfunction could be therapeutically targeted to reduce complications and sterile inflammation in CVID. In light of the increased morbidity and mortality that CVID patients with complications have compared to those with infections only, reducing non-infectious complications could elicit important clinical effects and increased survival in these patients.

9.9.4 Modulating via TMAO

In paper III, we collected blood samples, fecal samples and obtained food frequency questionnaires from CVID patients to investigate the interactions between diet, gut microbes, metabolites, autoimmunity and inflammation, and discovered increased levels of TMAO to be central. CVID patients' intake of meat, milk and dairy products were all within the expected range when compared to the reference population, thus not explaining their raised TMAO levels. However, CVID gut microbiota was abundant in *Gammaproteobacteria* when compared to controls, which correlated positively with elevated plasma levels of TMAO. Inflammatory cytokines TNF and IL-12 also correlated positively with TMAO levels, thus linking gut microbiome and the gut microbiota-dependent metabolite TMAO with systemic inflammation in CVID. Diet did not appear to have a substantial impact on TMAO concentration in CVID patients, however wine consumption associated with lower TMAO and intake of table sugar with higher TMAO concentrations. Hence, the abundance of *Gammaproteobacteria* in the gut appeared to play a larger role than diet alone in affecting the TMAO concentration. This could imply that gut microbiota composition determines the formation of important metabolites like TMA somewhat independently of dietary nutrients, however over time the dietary profile also shapes the composition of gut microbes, creating a causative chain. Paper III suggests that abundance of certain intestinal microbes can contribute to increased TMAO production, which negatively affects metabolic functions like RCT, thereby influencing or potentially even driving systemic inflammation in CVID. TMAO could thus be a therapeutic target to improve RCT and reduce inflammation in CVID patients.

10. Conclusions and future perspectives

10.1 Concluding remarks

With its chronic low-grade systemic inflammation and frequent occurrence of inflammatory and/or autoimmune manifestations in addition to immunodeficiency in the face of infections, CVID is an interesting disease model for investigating the crosstalk between the immune system, metabolism and gut microbes. We have identified a gut microbial dysbiosis in these patients which appears linked to systemic inflammation through the metabolite TMAO and altered lipid metabolism through reduced reverse cholesterol transport. However, a randomized controlled trial using rifaximin for two weeks to

alter gut microbial composition had no effect on systemic inflammation. More mechanistic studies are needed to validate our findings, but several potential targets for therapeutic interventions that could reduce inflammation and subsequently improve the patients' long term mortality risk have been discovered.

10.2 Future perspectives

To further examine the role of antibiotic alteration of gut microbiota with the aim to modulate the immune response, a different drug intervention than rifaximin or a longer intervention period would likely be needed. Azithromycin, which was considered as study drug for paper I, has previously shown effect on both macrophages and gut microbiota, thus might be a potent immune modulator. However, the ecological side-effects of Azithromycin make it less tempting to use in a study (316), and the effect on systemic inflammation could be unrelated to gut microbial changes as Azithromycin also acts systemically. Since there is currently no clear knowledge as to which intervention would influence the composition of gut microbial 'key' bacteria most, further research is needed to explore targeted gut microbial therapy. In the future we might be able to offer a more personalized medicine based on microbial composition (unique for each individual), genotype and clinical phenotype.

Based on the findings in paper II, a rational next step would be a randomized controlled trial assessing the effect of therapeutically improving HDL function in CVID patients. To achieve this, one could use apo A-1 mimetics which would increase serum cholesterol acceptor function for cholesterol from ABCA1 in RCT. Alternatively, CETP-inhibitors which reduce the rate of cholesteryl ester transfer from HDL to triglyceride rich lipoproteins and thus aid formation of large and slowly catabolized HDL particles (317), could be tried out. Adding statins to this intervention, thereby lowering LDL levels and potentially reducing levels of oxLDL entering macrophages to form foam cells, could be considered. However, for hitherto unknown reasons, monotherapy with CETP-inhibitors have demonstrated a larger increase in total cholesterol efflux capacity than dual therapy with CETP-inhibitors and statins, raising the possibility that statins actually reduce expression of ABCA1 and ABCG1 in macrophages secondary to reduced liver X-receptor (LXR) activity (317), thus potentially cancelling out the effect on foam cell formation.

Also, in paper II, reduced mRNA expression of ABCA1 transporters in PBMC was identified as a contributing factor to reduced RCT in CVID patients. Hence, as a proof of concept, it would be interesting to attempt gene editing of CVID macrophages to improve ABCA1 function and thereby potentially reduce systemic inflammation via RCT. Indeed, in a recent proof of principle study, CRISPR-Cas9 genome editing was used to achieve loss of ABCA1 expression in human pluripotent stem cell (hPSC)-derived macrophages, resulting in reduced cholesterol efflux from the $ABCA1^{-/-}$ macrophages and increased expression of proinflammatory cytokines (318). A similar set-up to achieve (hPSC)-derived macrophages with increased ABCA1 expression would allow for cholesterol efflux assays to identify and verify specific therapeutic targets, as well as evaluate *in vitro* consequences of an improved cholesterol efflux capacity, in CVID.

Our findings in paper III of elevated TMAO associated with dysbiosis of the gut microbiota and elevated levels of inflammatory markers in CVID could be further investigated by trying out a TMAO reducing diet. Meat, dairy products and eggs are the best known dietary nutrient precursors of TMA, thus a vegetarian diet, often also high in fiber, influencing the gut microbes to reduce metabolite conversion to TMA could be tried out in CVID patients with systemic inflammation. DMB, one of the

components in red wine, would be an interesting compound to incorporate into a clinical trial with the aim to lower TMAO levels and subsequently systemic inflammation. If such a translational research trial was to be successful, there is a high probability the results would get a warm welcome from many.

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12. Appendix

I

SCIENTIFIC REPORTS

OPEN

Rifaximin alters gut microbiota profile, but does not affect systemic inflammation - a randomized controlled trial in common variable immunodeficiency

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S. F. Jørgensen[1,2,3], M. E. Macpherson[1,2,3], T. Bjørnetrø[1,4], K. Holm[1,3,4], M. Kummen[1,4], A. Rashidi[1], A. E. Michelsen[1,3], T. Lekva[1], B. Halvorsen[1,3], M. Trøseid[1,2,3], T. E. Mollnes[5,6,7], R. K. Berge[8,9], A. Yndestad[1,3], T. Ueland[1,3], T. H. Karlsen[1,3,4], P. Aukrust[1,2,3], J. R. Hov[1,3,4] & B. Fevang[1,2]

Common variable immunodeficiency (CVID) patients have reduced gut microbial diversity compared to healthy controls. The reduced diversity is associated with gut leakage, increased systemic inflammation and ten "key" bacteria that capture the gut dysbiosis (dysbiosis index) in CVID. Rifaximin is a broad-spectrum non-absorbable antibiotic known to reduce gut leakage (lipopolysaccharides, LPS) in liver disease. In this study, we explored as a 'proof of concept' that altering gut microbial composition could reduce systemic inflammation, using CVID as a disease model. Forty adult CVID patients were randomized, (1:1) to twice-daily oral rifaximin 550 mg versus no treatment for 2 weeks in an open-label, single-centre study. Primary endpoints were reduction in plasma/serum levels of soluble (s) CD14, sCD25, sCD163, neopterin, CRP, TNF, LPS and selected cytokines measured at 0, 2 and 8 weeks. Secondary endpoint was changes in intra-individual bacterial diversity in stool samples. Rifaximin-use did not significantly change any of the inflammation or gut leakage markers, but decreased gut microbial diversity compared with no treatment ($p = 0.002$). Importantly, the gut bacteria in the CVID dysbiosis index were not changed by rifaximin. The results suggest that modulating gut microbiota by rifaximin is not the chosen intervention to affect systemic inflammation, at least not in CVID.

Over the last decade, numerous discoveries have highlighted the role of gut microbiota in critical processes to human health including digestion, absorption of nutrients, metabolism, growth, and immune responses[1]. Compositional and functional changes of the gut microbiome, referred to as dysbiosis, have emerged as an important contributor not only to intestinal diseases, but also systemic metabolic and inflammatory conditions such as obesity[2], diabetes[3], cardiac disease[4], HIV infection[5] and brain disorders[1]. Studies in mice have shown that bacteria and their products (e.g., lipopolysaccharide [LPS]) interact with the immune system both locally in the gut and systemically. The composition of the gut microbiota can also alter the balance between pro- and

[1]Research Institute of Internal Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital, Rikshospitalet, Norway. [2]Section of Clinical Immunology and Infectious Diseases, Department of Rheumatology, Dermatology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Norway. [3]Institute of Clinical Medicine, University of Oslo, Oslo, Norway. [4]Norwegian PSC Research Center, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway. [5]Research Laboratory, Nordland Hospital, Bodø, and Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, Tromsø, Norway. [6]Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway. [7]Department of Immunology, Oslo University Hospital, and University of Oslo, Oslo, Norway. [8]Department of Clinical Science, University of Bergen, Bergen, Norway. [9]Department of Heart Disease, Haukeland University Hospital, Bergen, Norway. J. R. Hov and B. Fevang contributed equally. Correspondence and requests for materials should be addressed to S.F.J. (email: s.f.jorgensen@medisin.uio.no)

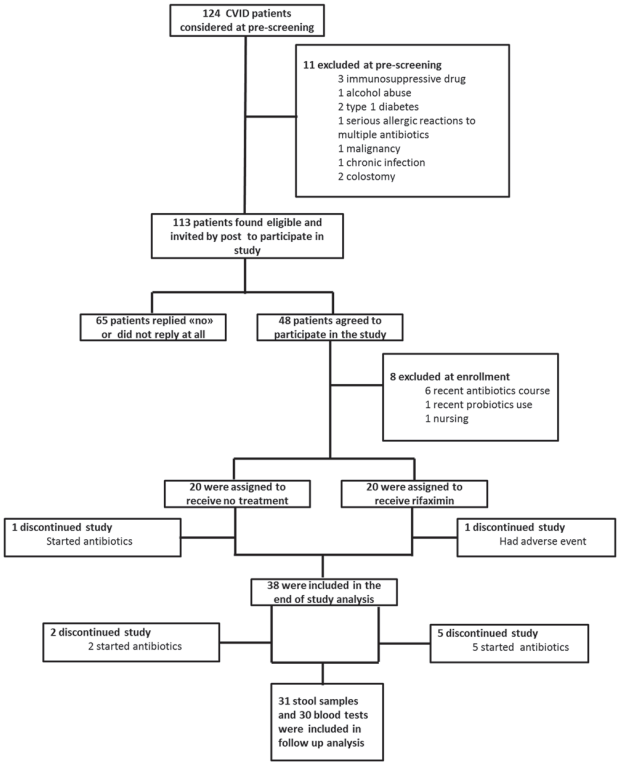

Figure 1. Trial Profile.

anti-inflammatory immune mediators and influence important metabolic pathways, sometimes being sufficient to modulate disease development and progression[6]. However, initial gut microbiota studies in human diseases have been cross-sectional and not able to establish causality, i.e. in principle the dysbiosis could be entirely secondary to disease.

We have recently shown that patients with common variable immunodeficiency (CVID) display a reduced microbial diversity[7]. CVID is the most common symptomatic primary immunodeficiency among adults, characterized by reduced levels of Immunoglobulin (Ig)G, IgM and/or IgA, and increased susceptibility to respiratory tract infections with capsulated bacteria[8]. Due to immune dysregulation, 74% of the patients also have one or more inflammatory and/or autoimmune complications, including enteropathy[9,10]. CVID patients have an underlying B-cell defect, but many patients also display T-cell dysfunction and evidence of systemic immune activation with elevated levels of inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-6, and soluble markers of monocyte/macrophage- (e.g., soluble [s]CD14) and T cell activation (i.e., sCD25)[11]. The reason for this persistent immune activation is not fully elucidated, but could at least partly reflect gut leakage mechanisms with a subsequent stimulation of innate immunity through interaction with Toll like receptors (TLRs) and related molecules[12]. We have previously reported increased plasma levels of LPS in CVID patients compared to healthy controls, negatively correlated with gut microbial diversity[7], suggesting that a less diverse gut microbiota is associated with increased bacterial translocation. We have therefore proposed that chronic inflammation in the gastrointestinal tract can cause a breach of the intestinal barrier with subsequent systemic immune activation, similar to what is observed in HIV infection[5].

In humans, interventional studies targeting the gut microbiota could provide evidence of a causal link between the intestine and human disease. In the present study, we used CVID as a disease model to therapeutically manipulate the gut microbiota to test our hypothesis that alteration of the microbiota affects systemic inflammation. The oral non-absorbable antibiotic rifaximin was chosen due to its bactericidal activity against a broad array of enteric pathogens and because it has been shown to reduce plasma levels of LPS in chronic liver disease with reduced risk of developing encephalopathy[13]. Importantly, there is negligible absorption of rifaximin from the gastrointestinal tract and thereby no direct antibacterial systemic effect which may bias the interpretation of a link between the gut and systemic inflammation[14].

Results

All adult CVID patients (n = 124) aged 18–74 years registered at the Section of Clinical Immunology and Infectious Diseases at Oslo University Hospital, Rikshospitalet, Oslo, Norway were pre-screened for inclusion. The Section functions as a national centre for diagnosis and treatment of primary immunodeficiency diseases for adults in Norway, and only a few (<10) patients are followed at other hospitals in Norway. Flowchart for recruitment of patients to the study is shown in Fig. 1. Out of 124 CVID patients, 11 were excluded at pre-screening

Baseline characteristics	All subjects (n = 40)	Rifaximin arm (n = 20)	No-treatment arm (n = 20)	p-value*
Age mean ± SD (range)	50 ± 12 (21–69)	47 ± 11 (21–63)	53 ± 12 (21–69)	0.07
Male, *n* (%)	15 (38)	8 (40)	7 (35)	1.00
Immunoglobulin therapy, *n (%)*:				0.06
s.c.[a]	30 (75)	14 (70)	16 (80)	
i.v.[b]	6 (15)	5 (25)	1 (5)	
s.c + i.v.	4 (10)	1 (5)	3 (15)	
Infection only, *n* (%)	8 (20)	7 (35)	1 (5)	0.04
Splenomegaly, n (%)	16 (40)	7 (35)	9 (45)	0.75
Organ-specific autoimmunity	8 (20)	5 (25)	3 (15)	0.70
Autoimmune cytopenia, n (%)	8 (20)	2 (10)	6 (30)	0.24
Enteropathy, n (%)	13 (33)	8 (40)	5 (25)	0.50
Lymphoid hyperplasia, n (%)	23 (56)	9 (45)	14 (70)	0.20
Granulomas, n (%)	6 (15)	3 (15)	3 (15)	1.00
Lymphocytic interstitial pneumonitis, n (%)	1 (2.5)	0	1(95)	1.00
Nodular regenerative hyperplasia, n (%)	1 (2.5)	0	1(95)	1.00
Overall non-infectious complications, n (%)	32 (80)	13 (65)	19 (95)	0.04

Table 1. Baseline characteristics of CVID patients in the Rifaximin study. [a]s.c., subcutaneously; [b]i.v., intravenously. *P value for rifaximin arm compared to no-treatment arm

Marker	Group	Baseline[a] (n = 40)	2 weeks[a] (n = 38)	8 weeks[a] (n = 30)	P value interaction[b]
CRP mg/L	No Int	3.5 (2.1–7.1)	4.7 (1.0–7.9)	3.2 (2.5–6.9)	0.029
	Rif	1.9 (0.86–4.80)	2.20 (1.2–4.8)	2.8 (1.9–5.4)*	
sCD14 ng/mL	No Int	3949 (3162–4456)	4032 (3290–4630)	3826 (2978–4459)	0.555
	Rif	3378 (3072–4219)	3639 (3241–4414)	3552 (2921–4485)	
LPS pg/ml	No Int	83 (78–91)	86 (78–92)	87 (78–94)	0.955
	Rif	87 (80–96)	90 (78–98)	90 (83–102)	
sCD25 ng/mL	No Int	1.44 (0.71–2.10)	1.54 (0.74–1.89)	1.33 (0.80–1.86)	0.636
	Rif	0.65 (0.51–1.43)	0.63 (0.54–1.18)	0.99 (0.44–1.55)	
sCD163 ng/mL	No Int	1699 (1350–2001)	1677 (984–1930)	1536 (1194–1940)	0.320
	Rif	971 (647–1353)	902 (749–1310)	1151 (793–1549)	
Neopterin nmol/L	No Int	11.18 (9.40–21.60)	13.17 (8.32–24.31)	10.37 (8.11–21.90)	0.058
	Rif	9.02 (6.71–14.65)	7.79 (6.37–17.24)	11.43 (6.66–21.78)	

Table 2. Soluble markers of inflammation in plasma in the "rifaximin" (Rif, n = 20) and in the "no intervention" group (No Int, n = 20). [a]Data are given in median (25–75 percentile). [b]The p value reflects the interaction between time and group from UNIANOVA. *$P < 0.05$ *vs*. baseline.

due to immunosuppressive drugs (n = 3), alcohol abuse (n = 1), type 1 diabetes (n = 2), serious multiple adverse reactions to antibiotics (n = 1), malignancy (n = 1), chronic herpes simplex virus infection (1) and colostomy (n = 2). Of the 113 CVID patients found eligible, 48 volunteered to participate in the study, and of these eight were not included due to recent antibiotic course (n = 6), recent probiotic use (n = 1) and lactation (n = 1). In the end 40 patients, aged 21–69 years (63% women), with CVID were enrolled in this study and randomized to rifaximin 550 mg bd versus no treatment for 14 days.

Baseline patient characteristics are presented in Table 1. Except for a lower frequency of patients with 'non-infectious complications' in the rifaximin group (P = 0.04), there were no significant differences between the rifaximin and the no intervention group at baseline (Table 1).

Effect of rifaximin on markers of systemic inflammation and gut leakage. Overall there was no effect of rifaximin use on markers of systemic inflammation or markers of gut leakage (i.e., LPS) (uniANOVA), except for CRP (Table 2). The significant change in CRP was driven by an increase in CRP from baseline to week 8 in the rifaximin group (Wilcoxon, P = 0.015) (Table 2). Furthermore, there was no effect of rifaximin treatment on mRNA levels of selected pro- and anti-inflammatory cytokines and chemokines in PBMC (i.e., IL-6, IL-8/CXCL8, IL-10, IL-12, TNF, TGF-β, MIG/CXCL9, IP-10/CXCL10; Supplementary Table S1). Also, the distribution of B and T cell subpopulations, as measured by 10 readouts, remained unchanged by rifaximin use, except for minor changes in the naive $CD4^+$ T cells reflecting a decrease in the no intervention group (Supplementary Table S2).

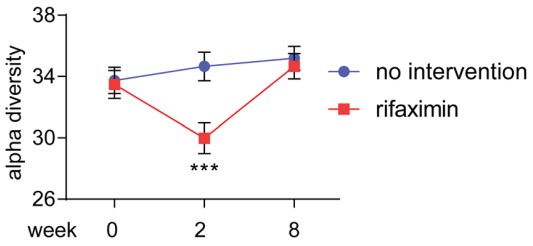

Figure 2. Comparing alpha diversity (phylogenetic diversity [Faith's PD]) in stool samples before (week 0), after (week 2) and at follow up (week 8) for a 2-week rifaximin course versus no treatment. ***P < 0.001 (UNIANOVA, baseline values at week 0 is corrected for values at week −14 [*baseline 2*]).

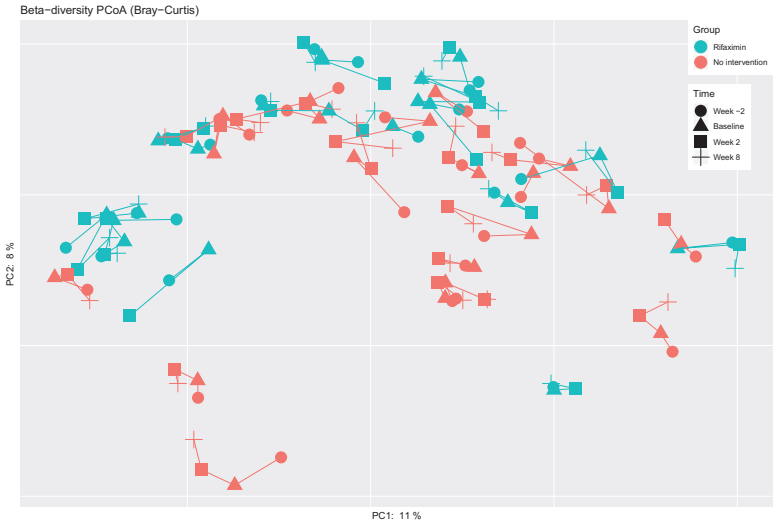

Figure 3. Beta diversity plot with each patient's samples at four time points for the rifaximin and no intervention group.

Effect of rifaximin on gut microbiota composition. In contrast to the lack of effect on markers of systemic inflammation, rifaximin treatment was associated with a significant change in microbial alpha diversity (Faith's PD; UNIANOVA P = 0.0002, Fig. 2). Thus, there was a significant decrease in microbial alpha diversity (Faith's PD: beta = −0.26, P = 0.002) after the first two weeks of the study, followed by a return to baseline six weeks after rifaximin was terminated (beta: 0.02 linear regression [baseline to week 8] P = 0.82, Fig. 2). Other measures of alpha diversity such as Chao1 (P = 0.03), Shannon index (P = 0.002) and observed OTUs (P = 0.002) were also significantly changed by rifaximin, whereas Simpson index (P = 0.10) was not (linear regression data for baseline to week 2 and baseline to week 8 are given in Supplementary Table S3). Global differences in the gut microbiota measured by beta diversity (i.e., to which degree different taxa are shared between individuals or groups of individuals) are illustrated in Fig. 3 at four time points for the two groups. The beta diversity (as measured by Bray-Curtis distances between paired samples at week 0 and week 2) was significantly higher in the rifaximin group compared to the no intervention group (Mann-Whitney P = 0.014, median 0.34 (IQR 0.32–0.40) and 0.27 (IQR 0.24–0.35), respectively, Fig. 4), but not between week 0 and week 8 (P = 0.46, median 0.32 (IQR 0.28–0.36) and 0.30 (IQR 0.25–0.38), respectively).

Sixteen bacterial taxa, at different taxonomic levels, were significantly changed by rifaximin use (Table 3, Fig. 5). Also, the direction of change (expressed as beta values from the linear regression analysis) for the different taxa from baseline and week 2 and 8 are shown in Table 3. None of the ten main bacteria previously shown to differentiate CVID patients and healthy controls, the *CVID specific dysbiosis index*[7], were significantly changed by rifaximin use (Supplementary Fig. S1), thus the *CVID dysbiosis index* was unchanged by rifaximin (Supplementary Fig. S2). Consistent with the data from our previous publication[7], the dysbiosis index in the present study correlated with the microbial diversity (Faith's PD) (rho = −0.42, *P* = 0.007, at *week 0* [n = 40]), suggesting that the abundance of these ten bacteria captures the microbial diversity in CVID also in this study.

Adverse effects during the study. Out of 20 patients in the rifaximin group, one patient experienced an allergic reaction classified as a moderate adverse event. The patient stopped rifaximin immediately and was treated with betamethasone and the symptoms resolved after treatment. Another eight adverse events [acne

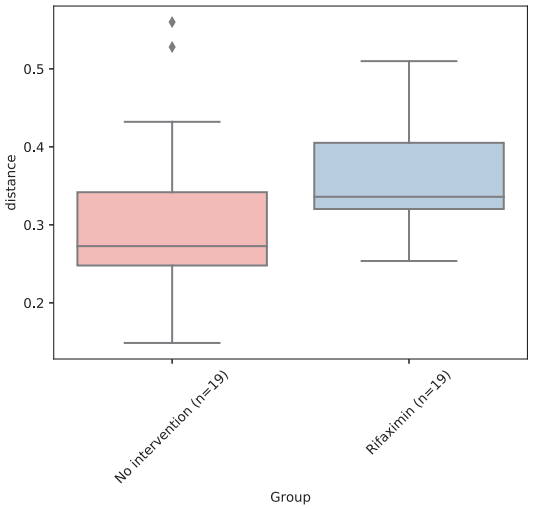

Figure 4. Bray-Curtis distances between (paired) samples at baseline and week 2 in the rifaximin and no intervention group.

Phylum	Class	Order	Family	*Genus*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*uncultured*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcaceae UCG-014*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcaceae UCG-002*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillibacter*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Anaerotruncus*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Lactonifactor*
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Anaerostipes*
Firmicutes	Clostridia	Clostridiales	Family XIII	*Family XIII UCG-001*
Firmicutes	Clostridia	Clostridiales	Family XI	*Ezakiella*
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteroidetes	Bacteroidia			
Bacteroidetes				

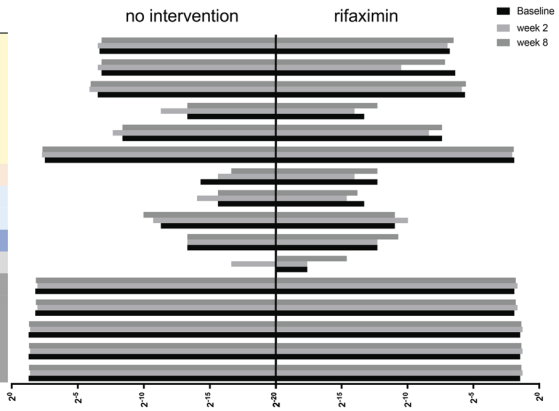

Figure 5. On the left are the names of the 16 taxa significantly changed by rifaximin (UNIANOVA) and on the right is the corresponding mean relative abundance of each specific taxa expressed (on a log2 scale) for the no intervention and the rifaximin group at baseline, week 2 and week 8. The colors to the left in the figure illustrate which taxa are in the same taxonomic family.

(n = 1), nausea (n = 1), dyspepsia (n = 1), bad taste in the mouth (n = 2), abdominal pain (n = 1), loose stools (n = 1) and urticaria (n = 1)] were reported at the end of study visit.

Discussion

In this 'proof of concept' study, rifaximin had no significant effect on markers of systemic inflammation or LPS as a marker of gut leakage in CVID. Microbial diversity in the gut and the abundance of multiple bacteria, however, were changed by rifaximin, but returned to baseline after the intervention. In contrast, the previously identified CVID specific dysbiosis index, which has been associated with increased markers of systemic inflammation and gut leakage, was not affected. It could be speculated that this causes the lack of anti-inflammatory effects of rifaximin in these patients.

Multiple studies of rifaximin have been performed for different conditions[15,16]. However, the majority of these studies have been performed in liver diseases, where rifaximin has been convincingly shown to reduce hepatic encephalopathy[17]. Studies in cirrhotic patients with minimal encephalopathy, alcoholic cirrhosis and fatty liver disease have all shown reductions in LPS levels after rifaximin treatment[13,18,19], also after only 2 weeks of treatment as in the present study[20]. However, the mechanisms leading to gut leakage in severe liver failure and CVID may differ. In the only previous study in immunodeficient patients (i.e. HIV-infected patients), rifaximin showed very limited effects on neither microbial translocation nor T cell activation compared with placebo in 65 HIV patients, but unfortunately, this study presented no data on gut microbiota composition[16].

The lack of effects observed on the primary endpoints cannot be discussed independently from the gut microbiota profile, where antibiotics would be expected to act. In the present study, a transient reduction of alpha

Taxa	Group	Baseline[a] (n = 40)	2 weeks[a] (n = 38)	8 weeks[a] (n = 31)	P value interaction[b]	Beta2[c] (week 2)	Beta2[c] (week 8)
Bacteroidetes	No Int	0.415 (0.388–0.441)	0.385 (0.357–0.413)	0.413 (0.384–0.443)	0.012	0.28*	0.08
	Rif	0.366 (0.340–0.393)	0.413 (0.385–0.441)	0.382 (0.348–0.415)			
Bacteroidetes.Bacteroidia	No Int	0.414 (0.388–0.441)	0.385 (0.357–0.412)	0.413 (0.383–0.443)	0.012	0.28*	0.08
	Rif	0.366 (0.340–0.393)	0.413 (0.385–0.440)	0.382 (0.348–0.415)			
Bacteroidetes.Bacteroidia.Bacteroidales	No Int	0.414 (0.388–0.441)	0.385 (0.357–0.412)	0.413 (0.383–0.443)	0.012	0.28*	0.08
	Rif	0.366 (0.340–0.393)	0.413 (0.385–0.440)	0.382 (0.348–0.415)			
Bacteroidetes.Bacteroidia.Bacteroidales. Bacteroidaceae	No Int	0.292 (0.267–0.316)	0.265 (0.239–0.290)	0.283 (0.255–0.310)	0.009	0.25**	0.12
	Rif	0.269 (0.245–0.294)	0.315 (0.289–0.340)	0.282 (0.251–0.312)			
Bacteroidetes.Bacteroidia.Bacteroidales. Bacteroidaceae. Bacteroides	No Int	0.292 (0.267–0.316)	0.265 (0.239–0.290)	0.283 (0.255–0.310)	0.009	0.25**	0.12
	Rif	0.269 (0.245–0.294)	0.315 (0.289–0.340)	0.282 (0.251–0.312)			
Firmicutes.Clostridia.Clostridiales. Family XI.Ezakiella	No Int	$-7.8E^{-20}$ ($-1.3E^{-5}$–$1.3E^{-5}$)	$1.1E^{-5}$ ($-2.2E^{-6}$–$2.4E^{-5}$)	$-4.3E^{-7}$ ($-1.5E^{-5}$–$1.4E^{-5}$)	0.029	−0.09	0.43
	Rif	$5.3E^{-6}$ ($-7.5\ E^{-6}$–$1.8E^{-5}$)	$5.0E^{-6}$ ($-8.3E^{-6}$–$1.8\ E^{-5}$)	$3.4E^{-5}$ ($1.8E^{-5}$–$5.0E^{-5}$)			
Firmicutes.Clostridia.Clostridiales. Family XIII.Family XIII UCG-001	No Int	$1.1E^{-4}$ ($-3.9E^{-6}$–$2.3E^{-4}$)	$9.7E^{-5}$ ($-2.2E^{-5}$–$2.2E^{-4}$)	$1.4E^{-4}$ ($7.5E^{-6}$–$2.6E^{-4}$)	0.028	0.04	0.15
	Rif	$2.3E^{-4}$ ($1.2E^{-4}$–$3.5E^{-4}$)	$2.5E^{-4}$ ($1.3E^{-4}$–$3.7E^{-4}$)	0.001 ($4.2E^{-4}$–0.001)			
Firmicutes.Clostridia.Clostridiales. Lachnospiraceae. Anaerostipes	No Int	$3.9E^{-4}$ ($-3.7E^{-7}$–0.001)	0.001 ($2.5E^{-4}$–0.001)	0.001 (0.001–0.001)	0.031	0.21	−0.25
	Rif	$4.9E^{-4}$ ($1.0E^{-4}$–0.001)	0.001 (0.001–0.002)	$4.6E^{-4}$ ($-3.5E^{-5}$–0.001)			
Firmicutes.Clostridia.Clostridiales. Lachnospiraceae. Lactonifactor	No Int	$2.6E^{-5}$ ($-9.7E^{-6}$–$6.2E^{-5}$)	$5.9E^{-5}$ ($2.2E^{-5}$–$9.6E^{-5}$)	$1.2E^{-5}$ ($-2.9E^{-5}$–$5.2E^{-5}$)	0.0003	−0.32*	0.37*
	Rif	$1.0E^{-4}$ ($6.4E^{-5}$–$1.4E^{-4}$)	$4.2E^{-5}$ ($5.0E^{-6}$–$8.0E^{-5}$)	$1.2\ E^{-4}$ ($7.0E^{-5}$–$1.6\ E^{-4}$)			
Firmicutes.Clostridia.Clostridiales. Peptococcaceae	No Int	$5.3E^{-5}$ ($-1.6E^{-5}$–$1.2E^{-4}$)	$2.5E^{-5}$ ($-4.7E^{-5}$–$9.6E^{-5}$)	$3.1E^{-6}$ ($-7.4E^{-5}$–$8.0E^{-5}$)	0.023	−0.15	0.08
	Rif	$2.6E^{-4}$ ($1.9\ E^{-4}$–$3.3E^{-4}$)	$5.1E^{-5}$ (-2–$0E^{-5}$–$1.2\ E^{-4}$)	$1.8\ E^{-4}$ ($9.6\ E^{-5}$–$2.7\ E^{-4}$)			
Firmicutes.Clostridia.Clostridiales. Ruminococcaceae	No Int	0.003 (0.002–0.005)	0.005 (0.004–0.007)	0.003 (0.001–0.005)	0.012	−0.19	−0.01
	Rif	0.007 (0.005–0.009)	0.003 (0.001–0.005)	0.006 (0.004–0.008)			
Firmicutes.Clostridia.Clostridiales. Ruminococcaceae. Anaerotruncus	No Int	0.003 (0.002–0.005)	0.005 (0.004–0.007)	0.003 (0.001–0.005)	0.013	−0.28	0.12
	Rif	0.007 (0.005–0.009)	0.003 (0.001–0.005)	0.006 (0.004–0.008)			
Firmicutes.Clostridia.Clostridiales. Ruminococcaceae. Oscillibacter	No Int	$1.5E^{-4}$ ($6.6E^{-6}$–$2.9E^{-4}$)	$4.0E^{-4}$ ($2.6E^{-4}$–0.001)	$1.2E^{-4}$ ($-3.6E^{-5}$–$2.8E^{-4}$)	0.028	−0.22	0.16
	Rif	$1.3E^{-4}$ ($-1.4E^{-5}$–$2.7E^{-4}$)	$5.4E^{-5}$ ($-9.2E^{-5}$–$2.0E^{-4}$)	$1.9E^{-4}$ ($8.2E^{-6}$–$3.6E^{-4}$)			
Firmicutes.Clostridia.Clostridiales. Ruminococcaceae. Ruminococcaceae UCG-002	No Int	0.011 (0.007–0.015)	0.017 (0.013–0.021)	0.015 (0.010–0.019)	0.014	−0.28*	−0.22
	Rif	0.025 (0.021–0.029)	0.017 (0.013–0.021)	0.019 (0.014–0.025)			
Firmicutes.Clostridia.Clostridiales. Ruminococcaceae. Ruminococcaceae UCG-014	No Int	0.009 (0.005–0.013)	0.011 (0.006–0.016)	0.008 (0.003–0.013)	0.018	−0.41**	−0.06
	Rif	0.012 (0.008–0.018)	0.001 (−0.004–0.006)	0.007 (0.002–0.013)			
Firmicutes.Clostridia.Clostridiales. Ruminococcaceae. uncultured	No Int	0.011 (0.009–0.013)	0.011 (0.009–0.013)	0.008 (0.005–0.010)	0.005	−0.08	0.28*
	Rif	0.009 (0.007–0.011)	0.008 (0.001–0.006)	0.012 (0.010–0.015)			

Table 3. Taxa that are significantly changed by rifaximin in stool samples from CVID patients. [a]Data are given in mean (95% confidence interval). [b]The p value reflects the interaction between time and group from UNIANOVA. [c]The beta coefficient and corresponding asterisk reflects the effect of rifaximin from linear regression with taxa at the indicated time point as dependent and treatment and baseline taxa as independent in a forced model. *$P < 0.05$, **$P < 0.01$.

diversity was observed together with changes in multiple taxa, including reduction of several Ruminococcaceae and increase of *Bacteroides*. In contrast, in a study by Ponziani *et al*. including 20 patients with different gastrointestinal and liver diseases, using 16S rRNA-based gut microbiota profiling, rifaximin increased *Lactobacilli* and decreased *Roseburia*, *Haemophilus*, *Veilonella* and *Streptococcus*. Furthermore, in two other studies including patients with liver cirrhosis, rifaximin use reduced *Veillonellaceae*[21], and increased *Eubacteriaceae*[13,21]. None of these changes overlap with the results of the current study, but these studies did not report measures of diversity. One important cause of non-overlapping results could be that the effect of rifaximin depends on the initial bacterial composition, which probably differs between CVID and intestinal or hepatic pathologies. Random variation or methodological differences could also in part explain the lack of similarities. In addition to phenotype, comparability between microbiota studies can be influenced by many factors like different baseline lifestyle, diet, geography, host genetics, as well as lab methods including choice of PCR primers and DNA extraction[22]. Since all samples in the present study were handled exactly the same way and were sequenced in the same MiSeq run, the influence of methodological variation on the results should be minimal.

Several factors could potentially explain the failure of rifaximin to influence the primary end-point. In a previous study from our group, a CVID specific dysbiosis index calculated from key bacteria separated patients from controls[7]. This CVID specific dysbiosis index has been found to correlate with circulating markers of systemic inflammation and gut leakage, and inversely to alpha diversity. Although rifaximin reduced alpha diversity in the present study, there was no significant impact on the bacteria constituting the CVID specific dysbiosis index, and it could be hypothesized that rifaximin not targeting the putative culprits could explain the lack of effect on systemic inflammation. Of particular interest would be to increase *Bifidobacterium*, which is reduced in CVID[7], and

that has been shown to improve gut barrier and reduce systemic LPS and inflammation in mice[23]. Unfortunately, *Bifidobacterium* was not increased by rifaximin in our study. Thus, although rifaximin altered alpha diversity, the potential "inflammatory" bacteria were not changed. However, additional studies, including intervention studies, are needed to causally link gut dysbiosis and systemic inflammation in CVID.

The strengths of the present study were a randomized controlled study design, which although open-label had blinded primary end-points. Secondly, we included both data of gut microbiota using metagenomic techniques *and* systemic inflammation, thereby expanding previous rifaximin studies in humans, irrespective of phenotype. The length of the intervention was an important possible limitation of the present study. Our aim was to administrate a sufficient dose and length of therapy to get the desired effect on the microbiota and at the same time reducing the likelihood of adverse events. Although the intervention was sufficient to induce a significant change in alpha- and beta diversity, and in 16 taxa, we cannot exclude that a longer intervention could result in more subtle changes to the gut microbial composition and levels of systemic inflammation than we were able to detect with a 2-week intervention. This important issue should be investigated in forthcoming studies.

The randomization of CVID patients was not stratified, and by chance, the patients randomized to rifaximin treatment had a smaller proportion of patients with non-infectious complications compared to the no intervention group. This could imply that the *rifaximin* group was healthier than the *no intervention* group, which may have influenced the results. In addition, the lack of placebo excluded the use of GI symptom-questionnaire for evaluation of symptomatic relief with rifaximin use.

Even though the alterations in the gut microbiota induced by rifaximin did not change systemic inflammation in CVID, other interventions such as probiotics, prebiotics or fecal transplantation are likely to have different impacts on the gut microbiota and, theoretically, systemic inflammation. There is therefore still an unused potential to manipulate the gut microbiota in CVID preferably targeting the ten bacteria making up the CVID dysbiosis index. The results herein may be of relevance to other diseases with systemic inflammation, and suggest that rifaximin is not the chosen intervention to affect systemic inflammation, although more data is needed to support this finding. In the future, other interventions should be trialled in patients with chronic systemic inflammation, to further explore the 'proof of concept' that manipulation of the gut microbiota can influence systemic inflammation also in humans.

Methods

Study design and participants. We did this randomized, open, prospective, single-center, clinical trial at Oslo University Hospital, Rikshospitalet, Oslo, Norway between Oct 8 2013, and Oct 20, 2014. All CVID patients aged 18 to 74 registered at our clinic were initially pre-screened for eligibility. CVID was defined as decreased serum levels of IgG, IgA and/or IgM by at least two standard deviations below the mean for age, and exclusion of other causes of hypogammaglobulinemia[24,25]. Exclusion criteria were: antibiotics in the last 12 weeks, history of allergic reaction to rifaximin, malignancy, impaired kidney function, pregnancy or lactation, on-going infection, use of probiotics in the last 6 months, immunosuppressive drugs, comorbidity that may influence with the patient's safety or compromise the study results (e.g., cardiovascular disorders, alcoholism, psychiatric disease, HIV infection), and polypharmacy (patient with an extensive medication list i.e. ten drugs or more). The study was approved by the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway (number 2013/1037) and the Norwegian Medicines Agency (EudraCT number: 2013-000883-27), and conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants. The trial is registered with clinicaltrials.gov, number NCT01946906 (registration date 20.09.2013).

Randomization and masking. The CVID patients were randomized into two groups (*rifaximin* and *no intervention*) using computer-generated randomization (1:1) to receive rifaximin (Norgine, Alanno Scalo, Italy) or no treatment. There was no placebo drug. A statistician, not otherwise involved in the trial, generated the random allocation sequence. A randomization list with a combination of permuted blocks of four (n = 10) and blocks of two (n = 5) was generated. The sequence was concealed by using sealed envelopes containing the group allocation until a decision to enrol a patient was made. Due to the estimated low number of participants in each arm, no stratification between the two treatment arms was performed. The persons conducting the laboratory work related to primary and secondary endpoints were blinded to which samples were receiving the active treatment.

Procedures. The remaining patients after pre-screening were invited to participate in the study (see Results). The trial involved three outpatient visits for all patients. The patients' eligibility to participate in the study was again evaluated at the first visit based on medical history, physical examination and blood tests (hematology, electrolytes, kidney function tests, liver function tests, human chorion-gonadotropin in fertile women). The patients enrolled in the study gave baseline blood test and fecal samples at visit 1. However, 14 days before this visit a fecal sample was sent by post, leaving two baseline stool assessments (*baseline* refers to fecal samples taken at visit 1 whereas *baseline 2* refers to fecal samples taken 14 days prior to visit 1). Participants allocated to *rifaximin* were asked to take rifaximin 550 mg every 12 hours for 2 weeks, whereas the participants receiving no intervention were followed in the same way as the group receiving treatment. A written material containing information about potential adverse effects of rifaximin and contact details to the investigators in case of moderate to serious adverse events was handed out to the participants receiving rifaximin at visit 1.

At the second visit, *the end of study visit*, the participants brought a fecal sample and had blood tests taken. Assessment of compliance was carried out by collecting the compliance diary and counting remaining tablets in the box for the participants in the *rifaximin* group. Furthermore, the patients were assessed for the development of any new symptoms (blood tests and physical examination) or treatment-related adverse events. The third visit

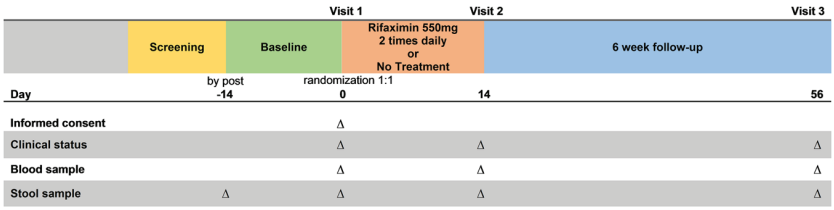

Figure 6. The study procedure.

at 8 weeks, *follow up visit*, included all the activities of the first and second visit except assessment of compliance. The study procedure is outlined in Fig. 6.

Primary and secondary outcome. Study endpoints were assessed at 2 weeks (i.e., study end) and at 8 weeks (i.e. follow up). The primary endpoint was time-dependent changes in inflammatory and anti-inflammatory mediators and markers of gut leakage in plasma, serum and leukocytes. This included sCD14, sCD25, sCD163, neopterin, CRP, LPS and selected cytokines. The key secondary end points were changes in gut microbial diversity abundance of specific bacteria as evaluated by 16S rRNA sequencing.

Stool collection and analysis. All stool samples were collected by the participants at home with a standardised collection device[26] and transferred to Stool Collection Tubes with Stool DNA Stabilizer (Stratec Biomedical, Birkenfeld, Germany)[27]. The first sample (2 weeks before visit 1 [*baseline 2*]) was returned by post together with a questionnaire reporting the sampling time, recent antibiotic and/or probiotic use, and medication. At visit 1 (*baseline*), 2 and 3, the stool samples (collected at home within the last 24 hours) were immediately stored at −20 °C, according to the manufacturer's recommendation, until DNA extraction. Bacterial DNA was extracted using the *PSP® Spin Stool DNA Plus Kit* (Stratec) and subjected to high-throughput sequencing of the 16S ribosomal RNA gene with dual-indexed barcodes according to an established protocol, followed by sequencing on an Illumina MiSeq (Supplementary Methods)[28].

Differences in microbial composition between groups were evaluated by measures of alpha diversity (within sample bacterial diversity), beta diversity (between sample bacterial diversity), dysbiosis index and the relative abundance of individual taxa (Supplementary Methods).

CVID dysbiosis index. We have previously published a specific CVID dysbiosis index that consists of the relative abundance of ten bacteria at different taxonomic levels, which captured the change in alpha diversity between CVID and healthy controls[7]. We, therefore, calculated the dysbiosis index for all samples at all four time points using the formula: Log_e [(sum of the relative abundances of bacteria increased in CVID)/(sum of the relative abundances of bacteria reduced in CVID)]. Bacteria which are increased in CVID include *Bacilli*, *Dorea*, *Roseburia*, *Gammaproteobacteria* and bacteria which are reduced in CVID include *Bifidobacterium*, *Odoribacteracea*, *Christensenellaceae*, *Blautia*, *Sutterella*, *Desulfovibrionace*[7].

Blood sampling protocol. Peripheral venous blood was drawn into sterile blood collection tubes without any additives (serum) or with EDTA as anticoagulant (plasma). The tubes were immediately immersed in melting ice, centrifuged within 15 minutes at 2000*g* for 20 minutes to obtain platelet-poor plasma or were allowed to clot at room temperature before centrifugation at 1000 *g* for 10 minutes (serum). Plasma and serum were stored at −80 °C in aliquots and thawed only once. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood isolated by gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway) within 1 hour after blood collection and PBMC pellets were immediately stored at −80 °C until mRNA analyses (Supplementary Methods).

Measurements of inflammatory and gut leakage markers. Serum levels of sCD14, neopterin, sCD163 and sCD25 were quantified in duplicate by enzyme immunoassays obtained from R&D Systems (Minneapolis, MN). LPS was analysed by Limulus Amebocyte Lysate chromogenic assay (Lonza, Walkersville, MD) according to the manufacturer's instructions, with the following modifications: Samples were diluted 5-fold to avoid interference with background colour, and preheated to 67 °C for 12 minutes prior to analysis to dissolve immune complexes. Serum levels of CRP were sampled together with safety blood samples via the routine hospital laboratory. B- and T-cell subpopulations were analysed by flow cytometry (Supplementary Methods).

Subgroup analysis. Clinical subgroups were classified as "Infection only" or "Complications" based on previously defined criteria[9] with one modification; CVID enteropathy was defined as persistent diarrhea after exclusion of gastrointestinal infection.

Statistical analysis. The primary objective of the statistical analysis was to compare the effect of rifaximin therapy with no therapy on gut leakage and inflammatory markers. This was an explorative study and we found that strict samples size calculation was inappropriate. However, we estimated an approximate 50% difference in changes in inflammatory markers between the two groups, and with 25 patients in each group we would have 95% power to detect a 50% (0.75) difference with an alpha of 0.05. Since CVID is a rare disease, we were only able

to recruit 20 patients in each group. Univariate Repeated measures ANOVA (UNIANOVA) was used to assess the effect of treatment focusing on the interaction between time and treatment group for the different markers, followed by paired samples t-test or Wilcoxon's Rank-Sum test for paired data if significant. Skewed variables were log transformed prior to regression analysis.

Differences in microbial composition between groups were evaluated by measures of alpha diversity (within individual bacterial richness), dysbiosis index and the abundance of individual taxa. Also, for alpha diversity, dysbiosis index and taxa UNIANOVA was used '*a priori*'. For the significant taxa, linear regression was performed with taxa at 2 or 8 weeks as dependent and baseline taxa and treatment group as independent variables in a forced model. When not specified otherwise, statistical analyses were performed with SPSS (IBM, Armonk, NY, USA). *P* values were two-sided and considered significant when <0.05.

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available due to Norwegian legislation about general data protection regulation, but are available from the corresponding author on reasonable request.

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Author Contributions
S.F.J., J.R.H., B.F., P.A. and T.H.K. had the initial idea for the study. S.F.J. developed the protocol and was responsible for the everyday management of the trial. S.F.J., M.E.M., P.A. and B.F. recruited patients. S.F.J., M.E.M., A.R., P.A. and B.F. obtained human samples and clinical data collection. S.F.J., M.E.M., T.B., K.H., M.K., A.E.M., T.L., B.H., M.T., T.E.M., R.K.B., A.Y., J.H.R., B.F. analyzed data and S.F.J., M.E.M., K.H., T.U., M.K., J.R.H. performed statistical analysis. P.A., T.H.K., J.R.H. and B.F. supervised the project. S.F.J., M.E.M., P.A., J.R.H. and B.F. drafted the manuscript. All authors revised the manuscript for critical content and approved the final version.

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Supplementary Information

Rifaximin alters gut microbiota profile, but does not affect systemic inflammation - a randomized controlled trial in common variable immunodeficiency

SF Jørgensen[1,2,3*], ME Macpherson[1,2,3], T Bjørnetrø[1,4], K Holm[1,3,4], M Kummen[1,4], A Rashidi[1], AE Michelsen[1,3], T Lekva [1], B Halvorsen[1,3], M Trøseid[1,2,3], TE Mollnes [5,6,7], RK Berge[8,9], A Yndestad [1,3], T Ueland[1,3], TH Karlsen[1,3,4],P Aukrust[1,2,3], JR Hov[1,3,4 §] and B Fevang[1,2 §].

1. Research Institute of Internal Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital, Rikshospitalet, Norway.
2. Section of Clinical Immunology and Infectious Diseases, Department of Rheumatology, Dermatology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Norway.
3. Institute of Clinical Medicine, University of Oslo, Norway.
4. Norwegian PSC Research Center, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital Rikshospitalet, Norway.
5. Research Laboratory, Nordland Hospital, Bodø, and Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, Norway.
6. Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Norway.
7. Department of Immunology, Oslo University Hospital, and University of Oslo, Norway.
8. Department of Clinical Science, University of Bergen, Norway.
9. Department of Heart Disease, Haukeland University Hospital,Norway.

[§]contributed equally

Supplementary tables:

Table S1. mRNA expression levels of cytokines and chemokines in PBMC of patients completing all study visits in the "rifaximin" (Rif, n=13) and in the "no intervention" group (No Int, n=15)

Cytokine	Group	Baseline	2 weeks	8 weeks	P value interaction
IL-10	No Int	4.9 (3.4-8.2)	4.4 (3.3-5.7)	5.4 (3.4-8.1)	0.40
	Rif	3.8 (3.4-5.4)	4.8 (3.3-5.3)	3.8 (2.3-4.5)	
IL-12	No Int	3.2 (1.6-5.4)	2.8 (1.8-3.6)	4.0 (2.5-7.1)	0.79
	Rif	2.7 (2.4-3.2)	3.0 (2.2-3.2)	2.7 (2.1-3.6)	
IL-6	No Int	26.6 (10.0-42.5)	20.6 (11.4-51.4)	27.8 (18.2-157.4)	0.27
	Rif	23.3 (14.9-33.9)	27.1 (17.4-36.6)	22.0 (15.8-32.0)	
TGF-beta	No Int	2.2 (2.0-2.9)	2.0 (1.5-3.0)	2.3 (1.6-3.3)	0.61
	Rif	2.8 (2.5-3.1)	2.5 (2.2-2.9)	2.4 (2.1-2.7)	
TNF	No Int	26.4 (17.7-118.0)	56.7 (14.5-85.5)	56.5 (13.1-114.9)	0.57
	Rif	33.1 (22.6-95.8)	39.4 (16.4-57.7)	36.5 (13.7-84.0)	
IL-8	No Int	24.3 (9.4-54.5)	25.5 (10.3-39.5)	24.8 (6.8-91.0)	0.80
	Rif	20.4 (12.5-38.2)	23.6 (10.1-33.3)	22.6 (7.2-41.7)	
IP-10	No Int	11.4 (5.2-19.1)	10.1 (6.2-19.5)	13.4 (8.0-49.8)	0.76
	Rif	9.3 (7.6-23.1)	6.2 (5.8-18.8)	10.9 (9.0-19.8)	
MIG	No Int	3.0 (1.5-4.8)	2.6 (1.6-4.3)	3.0 (1.7-5.6)	0.87
	Rif	2.5 (1.3-3.2)	2.6 (1.3-3.8)	2.1 (1.4-3.7)	

Data are given in median (25-75 percentile) at baseline (week 0), 2 weeks (end of study) and 8 weeks (follow-up). mRNA expression levels were quantified by real-time qPCR and related to the endogenous levels of beta-actin (arbitrary units; AU). The p value reflects the interaction between time and group from the UNIANOVA (see methods). IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; IP, interferon gamma-induced protein; MIG, monokine induced by gamma interferon

Table S2: Results from immunophenotyping of B- and T-cells in peripheral blood from CVID patients in the Rifaximin study

	Group	Baseline (n=40)	2 weeks (n=38)	8 weeks (n=30)	P value interaction
CD3[1]	No Int	1209 (799-1881)	958 (702-1691)	1261 (680-1801)	0.91
	Rif	1588 (1040-2218)	1768 (1120-2300)	1855 (1242-2069)	
CD4[1]	No Int	623 (380-856)	558 (319-879)	554 (403-893)	0.39
	Rif	730 (477-865)	752 (583-924)	831 (591-964)	
CD8[1]	No Int	606 (237-938)	504 (175-785)	394 (237-1040)	0.57
	Rif	816 (465-1148)	1009 (356-1171)	940 (487-1253)	
T naïve CD4 %[2]	No Int	28.7 (21.1-40.2)	26.0(14.9-40.0)*	29.3 (21.0-44.0)	0.038*
	Rif	33.7 (17.5-46.5)	39.0 (27.3-47.2)	32.2 (17.0-45.1)	
T regulatory cells %[2]	No Int	5.8 (3.6-6.5)	5.2 (3.5-7.6)	4.4 (3.1-7.6)	0.71
	Rif	5.7 (4.4-7.8)	6.4 (4.6-8.7)	5.2 (3.2-6.8)	
CD19[1]	No Int	104 (35-227)	87 (31-140)	119 (28-214)	0.12
	Rif	186 (81-383)	216 (79-470)	186 (42-463)	
CD19%[3]	No Int	8.5 (2.9-12.5)	7.8 (3.5-11.1)	8.3 (3.2-13.6)	0.28
	Rif	14.0 (3.8-17.0)	12.8 (3.8-17.4)	11.6 (3.5-16.0)	
B-classwitched %[4]	No Int	1.8 (0.4-8.1)	1.1 (0.2-7.0)	1.1 (0.2-3.8)	0.49
	Rif	1.7 (0.8-4.1)	1.0 (0.6-2.8)	1.3 (0.7-3.1)	
B-cd21low %[4]	No Int	14.9 (3.1-31.9)	15.4 (3.5-27.7)	15.3 (4.1-46.0)	0.17
	Rif	5.9 (3.1-13.5)	5.7 (2.8-21.6)	9.3 (3.4-25.0)	
B-transitional %[4]	No Int	2.7 (1.1-10.5)	2.9 (0.6-11.0)	2.0 (0.9-10.1)	0.45
	Rif	2.4 (0.9-5.0)	2.3 (1.5-4.8)	2.5 (1.3-9.9)	

Data are given in median (25 – 75 percentile) at baseline (week 0), 2 weeks (end of study) and 8 weeks (follow-up). The p value reflects the interaction between time and group from the UNIANOVA (see methods).

*P <0.05 vs. baseline

[1] x 10^{-6}/L [2]given as percentage of CD4+ T-cells, [3]given as percentage of total lymphocytes (x10^6/L), [4]given as percentage of CD19+ B-cells. CD3, CD4 and CD8 are given as actual measured levels x10^6/L.

Table S3: Effects on rifaximin on other alpha diversity measurements than Faith's PD

	Baseline to 2 weeks		Baseline to 8 weeks	
	P value	Beta	P value	Beta
Chao1	0.048	-0.18	0.72	-0.03
Shannon	0.003	-0.26	0.71	-0.04
Observed OTUs	0.007	-0.24	0.48	-0.06
Simpson	0.122	-0.18	0.75	0.04

P values and beta measurements are given for each alpha diversity using after 2 and 8 weeks compared with baseline. Linear regression was performed with alpha diversity at 2 or 8 weeks as dependent and baseline alpha diversity and treatment group as independent variables in a forced model

Supplementary Figures:

Figure S1

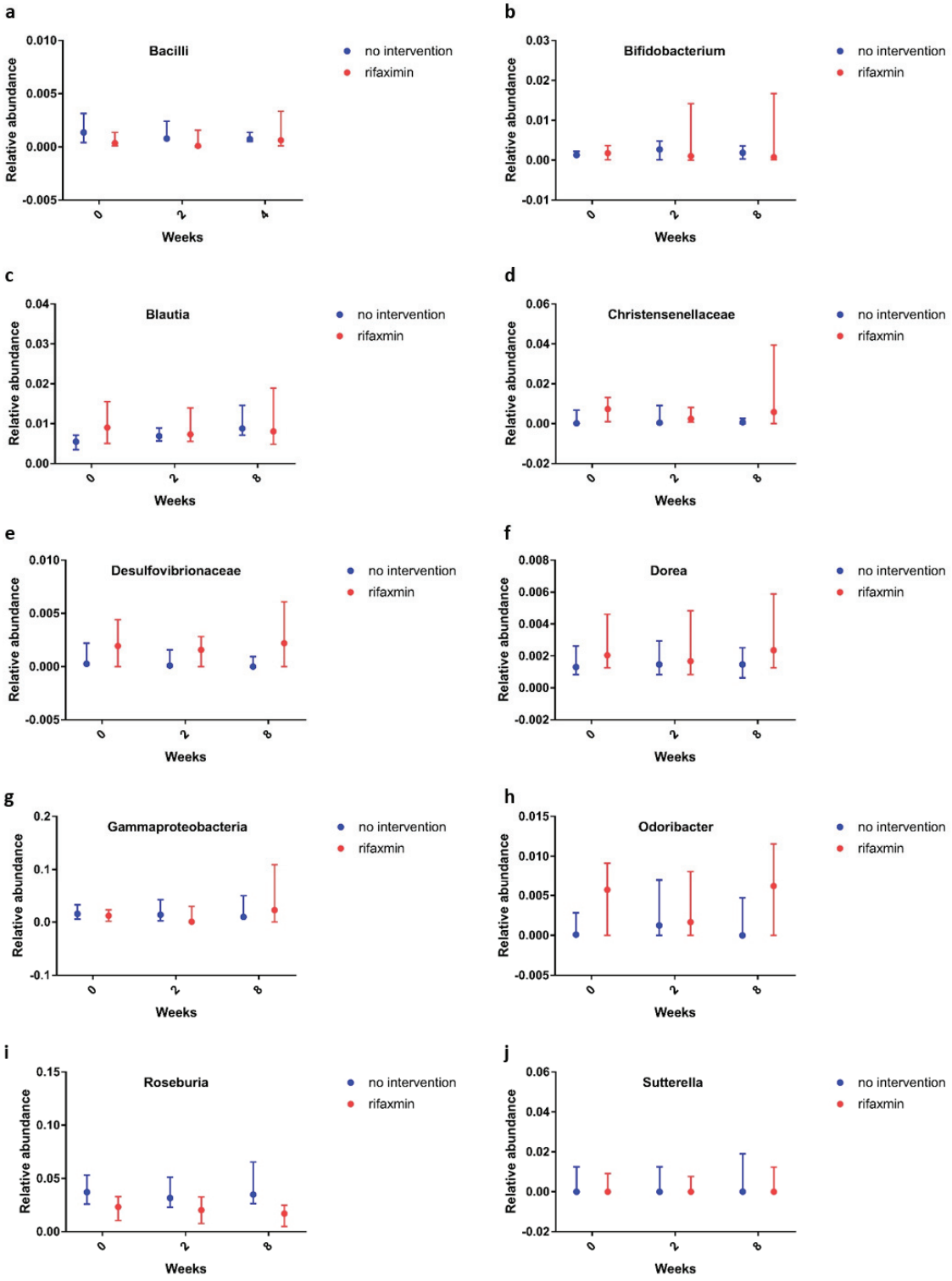

Figure S1: Different taxa in the CVID specific dysbiosis index from start of study (week 0) to end of study (week 2) and at follow up (week 8) in the rifaximin (n=20) and no intervention group (n=20). Univariate Repeated measures ANOVA (UNIANOVA) was used to assess the effect of treatment focusing on the interaction between time and treatment group for the different markers

Figure S2:

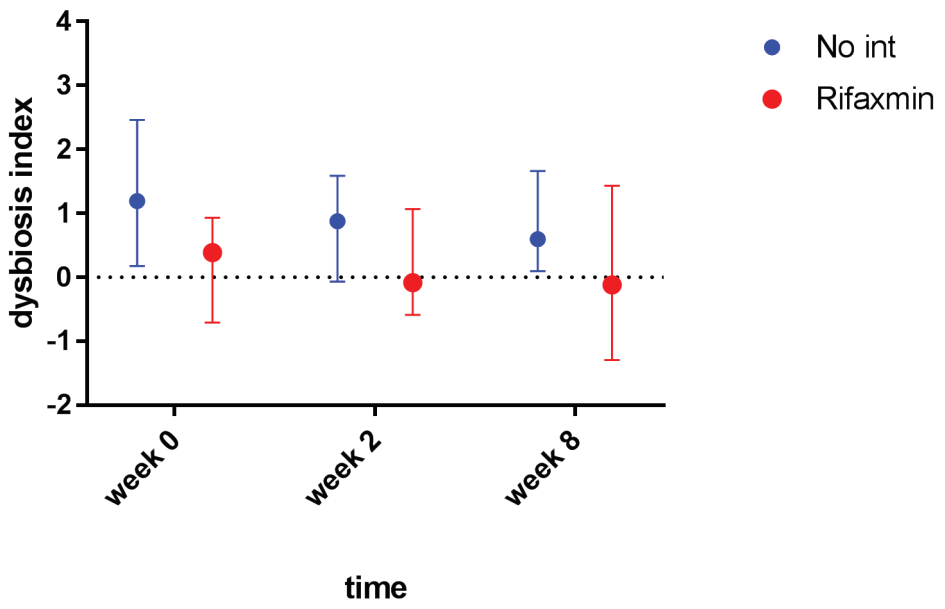

Figure S2: CVID specific dysbiosis index variation from start of study (week 0) to end of study (week 2) and at follow up (week 8) in the rifaximin (n=20) and no intervention group (n=20). Univariate Repeated measures ANOVA (UNIANOVA) was used to assess the effect of treatment focusing on the interaction between time and treatment group for the different markers

Supplementary methods

Gut microbiota analyses

Bacterial DNA was extracted using the *PSP® Spin Stool DNA Plus Kit (*STRATEC Biomedical, Birkenfeld, Germany. The DNA was subjected to high-throughput sequencing of the 16S ribosomal RNA gene with dual-indexed barcodes according to an established protocol.[1] Briefly, the hypervariable V3-V4 region was amplified with generic primers as described in detail,[1] including the gene specific primer sequences CCTACGGGAGGCAGCAG (forward) and GGACTACHVGGGTWTCTAAT (reverse) and up to 192 (24x8 barcodes) samples in parallel. Cleanup and normalization were performed using the SequalPrep Normalization Plate Kit (Life Technologies), followed by pooling and quality control. The final libraries were sequenced on an Illumina MiSeq (Norwegian Sequencing Centre, Oslo University Hospital Ullevål) using the v3 kit, allowing up to 300 basepairs paired-end sequencing.

Paired-end reads were quality trimmed with cutadapt version 1.13 and then merged using FLASH version 1.2.11. The merged reads were de-multiplexed and quality filtered using default values in Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1. Closed reference operational taxonomic unit (OTU) mapping to the Silva database (version 123, reference OTUs clustered at 97% sequence similarity) was performed using SortMeRNA version 2.0 through QIIME.

A rarefied OTU table (9525 reads per sample) was generated and OTUs with less than 2 reads to support it were discarded. Calculation of rarefied alpha diversity and beta diversity was performed in QIIME.

Cytokines and chemokines analysis in peripheral blood mononuclear cells (PBMC)

Total RNA was isolated from PBMC using RNeasy spin columns, as described by the manufacturer (Qiagen, Hilden, Germany). Isolated RNA was treated with DNase (Qiagen) and stored at −80 °C until further analysis. RNA concentrations and purity were assessed by spectrophotometer absorbance (NanoDrop ND-1000 Thermo Scientific, Wilmington, DE). 500 ng of RNA was reverse transcribed using q-Script cDNA Synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantification of mRNA

was performed using Brilliant III Ultra-Fast SYBR Green qPCR Mastermix (Agilent Technologies, Santa Clara, CA) on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the accompanying software SDS 2.4. All primer sequences can be provided upon request. As small volumes were used in the analyses of human samples, robot pipetting for RT-qPCR reactions was applied. For each transcript, RT-qPCR was conducted in duplicates. Target transcript levels were quantified by the comparative Ct method using the reference gene *β-actin* as endogenous control.

Flowcytometry

B- and T-cell subpopulations were analyzed by flow cytometry. For B-cell analysis the blood samples were washed twice before incubation with antibodies. T-cell analysis was performed in unwashed blood sample. Briefly EDTA-blood was incubated with optimally titrated antibodies for 15 minutes at room temperature, followed by erythrocyte lysis (BD FACSLysing Solution, Beckman Dickinson [BD], San Jose, CA). Data acquisition was performed on a Canto II flow cytometer (BD). For T-cells $1x10^5$ cells were acquired and for B-cells $1x10^6$ if possible. The following antibodies were used: Beckman Coulter ([BC], Brea, CA): CD31, CD45RO, CD28, CD45RA, CD127, CD19; BD: CD4, CD8, CD3, CD25, CD21, CD38, IgM, IgD; eBioscience (San Diego, CA): TCR alfa/beta; R&D Systems (Abigdon, UK): CXCR5; Dako (Glostrup, Denmark): CD27. The following subpopulations were determined: B-cells were gated as CD19+ and further subclassified as Naive (IgD+, CD27-), IgM memory (CD27+, IgD+, IgM+), class switched (CD27+, IgM-, IgD-), plasmablasts (CD19+dim, CD27++, CD38++), transitional (IgM++, CD38++) and CD21 low B cells (CD38 low, CD21 low).

T-cells were gated as CD3+ and further as naive CD4+ (CD4+, CD45RA+), recent thymic emigrants (CD4+, CD45RA+, CD31+), CD4+ memory (CD4+, CD45RO+), follicular like CD4+ (CD4+, CD45RO+, CCR5+), regulatory T-cells (CD4+, CD25++, CD127-), naive CD8+ (CD8+, CD27+, CD28+), CD8+ early effector memory (CD8+, CD27+, CD28-), CD8+ late effector memory (CD8+, CD27-, CD28-).

1. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013; **79**(17): 5112-20.

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Impaired HDL Function Amplifies Systemic Inflammation in Common Variable Immunodeficiency

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Magnhild E. Macpherson[1,2,3], Bente Halvorsen[1,3], Arne Yndestad[1], Thor Ueland[1,3,8], Tom E. Mollnes[6,7,8,9], Rolf K. Berge[4,5], Azita Rashidi[1], Kari Otterdal[1], Ida Gregersen[1,3], Xiang Y. Kong[1,3], Kirsten B. Holven[10,11], Pål Aukrust[1,2,3,8], Børre Fevang[1,2] & Silje F. Jørgensen[1,2,3]

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency, characterized by inadequate antibody responses and recurrent bacterial infections. Paradoxically, a majority of CVID patients have non-infectious inflammatory and autoimmune complications, associated with systemic immune activation. Our aim was to explore if HDL, known to have anti-inflammatory properties, had impaired function in CVID patients and thereby contributed to their inflammatory phenotype. We found reduced HDL cholesterol levels in plasma of CVID patients compared to healthy controls, particularly in patients with inflammatory and autoimmune complications, correlating negatively with inflammatory markers CRP and sCD25. Reverse cholesterol transport capacity testing showed reduced serum acceptance capacity for cholesterol in CVID patients with inflammatory and autoimmune complications. They also had reduced cholesterol efflux capacity from macrophages to serum and decreased expression of ATP-binding cassette transporter ABCA1. Human HDL suppressed TLR2-induced TNF release less in blood mononuclear cells from CVID patients, associated with decreased expression of transcriptional factor ATF3. Our data suggest a link between impaired HDL function and systemic inflammation in CVID patients, particularly in those with autoimmune and inflammatory complications. This identifies HDL as a novel therapeutic target in CVID as well as other more common conditions characterized by sterile inflammation or autoimmunity.

Common variable immunodeficiency (CVID) is a heterogeneous disease where the immune system fails to produce sufficient amounts of antibodies, resulting in reduced levels of immunoglobulin (Ig)G, IgM and/or IgA. It is the most common symptomatic primary immunodeficiency in adults with a prevalence of 1:25 000–1:50 000 in Caucasians[1]. Monogenic causes are identified in approximately 10–20% of CVID patients[2–4], but in most cases the etiology is unknown though might include polygenic variations in addition to epigenetic modifications[5,6]. The clinical hallmark of CVID is increased susceptibility to respiratory tract infections with encapsulated bacteria. Paradoxically, a large proportion of CVID patients also have autoimmune and non-infectious inflammatory complications such as lymphoid hyperplasia, granuloma, lymphoid interstitial pneumonitis, gastrointestinal inflammation and a variety of autoimmune disorders[7]. In line with this, CVID patients show signs of persistent systemic inflammation including monocyte/macrophage and T-cell activation, with increased levels of inflammatory mediators such as C-reactive protein (CRP), tumor necrosis factor (TNF), soluble (s) CD25 and sCD14[8–11].

[1]Research Institute of Internal Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital, Rikshospitalet, Oslo, Norway. [2]Section of Clinical Immunology and Infectious Diseases, Department of Rheumatology, Dermatology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Oslo, Norway. [3]Institute of Clinical Medicine, University of Oslo, Oslo, Norway. [4]Department of Clinical Science, University of Bergen, Bergen, Norway. [5]Department of Heart Disease, Haukeland University Hospital, Bergen, Norway. [6]Department of Immunology, Oslo University Hospital, Rikshospitalet, Oslo, Norway. [7]Research Laboratory, Nordland Hospital, Bodø, Norway. [8]Faculty of Health Sciences and K.G. Jebsen TREC, University of Tromsø, Tromsø, Norway. [9]Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway. [10]Department of Nutrition, Institute for Basic Medical Sciences, University of Oslo, Oslo, Norway. [11]Norwegian National Advisory Unit on Familial Hypercholesterolemia, Oslo University Hospital Rikshospitalet, Oslo, Norway. Correspondence and requests for materials should be addressed to M.E.M. (email: m.e.macpherson@studmed.uio.no)

	CVID cohort (n = 102)	Healthy controls (n = 28)	p-value
Age in years mean ± SD [min-max]	48 ± 15 [18–83]	42 ± 10 [28–65]	0.053*
Female (%)	54%	64%	0.328†
BMI mean ± SD [min-max]	25 ± 4 [17–39]	24 ± 3 [19–34]	0.232‡
IVIG (%)	18 (18%)	—	—
SCIG (%)	71 (70%)	—	—
IVIG and SCIG° (%)	12 (12%)	—	—
Infections only (%)	26 (26%)	—	—
Non-infectious complications (%)	76 (75%)	—	—

Table 1. Patient characteristics for CVID cohort and healthy controls. BMI: Body mass index. IVIG: Intravenous immunoglobulins. SCIG: Subcutaneous immunoglobulins. °One CVID patient did not receive any immunoglobulin substitution. *Mann Whitney test, †Pearson Chi square test, ‡Student's t-test.

In view of the wide variety of phenotypes in CVID, it is useful to subdivide the patient group into two main categories: those with infections only and those who also display non-infectious complications. Importantly, CVID patients with one or more of these non-infectious complications have an 11-fold increase in mortality[7]. Whereas IgG replacement therapy reduces the severity and frequency of infections, it has little effect on the inflammatory and autoimmune complications[12]. It is therefore of major importance to elucidate the mechanisms of these non-infectious complications in order to identify new targets for therapy in CVID[13].

The anti-inflammatory properties of high-density lipoprotein (HDL) are well established in atherosclerotic disorders, but have also been suggested in non-atherosclerotic conditions such as sepsis, autoimmune disorders and cancer[14–16]. *In vitro* and *in vivo* experiments have suggested an anti-inflammatory role for HDL by its ability to counteract toll-like receptor (TLR)-induced inflammation in macrophages via activating transcription factor 3 (AFT3)[17]. Decreased cholesterol efflux capacity from peripheral cells, which is influenced by HDL activity, has in murine models of dendritic cells been shown to result in autoimmunity and inflammation via increased intracellular inflammasome activity[18].

Based on the above, we hypothesized HDL levels and functional activity to be altered in CVID, possibly contributing to systemic immune activation and inflammation as well as development of autoimmunity. We explored this hypothesis both by conducting analyses of lipid profiles in a large CVID population and by running experimental studies of HDL function in relation to cholesterol efflux capacity and anti-inflammatory effects in CVID patients and controls. Here, we present novel insights into the role of HDL in systemic inflammation of CVID, findings that can be of relevance to other autoimmune and inflammatory disorders as well.

Results

Patients' characteristics. Clinical characteristics of the 102 CVID patients included in the lipid- and inflammatory analyses are shown in Table 1. In addition, a complete description of their autoimmune and inflammatory complications is given in Supplemental Table S1. Not all sub analyses and *in vitro* experiments were performed in all patients and so clinical characteristics of the 40 patients making up the smaller cohort, used for HDL subclass analyses and PBMC gene expression studies, and also the cohorts used for functional studies are shown in Supplemental Table S2. A complete overview of the CVID patients participating in the different analyses is given in Supplemental Table S3.

The CVID patients had decreased HDL cholesterol levels. We measured the total cholesterol, HDL cholesterol and LDL cholesterol levels in plasma from 102 CVID patients and 28 healthy controls. Compared to healthy controls, CVID patients had lower plasma HDL levels (1.66 [1.42, 1.84] vs 1.16 [0.91, 1.55], $p < 0.0001$, median [25th, 75th percentile] mmol/L, respectively) (Fig. 1), whilst LDL ($p = 0.99$) and total cholesterol levels ($p = 0.57$) showed no significant difference. Low HDL levels in CVID patients compared to controls were consistent after correcting for sex, BMI and age ($p < 0.001$).

In 32 of the CVID patients we had two HDL measurements (mean time gap between sample collection 21 months, range 8–28 months) and found no significant difference between HDL levels measured at the two different time points ($p = 0.42$), suggesting that decreased HDL levels is a stable feature in CVID (Supplemental Fig. S1).

Out of 102 CVID patients, seven (7%) were using statins and 18 (18%) had a disease history of cardiovascular disease (defined as hypertension requiring medical treatment [n = 16] or coronary artery disease [n = 2]). Importantly, HDL cholesterol levels in these patients did not differ from the other CVID patients (1.12 ± 0.13 in statin users vs 1.26 ± 0.04 in non-statin users; $p = 0.439$, and 1.15 ± 0.11 for patients with cardiovascular disease vs 1.28 ± 0.05 for those without cardiovascular disease; $p = 0.234$, HDL levels given in mean ± SEM mmol/L).

Low HDL cholesterol levels were associated with systemic inflammation and non-infectious complications in CVID. When dividing the CVID cohort into subgroups by phenotype, we found significantly lower HDL levels in CVID patients with non-infectious complications (n = 76) than in CVID patients with infections only (n = 26) (1.1 [0.9, 1.5] vs 1.4 [1.1, 1.9] respectively, $p < 0.01$, HDL results given as median [25th, 75th percentile] mmol/L) (Fig. 1). There was no significant difference between healthy controls and patients with infections only ($p = 0.09$), hence the low HDL levels in those with non-infectious complications were driving the difference between healthy controls and CVID patients overall ($p < 0.0001$).

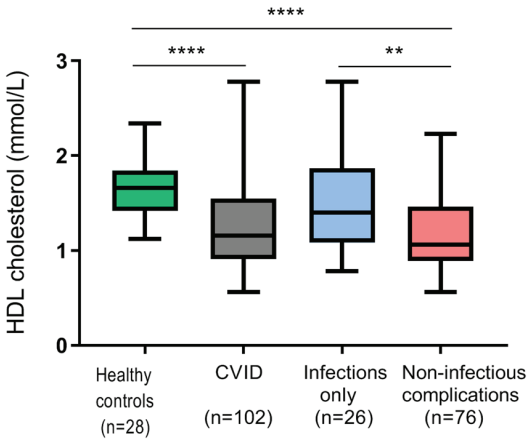

Figure 1. HDL cholesterol levels in CVID patients and controls including CVID subgroups. Plasma levels of HDL cholesterol in Common Variable Immunodeficiency (CVID) patients and controls. The CVID cohort is further divided into two subgroups: infection only and non-infectious complications. Results are given as boxes representing the 25th to 75th percentile with lines indicating median and whiskers min-max values; **$p < 0.01$, ****$p < 0.0001$ using Mann-Whitney test between groups.

Furthermore, we found a negative correlation between plasma levels of HDL cholesterol and sCD25 ($r = -0.59$, $p < 0.001$) and CRP ($r = -0.24$, $p = 0.018$) (Fig. 2a,b), but not with LPS ($r = -0.08$, $p = 0.407$) or sCD14 ($r = -0.16$, $p = 0.119$) in CVID patients. Combined, these findings suggest that the decreased levels of HDL cholesterol in CVID are associated with increased systemic immune activation and an inflammatory and autoimmune phenotype. Stepwise regression identified sCD25 (beta $= -0.52$, $p = 0.001$) and sex (beta $= -0.33$, $p = 0.034$) as the strongest predictors of HDL cholesterol levels in CVID (Supplemental Table S4).

HDL subclasses and Apo A-1 in CVID. HDL particles are heterogeneous and can be fractioned into subclasses defined by density or size reflecting differences in relative content of proteins and lipids: small (S), medium (M), large (L) and extra-large (XL). Hence, the HDL subclasses vary in composition and also differ in capacity of contributing to reverse cholesterol transport as well as anti-oxidative and anti-inflammatory activity[19]. Several studies have found lipid-free Apo A-1 and the smaller forms of HDL to be the main acceptors of cholesterol efflux via the ATP-binding cassette transporter (ABCA1) pathway in macrophages[20,21]. We therefore next measured plasma concentration of Apo A-1 and each HDL subclass in 40 CVID patients and 28 healthy controls (characteristics are given in Supplemental Table S2). CVID patients had significantly lower levels of M-HDL ($p = 0.025$), L-HDL ($p = 0.005$) and XL-HDL ($p = 0.041$), but not S-HDL ($p = 0.778$) (Supplemental Fig. S2). Importantly, Apo A-1, the main protein and functional component of HDL[19,22] was also significantly lower in our CVID patients compared to healthy controls ($p = 0.005$) (Fig. 3). Thus, although the normal proportion of S-HDL could suggest a normal reverse cholesterol transport (RCT) function, HDL particles in CVID had markedly reduced levels of Apo A-1, predicting impairment of function.

Serum from CVID patients had decreased cholesterol acceptor function. To further look into the functional capacity of HDL in CVID patients, we tested the cholesterol acceptor function of HDL in a subgroup of CVID patients ($n = 18$) and healthy controls ($n = 10$); characteristics are given in Supplemental Table S2. Serum was employed, as its cholesterol acceptance is mainly driven by HDL function and concentration. We exposed THP-1 macrophages to ^{14}C-cholesterol for 48 hours before cholesterol efflux was induced by serum from either CVID patients or controls. We found significantly decreased cholesterol acceptor capacity in serum from CVID patients compared to healthy controls (14.1% [13.0%, 15.3%] versus 15.0% [14.7%, 17.3%], $p < 0.05$, results given as median [25% percentile, 75% percentile]) (Fig. 4), illuminating reduced function of a key step in RCT in these patients. Furthermore, when dividing the patients into the two clinical subgroups, serum from CVID patients with non-infectious complications had significantly reduced cholesterol acceptor capacity (13.5% [12.7%, 14.7%]) compared to controls ($p = 0.003$), in contrast with those who suffered from infections only (15.3% [13.1%, 15.6%], $p = 0.46$, all results given as median [25% percentile, 75% percentile]) (Fig. 4). These findings suggest that CVID patients with autoimmune and inflammatory complications have decreased HDL function and not only decreased HDL levels.

CVID patients had decreased expression of ABCA1. Reversed cholesterol transport is not only dependent on HDL acceptor capacity, but also the cellular transport properties of the cholesterol-loaded cells. Further exploring cellular transport activity, we analyzed mRNA expression of RCT related genes measured by qPCR on PBMC from 40 CVID patients and 30 healthy controls (characteristics are given in Supplemental Table S2). We found significantly lower levels of ABCA1 mRNA in CVID patients than in healthy controls ($p < 0.0001$) (Fig. 5). There was no significant difference in mRNA expression of ABCG1 ($p = 0.17$), SR-A1 ($p = 0.55$) or SR-B1 ($p = 0.32$) between CVID patients and healthy controls, respectively (Fig. 5).

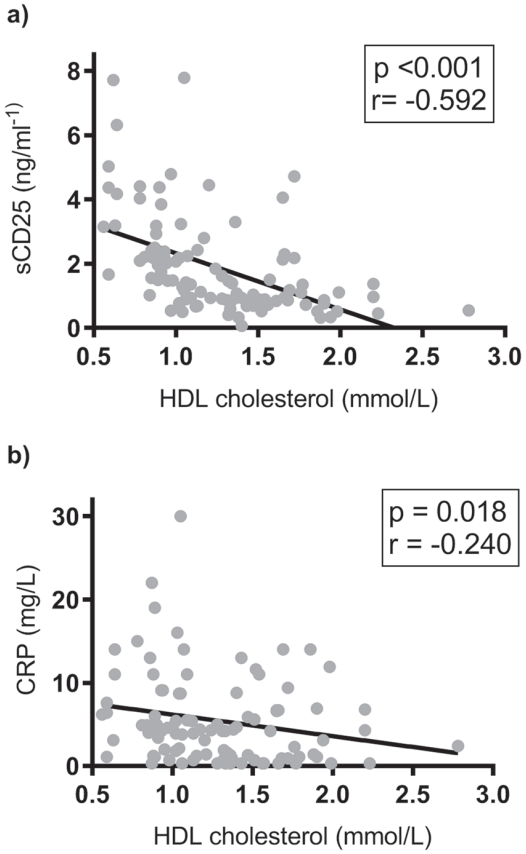

Figure 2. Correlation between HDL cholesterol levels and inflammatory markers in CVID. Panels show correlation between HDL cholesterol and sCD25 **(a)** and CRP **(b)** in CVID patients (n = 102). Comparisons are made by Spearman's rank correlation test. Spearman's rank correlation coefficient is referred to as r. Trend lines indicate negative correlation between variables sCD25 and HDL cholesterol as well as between CRP and HDL cholesterol.

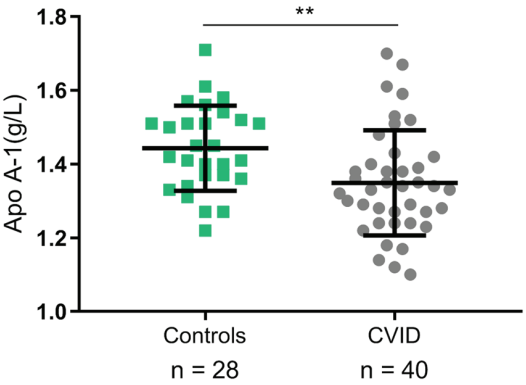

Figure 3. Apo A-1 levels in CVID patients and controls. The results are given as mean with error bars for SD. **p < 0.01 using unpaired Student's t-test between groups.

Cholesterol efflux capacity decreased in macrophages from CVID patients. In order to further assess reverse cholesterol transport capacity in CVID, we used monocyte derived macrophages from 11 CVID patients and 11 age and sex matched controls (in this particular experiment only CVID patients with non-infectious complications were included; Supplemental Table S2) and a uniform control serum (2.5%, v/v) as acceptor. We exposed macrophages to ^{14}C-cholesterol for 48 hours for cholesterol loading followed by overnight stabilization in medium without additives before efflux was analyzed. Whereas there was no difference in cholesterol loading between macrophages from CVID patients and controls (data not shown), macrophages from

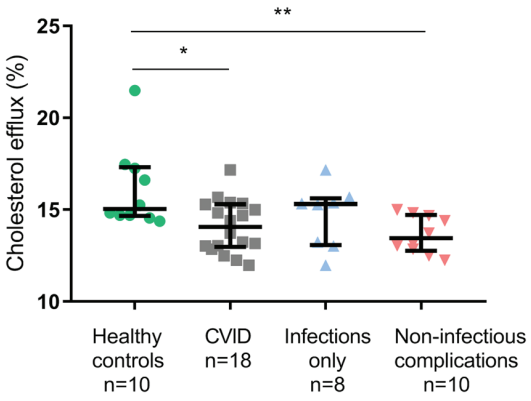

Figure 4. Cholesterol efflux from THP-1 macrophages to serum in CVID patients and controls. The results are given as median with interquartile range showing cholesterol efflux in CVID patients and healthy controls as well as in the CVID subgroups: infection only and non-infectious complications. *$p < 0.05$, **$p < 0.01$ using Mann-Whitney test between groups.

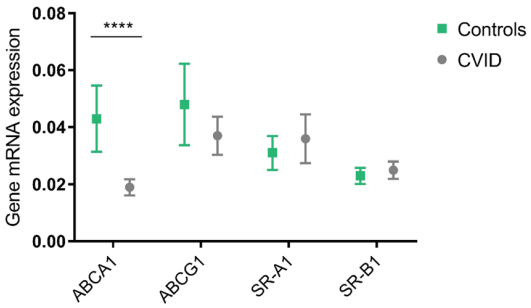

Figure 5. Reverse cholesterol transport related gene expression in PBMC from CVID patients and controls. mRNA expression in PBMC from CVID patients ($n = 40$) and healthy controls ($n = 30$) of the genes: ABCA1 (****$p < 0.0001$), ABCG1 ($p = 0.17$), SR-A1 ($p = 0.55$) and SR-B1 ($p = 0.32$) involved in reverse cholesterol transport from peripheral cells, using Mann-Whitney test between groups. Results given as mean with 95% CI. mRNA levels were quantified by qPCR and values given in relation to the reference genes β-actin and GAPDH.

CVID patients had significantly reduced cholesterol efflux capacity as compared to macrophages from healthy controls (14.6% [12.2%, 18.2%] vs 16.3% [13.0%, 21.3%], $p = 0.04$, results given as median [25% percentile, 75% percentile]) (Fig. 6), consistent with our findings of low ABCA1 expression in CVID patients.

Impaired anti-inflammatory effect of HDL on TLR stimulation of PBMC from CVID patients.

HDL has recently been shown to attenuate TLR mediated inflammatory response in macrophages as assessed by IL-6 and TNF measurements, at least partly mediated by the transcriptional regulator ATF3[17]. Thus, we next examined the interaction between HDL and ATF3 in PBMC from CVID patients and controls. PBMC from 40 CVID patients had decreased ATF3 mRNA levels compared to PBMC from 30 healthy controls ($p < 0.0001$, Fig. 7a) (cohort characteristics given in Supplemental Table S2). We explored this further by incubating freshly isolated PBMC from six CVID patients and six age and sex matched healthy controls (in this particular sub-study only CVID patients with an inflammatory phenotype were included; Supplemental Table S2) in the presence of different concentrations of human HDL for 6 hours. We then stimulated the cells with TLR4 (LPS) and TLR2 (Pam3Cys) ligands for 12 hours, and found a suppressive effect of HDL on TNF release in Pam3Cys exposed cells in healthy controls (Fig. 7b). Notably, however, the suppressive effect of HDL on TNF release was significantly decreased in cells from CVID patients stimulated with TLR2 ligand Pam3Cys (Fig. 7b), but not with TLR4 ligand LPS. The same effect was not observed for IL-6 release (Supplemental Fig. S3).

Discussion

In the present study, we show decreased levels of HDL cholesterol associated with systemic inflammation and inflammatory and autoimmune complications in CVID. Our functional studies demonstrate impaired HDL cholesterol acceptor function and impaired reverse cholesterol transport from macrophages in CVID, likely related to decreased levels of Apo A-1 and low mRNA expression of ABCA1 in these patients. Furthermore, our findings suggest attenuated anti-inflammatory effects of HDL on PBMC from CVID patients, which could reflect their decreased ATF3 expression. This study proposes new pathogenic mechanisms for the clinically significant

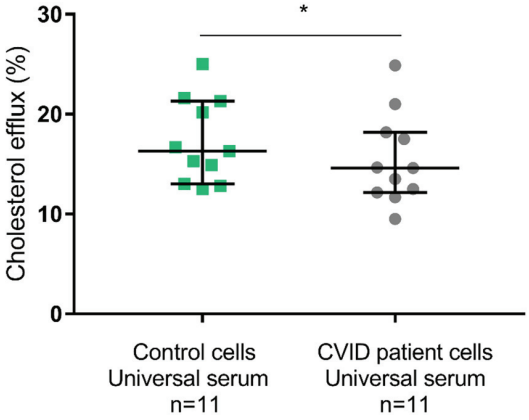

Figure 6. Cholesterol efflux from CVID and control macrophages to universal serum. Cholesterol efflux from monocyte-derived macrophages from CVID patients with non-infectious complications and age- and sex matched healthy controls to universal serum. Results given with bars for median and interquartile range, *$p < 0.05$ using Wilcoxon matched pair signed rank test.

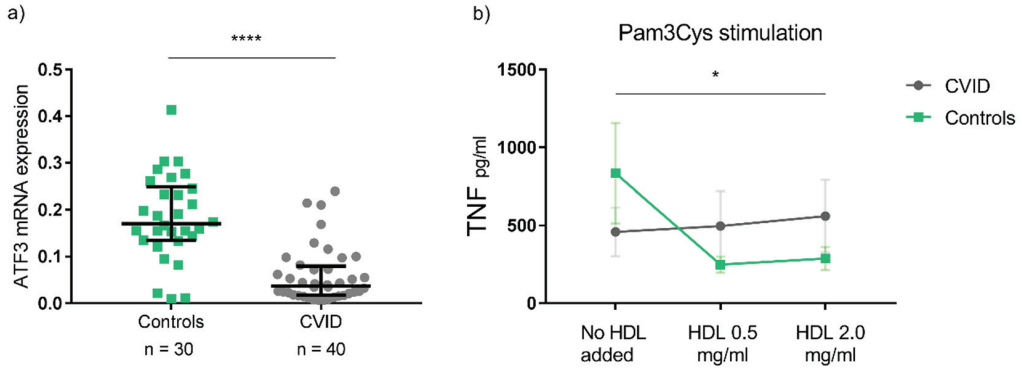

Figure 7. ATF3 mRNA levels and HDL cholesterol effects on TLR2-stimulated TNF release from mononuclear cells in CVID patients and controls. (**a**) ATF3 mRNA expression in CVID patients and healthy controls; results given with bars for median and interquartile range, ****$p < 0.0001$ using Mann-Whitney test between groups. mRNA levels were quantified by qPCR and values given in relation to the reference genes β-actin and GAPDH. (**b**) Effect of increasing HDL cholesterol concentration on TLR2- (Pam3Cys) stimulated TNF release from mononuclear cells in CVID ($n = 6$) and controls ($n = 6$); *$p < 0.05$. Results illustrated as median with variance, p-values calculated using repeated measures ANOVA analysis. The difference between CVID patients and the control group in TNF release at baseline from mononuclear cells after Pam3Cys stimulation was found non-significant ($p = 0.389$) when performing Student's t-test on log-transformed datasets.

systemic immune activation and non-infectious complications in CVID, expanding the role of HDL as an important modulator of inflammation.

One study has previously shown reduced HDL cholesterol and Apo A-1 levels in 18 patients with CVID and six patients with X-linked agammaglobulinaemia compared to 12 healthy controls, with a negative correlation between plasma levels of HDL and TNF in these patients[23]. Herein we extend these findings by showing decreased HDL cholesterol levels in a substantially larger study population, and further demonstrate that the reduced HDL cholesterol levels are stable during temporal testing. Importantly, the lowest HDL levels were seen in CVID patients with autoimmune and inflammatory complications. This suggests that low HDL levels are related to sterile inflammation rather than infectious-driven inflammation, supported by the detection of sCD25, a robust marker of T cell activation, as the strongest predictor for low HDL levels. In line with our findings, previous studies have found low HDL levels associated with disease activity and systemic inflammation in autoimmune diseases such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus and Sjogren's syndrome[24–27].

The detailed study of HDL particle composition gave some discordant results showing normal proportions of the small HDL particles but decreased levels of Apo A-1 in CVID, both particles of major importance to HDL function. However, our studies using control and patient serum to reflect HDL acceptor capacity for cholesterol in RCT showed significantly impaired HDL function in CVID, primarily reflecting impaired cholesterol acceptor

capacity in CVID patients with non-infectious complications. The consequence of this impaired HDL acceptor function was augmented by decreased cholesterol efflux from macrophages in CVID patients, likely related to decreased expression of the ATP-binding cassette transporter ABCA1.

A major finding in the present study was the association between decreased levels and function of HDL and systemic inflammation. These correlations could also be supported by functional studies. The combined impairment of cholesterol acceptor function in serum and reduced reverse cholesterol transport from macrophages likely leads to lipid accumulation in these cells. A similar pattern in dendritic cells has been demonstrated to promote a TLR mediated inflammatory response[28]. Additionally, we found that PBMC from CVID patients had lower expression of transcription factor ATF3 mRNA along with a modestly attenuated anti-inflammatory effect of HDL as assessed by TNF release in TLR2 activated PBMC. ATF3 has been found to be vital in the protective effect of HDL against TLR induced inflammation[17]. Increased TNF levels are typical of the inflammatory CVID phenotype[8], and our findings could relate this phenotype to impaired cellular HDL responses.

A rational line of reasoning could suggest the chronic systemic inflammation found in CVID patients would lead to increased incidence of cardiovascular disease in this group. However, no clear evidence has been found to substantiate this hypothesis[29]. American and European registry data have rather identified lung disease and autoimmune manifestations as the most prevalent complications to CVID[7,30]. It has however been speculated that atherosclerosis is present but does not amount to clinical levels substantial enough to cause cardiovascular disease due to the reduced life expectancy in CVID patients with non-infectious complications[29].

The reasons for the decreased HDL levels and function in CVID patients are, at present, not clear. We have previously shown GI tract inflammation in a large proportion of CVID patients[31] which could influence lipid metabolism. Patients with CVID enteropathy have been found to have low mucosal IgA levels in the duodenum, which has been suggested could shift intestinal molecular pathways from appropriate lipid metabolism to enteropathy-inducing immune processes[32]. The gut microbiota has previously been shown to contribute to variation in blood lipids[33] and alteration of the gut microbiota composition in CVID has been linked to systemic inflammation[34]. Importantly, whereas inflammation *per se* could have contributed to the decreased HDL levels[35], decreased HDL levels may further increase inflammation in CVID patients, hence representing a pathogenic loop. In a clinical setting, such a loop could be targeted therapeutically by Apo A-1 mimetics[36], cholesteryl transfer protein (CETP)-inhibitors[37], statins or a combination thereof.

Based on the prevalence of CVID, the total number of patients in the cholesterol analysis part of the study was large and the study included robust characteristics of HDL composition and function, viewed as a major strength of this study. Still there are some limitations, including a rather low number of patients in some of the functional sub-studies. Furthermore, the blood samples were not obtained at a fasted state. We do however suggest that this should only have minor influences on the HDL data. Finally, we lack protein data on some important molecules like ABCA1 and ATF3, which could have been useful as mRNA may not necessarily reflect the protein expression of these molecules.

In conclusion, the present study shows novel data demonstrating marked disturbances in HDL cholesterol levels and HDL function in CVID, with HDL levels inversely correlating with inflammatory markers and a more severe phenotype in these patients. Furthermore, it shows reduced HDL function via impaired reverse cholesterol transport in CVID patients, which appears to be an important factor contributing to their systemic inflammation and autoimmune and inflammatory manifestations. The identification of HDL function as a target for treatment to reduce inflammatory and autoimmune complications in CVID should be further explored. Our findings support a link between a dysregulated immune system, lipid metabolism, inflammation and autoimmunity in humans. Indeed, the findings of this study could be transferable and therapeutically useful to other and more common inflammatory and autoimmune diseases.

Methods

Study population. CVID patients were recruited from the outpatient clinic at the Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, which functions as a national center for diagnosis and treatment of adults with primary immunodeficiency diseases in Norway. CVID was defined as decreased serum levels of IgG, IgA and/or IgM by a minimum of two standard deviations below the mean for age, whilst excluding other causes of hypogammaglobulinemia. Patients with ongoing acute infections and patients using immunosuppressive drugs were excluded, whereas use of statins was not an exclusion criterion. The larger cohort of 102 CVID patients was recruited from November 2011 to December 2012 and the smaller cohort of 40 CVID patients was recruited between October 2013 and October 2014 as previously described[34,38] (Supplemental Fig. S4). For comparison, we included 30 healthy controls based on disease history and on no regular medications. Blood was drawn for PBMC isolation, serum and plasma samples from all controls. However, technical issues with the plasma samples from two healthy controls lead to a reduced number of controls (n = 28) for the plasma analyses. Supplemental Table S3 gives a detailed overview of which CVID patients contributed to the different functional studies and which were also part of the large and small cohorts. For cellular studies on macrophage cholesterol efflux and the effect of HDL on TLR-stimulated release of TNF and IL-6 from macrophages, we specifically wanted to focus on the possible pathological mechanisms in patients with systemic inflammation and autoimmunity and therefore selected CVID patients with known non-infectious complications for these particular analyses.

Blood sampling. Plasma for lipid analyses was sampled into EDTA collection tubes, immediately immersed in melting ice and centrifuged within 30 minutes at 2,500 *g* for 20 minutes prior to being stored at −80 °C until analysis. For patients on intravenous Ig therapy, blood samples were collected just prior to infusion.

Analyses of lipid and inflammatory parameters. Total cholesterol, HDL cholesterol, Apo A-1 and low density lipoprotein (LDL) cholesterol were measured enzymatically on a Hitachi 917 system (Roche Diagnosis GmbH, Mannheim, Germany) using the cholesterol (cholesterol CHOD-PAP), HDL-cholesterol plus (catalog no. 04713257190) and LDL-cholesterol plus (catalog no. 04714423190) kits from Roche Diagnostics. CRP levels were analysed via the routine hospital laboratory on the day of sampling using a high-sensitivity method. Plasma levels of sCD14 and sCD25 were quantified in duplicate by enzyme immunoassays obtained from R&D Systems (Minneapolis, MN). Endotoxin was analysed by Limulus Amebocyte Lysate chromogenic assay (Lonza, Walkersville, MD) according to the manufacturer's instructions, with the following modifications: samples were diluted 10-fold to avoid interference with background colour and preheated to 68 °C for 10 minutes prior to analysis to dissolve immune complexes. Supernatant TNF and IL-6 levels were analyzed with a V-plex Proinflammatory Panel 1 kit from Meso Scale Discovery (Meso Scale Diagnostics, LLC, 1601 Research Blvd. Rockville, MD) using QuickPlex SQ120.

Characterizing HDL sub-fractions. EDTA plasma samples were quantified using a commercial high-throughput proton NMR metabolomics platform (Nightingale Health Ltd, Helsinki, Finland). Details of the experimentation and applications of the NMR metabolomics platform have previously been described[39]. The mean size for the HDL particles was calculated by weighting the corresponding subclass diameters with their particle concentrations, and the four HDL subclass sizes were defined as 14.3 nm (XL), 12.1 nm (L), 10.9 nm (M), and 8.7 nm (S).

Isolation of PBMC. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway) within 1 hour after blood collection. PBMC were used for further *in vitro* experiments immediately following isolation or stored as pellet in −80 °C for PCR analyses.

Quantitative real-time (RT)-PCR analyses. Total RNA was isolated from PBMC using RNeasy spin columns as described by the manufacturer (Qiagen, Hilden, Germany). Isolated RNA was treated with DNase (Qiagen) and stored at −80 °C for later analysis. RNA concentrations and purity were assessed by spectrophotometer absorbance (NanoDrop ND-1000 Thermo Scientific, Wilmington, DE). 500 ng of RNA was loaded into the cDNA synthesis using q-Script cDNA Synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantification of mRNA was performed using Perfecta SYBR Green qPCR Fast Mastermix (Quanta Bioscience) on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the accompanying software SDS 2.4. All primer sequences can be provided upon request. As small volumes were used in the analyses of human samples, robot pipetting for RT-qPCR reactions was employed. For each transcript, RT-qPCR was conducted in duplicate. Target transcript levels were quantified by the comparative Ct method using the average Ct-median value from reference genes β-actin and GAPDH as endogenous control.

Functional studies. *Serum as cholesterol acceptor*. The human monocytic cell line THP-1 monocytes (ATCC, Chicago, IL) were differentiated to THP-1 macrophages using 12-myristate 13-acetate (PMA, Sigma) (100 nM). After 24 hours the macrophages were loaded with ^{14}C-cholesterol (0.5 μCi/ml [18.5 mBq/l] American Radiolabel Chemicals, Saint Louis, MO) in regular growth medium added oxidized LDL (10 μg/ml) and further grown for 48 hours. After overnight incubation in sterile 0.2% (wt/v) human serum albumin in RPMI1640, the cholesterol acceptor capacity was measured adding 2.5% (v/v) heat-inactivated serum (in RPMI-1640 medium) from CVID patients and healthy controls to the lipid laden THP-1 macrophages. After 3 hours incubation, the medium was collected and ^{14}C-cholesterol measured by liquid scintillation counter (TRI-CARB 2300 TR Scintillation Counter [Packard, Waltham, MA]). Total cell-associated ^{14}C-cholesterol loading of THP-1 macrophages was assessed in a parallel set up. Percentage efflux was calculated using the equation: $[DPM_{medium}/(DPM_{(cell+medium)})] \times 100$.

Cholesterol efflux capacity. Cell viability in the PBMC suspensions was tested using Countess™ automated cell counter, before seeding onto a 24-well plate (Nunclon™ Delta Surface, Thermo Fisher scientific, MA). The cells were incubated for an hour, washed twice, and plastic adhered monocytes were further stimulated with TNF 10 ng/ml (R&D Systems) for the mononuclear cells to differentiate into a macrophage-like phenotype. After two days, the macrophage-like cells were loaded with ^{14}C-cholesterol (0.5 μCi/ml [18.5 mBq/l]) in regular growth medium added oxidized LDL (10 μg/ml). After 48 hours lipid loading, the cells were incubated overnight with 0.2% (wt/v) human serum albumin in RPMI1640. The patient and control cells' efflux capacity were assessed using 2.5% heat inactivated serum from one healthy individual as detailed above.

ATF3 and anti-inflammatory reprogramming. PBMC were isolated as described above. The cells were pre-treated for 6 hours with HDL (0.5 mg/ml, 2 mg/ml and no HDL, respectively [purified human HDL, Kalen Biomedical, LLC, Germantown, MD]). They were then stimulated overnight with the TLR4 ligand LPS from *Escherichia coli* 026:B6 (2.5 ng/ml) and TLR2 ligand Pam3Cys (0.8 μg/ml), before supernatant levels of TNF and IL-6 were measured as described above.

Statistics. The datasets for HDL analyses, including HDL subclasses, were not normally distributed as per Kolmogorov-Smirnov normality tests and the data was therefore log transformed. Where normal distribution was achieved, we continued with two-tailed multivariate testing. Where normal distribution was not achieved, we applied Mann-Whitney non-parametric testing, all tests with significance level 0.05. We ran bivariate correlation analyses in addition to forced and stepwise linear regression analyses to investigate the connections between lipid

levels and inflammatory markers. For the longitudinal data, we compared two different time points using related samples Wilcoxon signed rank test. For the functional studies with age and sex matched controls we applied matched pair Wilcoxon signed rank test, and where repeated measurements were done under various HDL concentrations we used repeated measures ANOVA analysis.

Study approval. The study was approved by the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway (number 2012/521, 2013/1037) and conducted in accordance with the Helsinki Declaration. All participants provided written, informed consent. The study participants were given a study number which was used for handling samples and analyses throughout the study.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

M.E.M., B.H., A.Y., P.A., B.F. and S.F.J. designed the research studies. M.E.M., B.H., A.Y., T.E.M., R.K.B., A.R., K.O. and I.G. conducted the experiments. X.Y.K. and K.B.H. acquired data. M.E.M., B.H. and T.U. analyzed the data. M.E.M., P.A., B.F. and S.F.J. wrote the manuscript. MEM prepared figures. All authors have revised the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-45861-1.

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SUPPLEMENTAL MATERIAL

IMPAIRED HDL FUNCTION AMPLIFIES SYSTEMIC INFLAMMATION IN COMMON VARIABLE IMMUNODEFICIENCY

Magnhild E. Macpherson*, Bente Halvorsen, Arne Yndestad, Thor Ueland, Tom E. Mollnes, Rolf K. Berge, Azita Rashidi, Kari Otterdal, Ida Gregersen, Xiang Y. Kong, Kirsten B. Holven, Pål Aukrust, Børre Fevang, Silje F. Jørgensen.

*Corresponding author: Magnhild E. Macpherson, Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, P.Box 4950 Nydalen, 0424 Oslo, Norway. E-mail: m.e.macpherson@studmed.uio.no Telephone: +47 23070000

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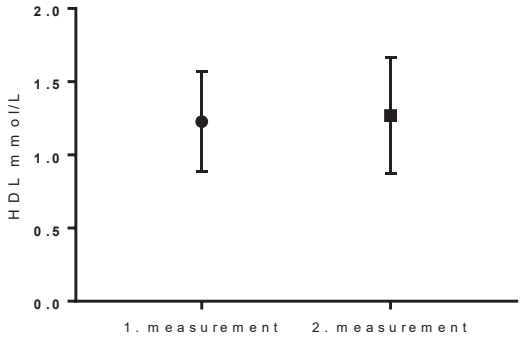
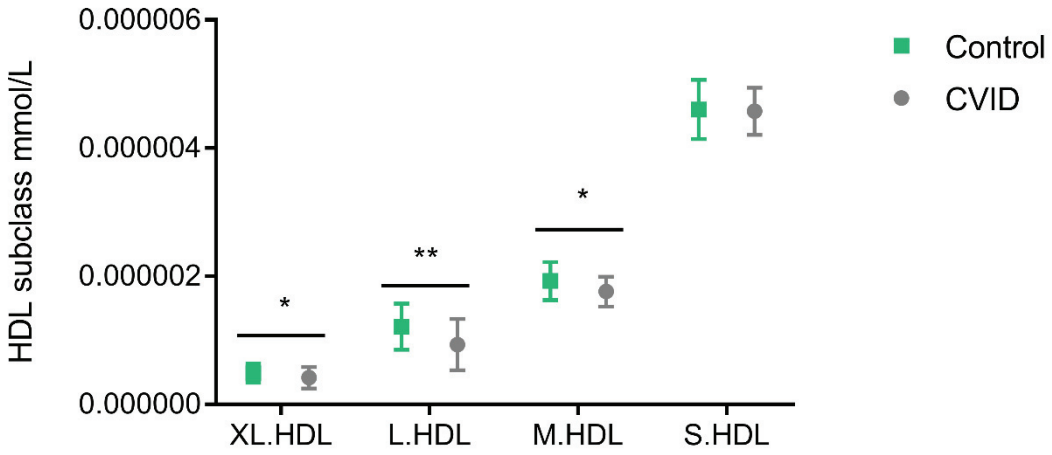


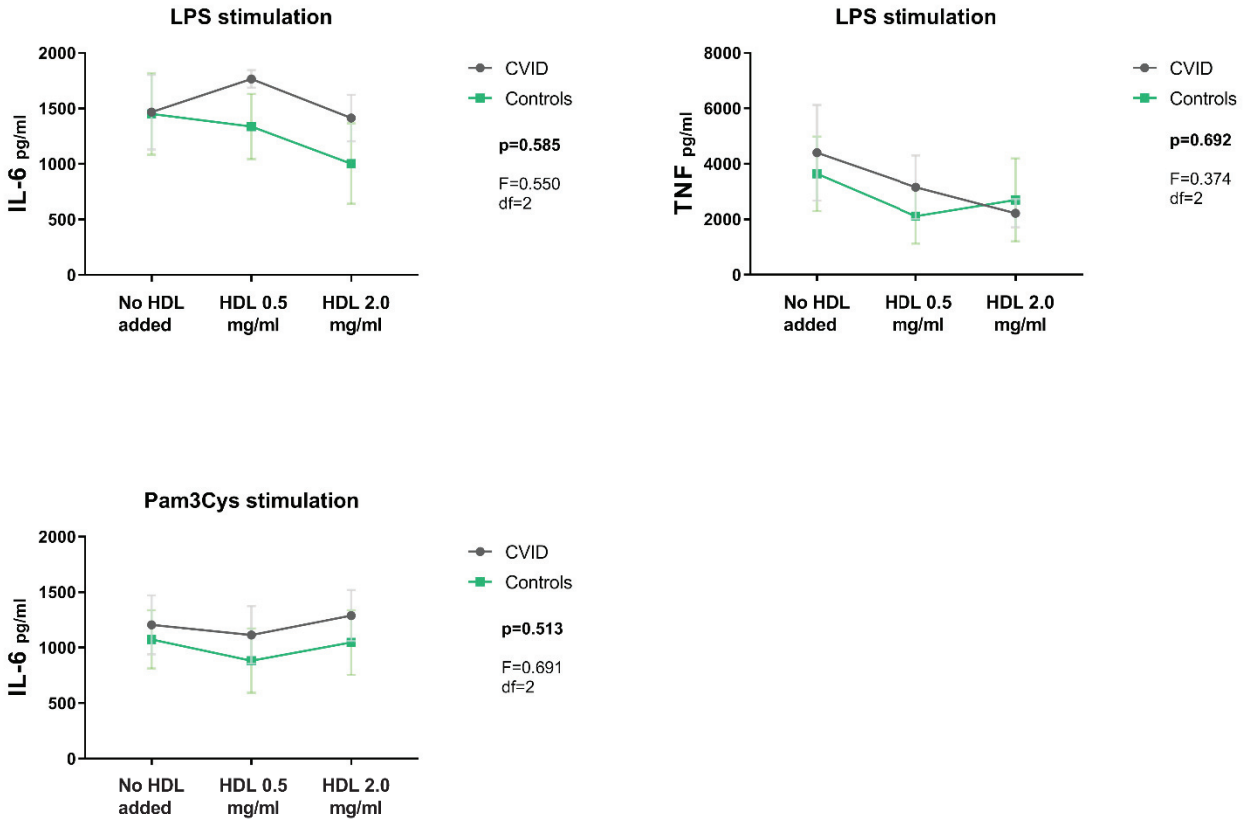
Supplemental Figure S1: HDL measurements stable over time in CVID patients

Longitudinal data for HDL in CVID replication cohort (n=32). p=0.42 using Wilcoxon matched-pair signed rank test. Data presented as mean with SD.



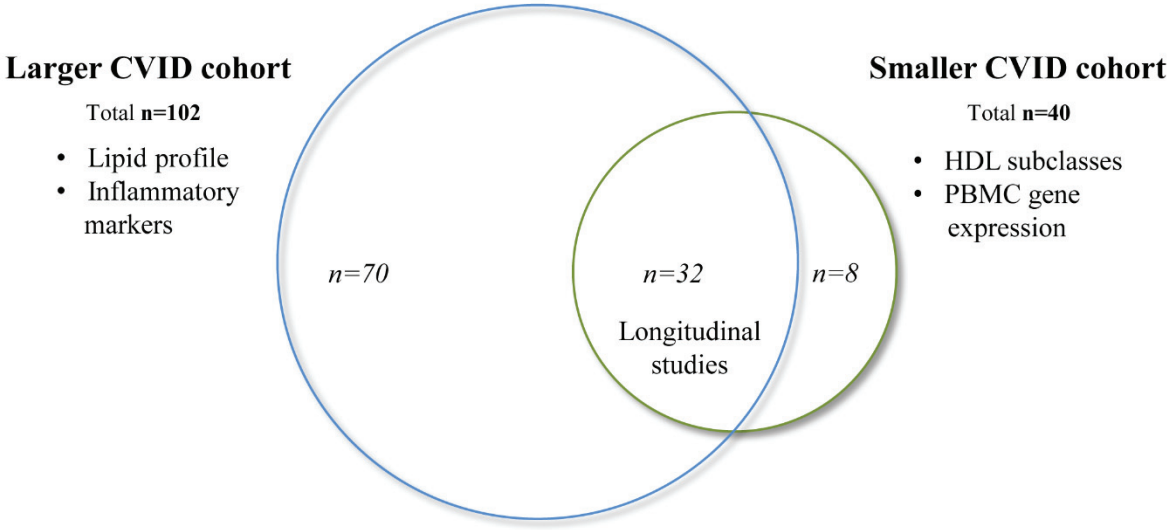
Supplemental figure S2: HDL subclasses in CVID patients and controls

CVID patients (n=40) have significantly lower levels of XL.HDL (*p<0.05), L.HDL (**p<0.01) and M.HDL (*p<0.05) than healthy controls (n=28), p-values calculated using Mann-Whitney test between groups. They also have lower levels of S.HDL than controls, but this difference does not reach statistical significance. Data presented as mean with SD.



Supplemental Figure S3: HDL effects on TLR4-stimulated TNF and IL-6 release and TLR2-stimulated IL-6 release from mononuclear cells from CVID patients (n=6) and healthy controls (n=6)

Results given as median with variance, p-values calculated using repeated measures ANOVA analysis.



Supplemental Figure S4: Larger and smaller CVID patient cohorts

The larger CVID patient cohort (n=102) was collected between November 2011 and December 2012 and the smaller CVID cohort (n=40) was collected between October 2013 and October 2014. For both cohorts the patients were recruited from the outpatient clinic at the Section of Clinical Immunology and Infectious Diseases at Oslo University Hospital Rikshospitalet. The cohorts were somewhat overlapping, allowing for longitudinal studies of HDL-levels over a range of 8-28 months in these 32 patients. In the larger cohort, 74.5% had non-infectious complications, whereas in the smaller cohort 80% of the patients had non-infectious complications.

Supplemental table S1: Phenotype characteristics for CVID cohort

Phenotype	**Complications to CVID (n=102)** Number (%)
Infection only[A]	26 (25.5%)
Non-infectious complications[B]	76 (74.5%)
Splenomegaly	47 (46.1%)
Enteropathy	30 (29.4%)
Granulomas	15 (14.7%)
Organspecific autoimmunity	24 (23.5%)
Autoimmune Cytopenias	22 (21.6%)
Lymphoid Hyperplasia	48 (47.1%)
Lymphoma	1 (1.0%)
Nodular regenerative hyperplasia, liver	4 (3.9%)
Lymphocytic interstitial pneumonia	1 (1.0%)

[A]Infection only; recurrent bacterial airway infections as only clinical manifestation of CVID. [B]Non-infectious complications; exhibit one or more of the below listed autoimmune and inflammatory complications of CVID in addition to recurrent bacterial airway infections. CVID: common variable immunodeficiency.

Supplemental Table S2: Overview of CVID patients and healthy controls contributing to the different analyses and functional studies

HDL subclass analyses

	CVID patients (n=40)	Controls (n=28)	p-value
Age mean ± SD [min-max]	48 ± 12 [18-67]	42 ± 10 [28-65]	0.032[A]
Female number (%)	25 (63%)	18 (64%)	0.881[B]
BMI mean ± SD [min-max]	26 ± 5 [17-38]	24 ± 3 [19-34]	0.077[C]
Non-infectious complications (%)	80%	-	N/A

Gene expression analyses

	CVID patients (n=40)	Controls (n=30)	p-value
Age mean ± SD [min-max]	48 ± 12 [18-67]	43 ± 11 [28-65]	0.083[A]
Female number (%)	25 (63%)	18 (60%)	0.832[B]
BMI mean ± SD [min-max]	26 ± 5 [17-38]	24 ± 3 [19-34]	0.047[C]
Non-infectious complications (%)	80%	-	N/A

Serum cholesterol acceptor studies

	CVID patients (n=18)	Controls (n=10)	p-value
Age mean ± SD [min-max]	46 ± 10 [18-63]	43 ± 11 [28-64]	0.474[A]
Female number (%)	10 (56%)	6 (60%)	0.820[B]
BMI mean ± SD [min-max]	26 ± 5 [17-37]	24 ± 2 [22-27]	0.439[A]
Non-infectious complications (%)	56%	-	N/A

Macrophage cholesterol efflux studies

	CVID patients (n=11)	Controls (n=11)	p-value
Age mean ± SD [min-max]	39 ± 10 [22-54]	41 ± 9 [29-59]	0.717[A]
Female number (%)	9 (82%)	9 (82%)	1.000[B]
BMI mean ± SD [min-max]	26 ± 6 [19-39]	22 ± 2 [19-27]	0.081[A]
Non-infectious complications (%)	100%	-	N/A

HDL effects on TLR-stimulated IL-6 and TNF release in macrophages

	CVID patients (n=6)	Controls (n=6)	p-value
Age mean ± SD [min-max]	40 ± 9 [31-53]	41 ± 7 [33-49]	0.914[A]
Female number (%)	5 (83%)	5 (83%)	1.000[B]
BMI mean ± SD [min-max]	23 ± 5 [19-33]	22 ± 2 [19-25]	1.000[C]
Non-infectious complications (%)	100%	-	N/A

[A]Student's t-test, [B]Pearson Chi square test, [C]Mann Whitney test.

CVID: common variable immunodeficiency. BMI: Body mass index.

Supplemental Table S3: Overview of CVID patients contributing to the different cohorts

Patient	**Larger cohort** (n=102)	**Smaller cohort** (n=40)	**Serum cholesterol acceptor study** (n=18)	**Cholesterol efflux from macrophages study** (n=11)	**HDL level, cytokine release study** (n=6)
1-14	X	X	X		
15	X	X		X	X
16	X	X		X	
17	X	X			X
18	X			X	X
19	X			X	
20	X			X	
21-36	X	X			
37-102	X				
103		X			
104		X			
105		X	X		
106		X	X		
107		X	X		
108		X	X		
109		X		X	
110				X	
111				X	
112				X	
113					X
114					X
115					X
116				X	
117				X	

Supplemental Table S4: Stepwise regression analysis of variables that could predict HDL levels in the CVID cohort (n=102)

Variable	Beta coefficient	p-value
sCD25	-0.52	0.001*
Sex	-0.33	0.034*
BMI	-0.21	0.154
CRP	-0.21	0.194
LPS	-0.19	0.226
Age	0.13	0.413
Smoking	-0.10	0.515
sCD14	0.10	0.583

*p-value <0.05. Soluble CD25 and sex are the strongest predictors of HDL levels.

CVID: common variable immunodeficiency. BMI: Body mass index. sCD25: soluble CD25. CRP: C-reactive protein. sCD14: soluble CD14.

III

GUT MICROBIOTA-DEPENDENT TRIMETHYLAMINE N-OXIDE LINKED TO INFLAMMATION IN COMMON VARIABLE IMMUNODEFICIENCY

Magnhild E. Macpherson[1-3], Johannes R. Hov[1,3,4,5], Thor Ueland[1,3,6], Tuva B. Dahl[1,3,7], Martin Kummen[1,3,4], Kari Otterdal[1], Kristian Holm[1,3,4], Rolf K. Berge[8], Bente Halvorsen [1,3], Pål Aukrust[1-3], Børre Fevang[1,2], Silje F. Jørgensen[1-3]

1. Research Institute of Internal Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital, Rikshospitalet, Oslo, Norway.

2. Section of Clinical Immunology and Infectious Diseases, Department of Rheumatology, Dermatology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Oslo, Norway.

3. Institute of Clinical Medicine, University of Oslo, Oslo, Norway.

4. Norwegian PSC Research Center, Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway

5. Section of Gastroenterology, Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway

6. Faculty of Health Sciences and K.G. Jebsen TREC, University of Tromsø, Tromsø, Norway.

7. Department of Microbiology, Oslo University Hospital HF, Rikshospitalet

8. Department of Clinical Science, University of Bergen, Bergen, Norway.

Abstract

Background: Recurrent infections is the clinical hallmark of common variable immunodeficiency (CVID). However, the majority of patients also have inflammatory and autoimmune complications of unknown etiology. We have previously shown systemic inflammation associated with gut microbial dysbiosis in CVID, including increased abundance of *Gammaproteobacteria*. The gut microbial dependent metabolite trimethylamine N-oxide (TMAO) has been linked to systemic inflammation in other diseases. We hypothesized that TMAO contributes to systemic inflammation in CVID through the gut-microbial axis.

Methods: We measured plasma concentrations of TMAO and inflammatory markers in 104 CVID patients and 30 healthy controls. In addition, we related TMAO levels to gut microbiota profiles available from a previous study (n=40) and a food frequency questionnaire.

Results: CVID patients had higher plasma concentrations of TMAO and its precursors than controls (TMAO 5.0 [2.9-8.6] vs 3.2 [2.2-6.3], p=0.022); carnitine (39.4 [33.4-45.3] vs 34.6 [29.0-38.7], p=0.005); choline (10.3 [8.6-12.2] vs 8.1 [7.0-9.0], p<0.001), betaine (39.7 [30.1-52.8] vs 32.6 [26.1-45.2], p=0.011), all results given as median with IQR. TMAO correlated positively with the prototypic pro-inflammatory cytokines tumor necrosis factor (TNF) (p=0.008, rho=0.259) and interleukin (IL)-12 (p=0.012, rho=0.245) known to be involved in the pathogenesis of CVID-related autoimmune complications. Furthermore, gut microbial abundance of *Gammaproteobacteria* in CVID patients correlated positively with TMAO levels (p=0.021, rho=0.363). Dietary factors had limited impact on plasma TMAO and related metabolites, except negative and positive correlations observed between TMAO and wine consumption (p=0.023, rho=-0.369) and table sugar consumption (p=0.006, rho=0.437), respectively.

Conclusion: CVID patients have increased plasma concentration of the gut microbiota-dependent metabolite TMAO associated with increased inflammatory markers and an abundance of the TMA producing *Gammaproteobacteria* in the gut, suggesting that TMAO could be linked to systemic inflammation in CVID through the gut microbial axis.

Introduction

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency among adults with an estimated prevalence of between 1:25.000 and 1:50.000[1], comprising a clinically and immunologically heterogeneous group. A maturation defect in the B-cell development to plasma cells results in impaired production of immunoglobulins (Ig), leading to the clinical hallmark of CVID; recurrent infections with encapsulated bacteria in the respiratory tract. In addition, ~70% of CVID patients have autoimmune and inflammatory complications[2,3], associated with persistent systemic inflammation and immune activation reflecting abnormalities in other immune cells such as monocytes/macrophages and T-cells[4-8]. At present, however, the mechanisms leading to systemic sterile inflammation and autoimmunity in CVID are not fully understood.

The gut microbiota composition has been linked to systemic inflammation and metabolic disturbances in various systemic metabolic and autoimmune disorders[9-11]. We have previously shown that reduced overall bacterial diversity, together with an abundance of certain 'key' bacteria responsible for gut microbiome dysbiosis in CVID, is associated with signs of systemic immune activation[12]. However, the mechanisms by which an altered gut microbiota translates into systemic inflammation in these patients are not clear.

Carnitine and choline acquired through diet is metabolized in the gut by the microbiota, where carnitine may be converted to the intermediate metabolite γ-butyrobetaine (γBB), before further conversion to trimethylamine (TMA)[13,14]. TMA can then be absorbed from the gut and translocate to the liver via the portal circulation, where it is oxidized to trimethylamine N-oxide (TMAO) by flavin-containing monooxygenases[15,16]. Meat, fish and eggs are particularly rich in choline. Red meat and dairy products are also the main sources of carnitine from the diet, but carnitine can additionally be endogenously

synthesized from trimethyllysine (TML) via γBB[17]. This carnitine –TMAO pathway is illustrated in Figure 1. Several studies, primarily in atherosclerotic and related metabolic disorders, have shown an association between TMAO and systemic inflammation[18-20]. Moreover, recent studies suggest that TMAO is not only a biomarker, but also a potential mediator of inflammation and endothelial cell activation[21-24].

We hypothesized that TMAO could represent a link between altered gut microbiota and systemic inflammation in CVID. To explore this, we measured TMAO and the other metabolites in the carnitine-TMAO pathway in CVID patients and healthy controls, before relating these metabolites to markers of systemic inflammation, gut microbiota composition and diet.

Results

Patient characteristics

We recruited 104 CVID patients consecutively over 13 months from the outpatient clinic at Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet. For comparison, we included 30 healthy controls (Table 1). In addition, n=40 CVID patients from a previous intervention trial targeting the gut microbiota were included[25] (Table 1), out of which n=32 were also included in the main study cohort at a previous time point. For this subset (n=40), coinciding stool samples and blood samples were collected for comparison of gut microbiota and circulating metabolites. These patients were also asked to fill in a food frequency questionnaire (FFQ), which included dietary supplements (completed by 38 of the 40 patients).

Metabolites in the carnitine-TMAO pathway in CVID and healthy controls

The CVID patients had significantly elevated plasma levels of TMAO (p=0.022, Figure 2a) and the TMA precursor carnitine (p=0.005, Figure 2b) as compared to healthy controls. In addition to carnitine, choline and betaine are TMA precursors and these metabolites were also elevated in CVID patients compared to healthy controls (p<0.001 and p=0.011, respectively; Figure 3a-b). Finally, CVID patients had raised levels of the endogenous carnitine precursor TML (p=0.011, Figure 3c), along with γ-BB (p=0.023, Figure 3d) which is both a precursor to endogenous carnitine and a metabolite from dietary carnitine. Thus, our data show that all examined metabolites of the carnitine-TMAO pathway are elevated in CVID patients as compared with healthy controls, including the end-product TMAO.

TMAO in relation to systemic inflammation

We and others have previously demonstrated increased inflammatory cytokines such as interleukin (IL)-6, IL-8, IL-12, and tumor necrosis factor (TNF) in CVID[26-28]. These cytokines were all markedly elevated in the CVID patients (n=104) compared with controls[12]. We therefore

selected these inflammatory markers to explore the association between systemic inflammation and TMAO. We found plasma levels of TMAO to be positively associated with plasma levels of TNF (p=0.008, rho=0.259) and IL-12 (p=0.012, rho=0.245; Figure 4), but not IL-6 (p=0.263, rho=0.111) or IL-8 (p=0.214, rho=0.123), linking TMAO to systemic inflammation in CVID.

Metabolites of the carnitine-TMAO pathway in relation to clinical characteristics of the CVID patients

When dividing the CVID patients (n=104) into two major subgroups by phenotype (i.e., infection only [n=25] and patients with inflammatory and autoimmune manifestations [n=79]), we found no differences in any of the measured metabolites between the two phenotypes (Supplemental Table S1). Both age and sex were factors found to correlate significantly with metabolites in the carnitine-TMAO pathway in our dataset and all p-values presented above comparing levels in CVID patients and controls were therefore adjusted to take into account these factors. BMI has previously been found to correlate positively with TMAO in some studies[29,30], but we did not find this in our dataset (Supplemental Table S2). Finally, kidney and liver function could potentially influence TMAO levels, but we found no significant difference between the patients and controls for these parameters (estimated glomerular filtration rate [eGFR] median <60 vs >60, p=0.277 and ALT median 23 vs 22, p=0.747), nor did they correlate significantly with TMAO in our patient cohort (Supplemental Table S2).

Metabolites of the carnitine-TMAO pathway in relation to gut microbiota

We found that plasma levels of lipopolysaccharide (LPS), a marker of gut leakage, were significantly correlated with TMAO (p=0.034, rho=0.208) and the TMA precursors carnitine (p=0.008, rho=0.257) and γ-BB (p=0.029, rho=0.214) in our CVID patients (n=104), indicating

a relation between gut leakage and TMAO pathway metabolites (Figure 5a-c). We have previously shown increased LPS to correlate with reduced bacterial richness in CVID. Using existing gut microbiota data from our CVID patients (n=40), we further explored if specific bacterial taxa previously found to differentiate between CVID patients and healthy controls correlated with the different carnitine-TMAO pathway metabolites in plasma. These included the ten taxa making up the CVID dysbiosis index found to capture CVID dysbiosis in the gut[12]: *Bacilli, Dorea, Roseburia, Gammaproteobacteria* (increased in CVID), and *Bifidobacterium, Odoribacteracea, Christensenellaceae, Blautia, Sutterella, Desulfovibrionacea* (reduced in CVID). In addition, we added five taxa identified to differ between CVID and healthy controls in another publication using a different statistical approach (ANCOM)[31]: *Hungatella, Flavonifractor, Veillonella* and *Escherichia-Shigella* (increased in CVID) and *Christensenellaceae R-7 group* (reduced in CVID). We found the abundance of *Gammaproteobacteria* in stool samples from CVID patients to correlate positively with levels of TMAO in plasma (p=0.021, rho=0.363; Figure 6a). On genus level, we found positive correlations between abundance of *Escherichia-Shigella* in stool samples and plasma levels of TMAO (p=0.031, r=0.342; Figure 6b) and γ-BB (p=0.046, r=0.318) in CVID patients. *Blautia*, a *Gram-positive* bacterial genus previously shown to be reduced in CVID patients, was negatively correlated with the TMA precursor betaine (p=0.037, r=-0.331). Collectively, our findings suggest that a gut microbiome more abundant in *Gram-negative* bacteria, harboring important pathogens in this patient group, is associated with elevated plasma levels of metabolites involved in the carnitine-TMAO-pathway.

Metabolites of the carnitine-TMAO pathway in relation to diet

A detailed self-reported food frequency questionnaire was obtained from 38 CVID patients. CVID patients did not appear to consume more meat, fish, eggs or milk/yoghurt compared to

a reference population[32] (Supplemental Figure S1). However, wine consumption correlated negatively with TMAO (p=0.023, rho=-0.369), carnitine (p=0.005, rho=-0.442), betaine (p=0.014, rho=-0.395), choline (p=0.004, rho=-0.460), γ-BB (p=0.001, rho=-0.507) and TML (p=0.016, rho=-0.388). Furthermore, when separating wine consumption into red wine and white wine, the negative correlation with TMAO, corrected for age and sex, in CVID patients remained significant for red wine (p=0.018, β=-0.332) but not for white wine (p=0.420, β=-0.120). We also found that intake of table sugar, but not total sugar consumption, was associated with increased plasma levels of TMAO (p=0.006, rho=0.437), γ-BB (p=0.045, rho=0.327), betaine (p=0.016, rho=0.389) and choline (p=0.005, rho=0.445; Table 2). Dietary intake of meat, fish, dairy products and fiber were not found significantly associated with any of the carnitine-TMAO pathway metabolites in plasma.

Metabolites of the carnitine-TMAO pathway are stable over an 8-week period

To study the temporal course of carnitine-TMAO pathway metabolites in our patient cohort, we measured the levels in 20 CVID patients (mean age 51.2 years ± 11.8 [SD], 13 (65%) women) three times over eight weeks. Sixteen patients completed all three measurements. Applying the Friedman test, we discovered no significant changes in levels of TMAO (p=0.444), carnitine (p=0.144), betaine (p=0.174), choline (p=0.472), γ-BB (p=0.646) or TML (p=0.570) amongst the patients (Supplemental Figure S2). This suggests that the plasma levels of these metabolites are relatively stable over time in our CVID cohort, rather than the results reflecting random fluctuations.

TMAO in relation to antibiotic use

It has been shown that short-term antibiotic use reduces TMAO levels in plasma of both healthy humans and mouse models of cardiovascular disease[33,34]. Due to an increased burden of infections, CVID patients use more antibiotics per year compared to the general

population[35]. We wanted to explore if antibiotic use had an effect on TMAO levels in CVID. Forty of our 104 CVID patients had reported the number of antibiotic courses they took in the last year, representing a subgroup previously described[12]. We found the number of antibiotic courses in the last year to correlate negatively with plasma levels of TMAO (p=0.033, rho=-0.339) (Supplemental Figure S3). Thus, it appears that not only short-term antibiotic use, but also the accumulative effect of repeated antibiotic use over a year affects TMAO levels. Despite this, CVID patients overall have raised TMAO levels compared to controls.

Discussion

Our study shows that CVID patients have elevated plasma levels of TMAO and its precursors carnitine, choline, betaine, y-BB and TML compared to healthy controls. Furthermore, the elevated levels of TMAO are positively correlated with prototypical CVID inflammatory markers TNF and IL-12. Moreover, we found the abundance of certain *Gram-negative* bacteria in the gut of CVID patients to associate with increased levels of TMAO. Our findings suggest a link between altered gut microbiota, TMAO production and systemic inflammation in CVID patients (Figure 7).

Studies have indicated an association between TMAO and inflammation in various diseases and particularly in metabolic and cardiovascular disorders[34,36,37]. The present study is, to the best of our knowledge, the first study to show a similar pattern in CVID. Here, we show that CVID patients have higher levels of the microbiota-dependent metabolite TMAO and its precursors in plasma compared to healthy controls, significantly correlated with the prototypical inflammatory cytokines TNF and IL-12, both known to be elevated in CVID[27,28]. Furthermore, our results show that intake of food known to increase TMAO and its related metabolites cannot explain the increased TMAO concentration seen in CVID patients compared to controls. Moreover, other mechanisms than diet, such as gut microbes, seem largely responsible for the increased TMAO levels in CVID, although certain dietary factors such as red wine appear to be able to affect TMAO in a suppressive way whereas dietary table sugar may elevate TMAO.

It has been proposed that TMAO is not only a marker but also a mediator of inflammation[38]. Mechanistic studies have demonstrated TMAO to trigger activation of the NLRP3 inflammasome in endothelial cells, involving increased production of reactive oxygen species[21,22]. Additionally, mice studies have demonstrated that dietary supplements of TMAO

result in reduced reverse cholesterol transport *in vivo*[39]. In line with this, we have recently shown that CVID patients with non-infectious complications have impaired reverse cholesterol transport, potentially contributing to systemic inflammation in these patients[40]. Our findings suggest that TMAO could contribute to this link between metabolic disturbances and inflammation in CVID.

Plasma levels of TMAO can be influenced by several factors including age, sex, BMI, diet, liver and kidney function. Herein, we show that CVID patients have raised TMAO levels compared with healthy controls also when adjusting for age and sex. Furthermore, we find a positive association between abundance of *Gammaproteobacteria* and *Escherichia-Shigella* in the gut microbiome of CVID patients and levels of TMAO in plasma. Supporting our findings, *Gammaproteobacteria* have previously been shown to produce TMA in the gut of HIV patients[41]. Moreover, several strains of *Escherichia coli* have been identified to degrade choline or carnitine to TMA by containing the catalytic enzymes *cut*C/D or *cnt*A/B[14]. Thus, with the CVID gut microbiome abundant in *Gammaproteobacteria,* this could contribute to elevated plasma levels of TMAO and subsequently increased systemic inflammation. Based on the present findings we suggest that the TMAO pathway could be a missing link between altered gut microbiota and systemic inflammation in CVID.

The gut microbiota is capable of rapidly adapting to our dietary intake[42,43], which is a key determinant of gut microbial composition. In the present study, there was no association between the main components of the diet (i.e., meat, fish, dairy products and fiber) and TMAO in the CVID patients. The negative correlation between consumption of red wine and plasma TMAO is however noteworthy, since red wine may contain compounds inhibiting of microbial TMA formation, reducing the potential for hepatic TMAO synthesis. The choline analogue

DMB (3,3-dimethyl-1-butanol), which can be found in red wine, has been shown to significantly reduce plasma levels of TMAO in mice[44], and has been used as targeted therapy to reduce gut microbial TMA formation and experimental atherosclerosis[45]. These observations suggest that studies of whether red wine may reduce TMAO in humans are warranted and could have therapeutic implications.

The strengths of this study, compared to other TMAO studies, are the inclusion of gut microbiota and dietary data allowing us to investigate carnitine-TMAO pathway metabolites in more detail. In addition, the total number of patients in the TMAO metabolite and inflammatory marker analyses was substantial considering the prevalence of CVID. Nevertheless, it also has its limitations, including not having dietary information and gut microbial data on all CVID patients. Moreover, associations do not necessarily signify causal relationship, thus more mechanistic studies are also needed to further explore the role of TMAO in persistent inflammation and immune activation such as seen in CVID.

In conclusion, the TMAO concentration is elevated in CVID patients compared to healthy controls and correlates with markers of systemic inflammation. TMAO could be a biomarker for inflammation in CVID, but potentially also a mediator of inflammation in these patients. Certain 'key' bacteria with increased prevalence in the gut microbiome of CVID patients are associated with raised TMAO, suggesting the gut microbiota could be therapeutically targeted through diet or medical interventions to reduce TMAO formation and thus systemic inflammation. This concept could be applicable to other inflammatory conditions characterized by systemic inflammation, increased TMAO and gut dysbiosis in future studies.

Methods

Ethics

The Regional Committee for Medical and Research Ethics approved the study protocol. All study participants signed a written, informed consent.

Patient and control cohorts

CVID patients were recruited from the outpatient clinic at the Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital (OUH) Rikshospitalet. CVID was defined as decreased serum levels of IgG, IgA and/or IgM by a minimum of two standard deviations below the mean for age, and exclusion of other causes of hypogammaglobulinemia[46].

Blood sampling protocol

Peripheral venous blood was collected into sterile blood collection tubes without additives (serum) or with EDTA as an anticoagulant (plasma). The tubes were immediately immersed in melting ice, centrifuged within 15 minutes at 2000*g* for 20 minutes to obtain platelet-poor plasma or were left to clot at room temperature prior to centrifugation at 1000*g* for 10 minutes (serum). Plasma and serum were stored at -80°C in multiple aliquots.

Analyses of inflammatory markers

Plasma levels of tumor necrosis factor (TNF) and interleukin (IL) -6, IL-8 and IL-12 were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA). The samples were analyzed on a Multiplex Analyzer (Bio-Rad Laboratories) according to instructions from the manufacturer. Lipopolysaccharide (LPS) was analyzed by Limulus Amebocyte Lysate chromogenic assay (Lonza, Walkersville, MD) according to the manufacturer's instructions, with the following modifications: Samples were diluted 5-fold to avoid interference with background color, and preheated to 67 °C for 12 minutes prior to analysis to dissolve immune complexes.

Measurement of carnitine, TMAO and related metabolites

Free carnitine and the precursors for carnitine, TML and yBB, were analyzed in plasma using MS/MS as described previously[47] with some modifications of the high-performance liquid chromatography (HPLC) conditions: The LC system was an Agilent (Waldbronn, Germany) 1200 Series with binary pump, variable volume injector, and a thermostated autosampler. HPLC separation was conducted at 30 °C using a gradient solvent mixture. Mobile phase A was made of 10 mM ammonium acetate and 12 mM heptafluorobutyric acid (HFBA) in water, and mobile phase B was made of 10 mM ammonium acetate and 12 mM HFBA in methanol. The gradient was B 0.1 min 20%, flow 0.2 mL/min; B 4 min 20–90%, flow 0.2 mL/min; B 14 min 90%, flow 0.2 mL/min; B 10 min 2%, flow 0.6 mL/min; B 0.1 min 20%, flow 0.2 mL/min. A Phenomenex Luna C8 column (5 µm, 150 × 2 mm) equipped with a Phenomenex C18 pre-column, (4.0 × 2.0 mm) was used. Two µl of the sample were injected. Levels of TMAO, choline and betaine were measured using the same assay as used to determine the concentrations of carnitine. Stable isotope dilution liquid chromatography–tandem mass spectrometry (LC/MS/MS) was used for the quantification of TMAO, choline and betaine; all three were monitored in positive liquid chromatography–tandem mass spectrometry (MRM) MS mode using characteristic precursor–product ion transitions: m/z 76→58, m/z 104→60 and m/z 118→58, respectively. The internal standards TMAO-trimethyl-d9 (d9-TMAO), choline-trimethyl-d9 (d9-choline) and betaine-trimethyl-d9-methylene-d2 (d11-betaine) were added to plasma samples before protein precipitation and were similarly monitored in MRM mode at m/z 85→66, m/z 113→69 and m/z 129→66, respectively. Various concentrations of TMAO, choline and betaine standards and a fixed amount of internal standards were spiked into 4% bovine serum albumin to prepare the calibration curves for the quantification of plasma analytes. All stable isotope-labelled

internal standards were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Microbiota analyses

Participants collected stool samples at home within 24 hours prior to their hospital visit, or alternatively at the hospital, with a standardized collection device[48]. The samples were then transferred to Stool Collection Tubes with Stool DNA Stabilizer (Stratec Biomedical, Birkenfeld, Germany)[49] and immediately stored at at least -20°C according to the manufacturer's recommendations until DNA extraction. Bacterial DNA was extracted using PSP Spin Stool DNA Plus Kit (Stratec) before being subjected to high-throughput sequencing of the 16S ribosomal RNA gene with dual-indexed barcodes according to an established protocol[50], followed by sequencing on an Illumina MiSeq. Briefly, the hypervariable V3-V4 region was amplified with generic primers as described in detail[50], including the gene specific primer sequences CCTACGGGAGGCAGCAG (forward) and GGACTACHVGGGTWTCTAAT (reverse) and up to 192 (24x8 barcodes) samples in parallel. Cleanup and normalization were performed using the SequalPrep Normalization Plate Kit (Life Technologies), followed by pooling and quality control. The final libraries were sequenced on an Illumina MiSeq (Norwegian Sequencing Centre, Oslo University Hospital Ullevål) using the v3 kit, allowing up to 300 basepairs paired-end sequencing. Paired-end reads were quality trimmed with cutadapt version 1.13 and then merged using FLASH version 1.2.11. The merged reads were de-multiplexed and quality filtered using default values in Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1. Closed reference operational taxonomic unit (OTU) mapping to the Silva database (version 123, reference OTUs clustered at 97% sequence similarity) was performed using SortMeRNA version 2.0 through QIIME. A rarefied OTU table

(9525 reads per sample) was generated and OTUs with less than 2 reads to support it were discarded.

Food frequency questionnaire

CVID patients were asked to complete a self-administrated validated Norwegian food frequency questionnaire designed to reflect dietary habits over the past year[51,52]. The questionnaire offers multiple choice alternatives and also the opportunity to provide supplementary information regarding specific dietary restrictions or habits. It covers 180 food items and has serving size alternatives specified in households units, which is then converted to grams per day using a software developed at the Institute for Nutrition Research, University of Oslo[51]. We selected food items that had previously been described to be associated with metabolites in the carnitine-TMAO pathway; meat, fish, egg and dairy products[15], and food items relevant to gut microbiota and inflammation: fiber, protein, wine and sugar[53-55] for association analyses with TMAO.

Statistical analyses

The datasets for TMAO pathway metabolites were not normally distributed and so the data was log transformed. Where normal distribution was achieved, we continued with multivariate testing and where normal distribution was not achieved, we applied Mann-Whitney non-parametric testing. Depending on the data distribution, we performed correlation analyses with either Spearman's rank correlation test (correlation coefficient rho) or Pearson's correlation test (correlation coefficient r). Additionally, stepwise linear regression analysis was performed to adjust for variables such as age and sex. For the longitudinal data, we compared datasets from three different time points using the Friedman test. Calculations were performed in SPSS (version 24, IBM, NY).

Conflict of interest

JRH has served on Advisory boards for Orkla Health and Novartis, and received research support from Biogen, all unrelated to the present study.

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Figures

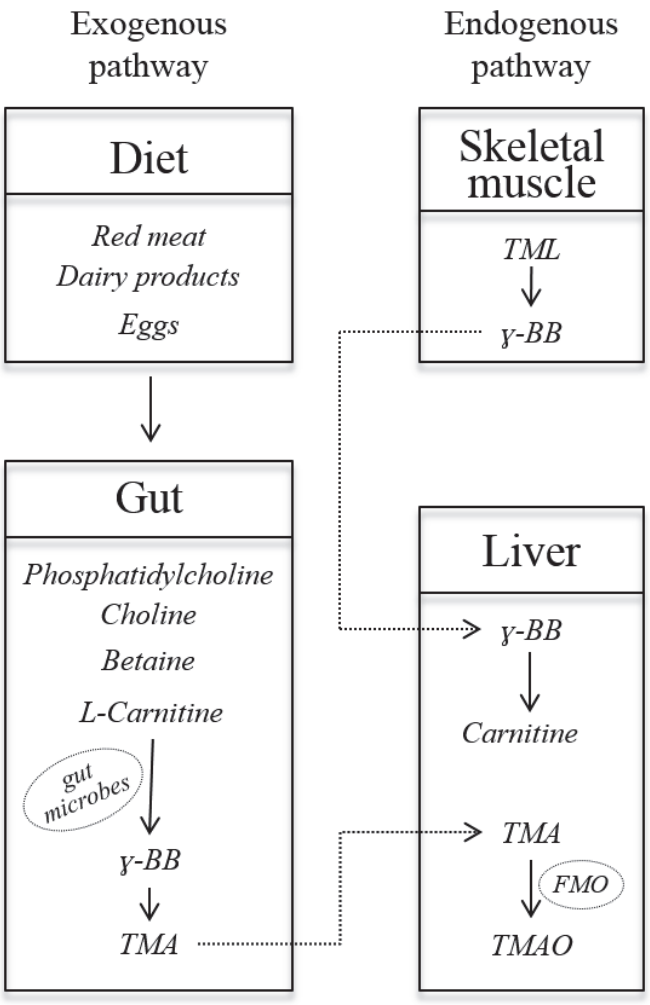


Figure 1: The carnitine - TMAO pathway

Exogenous and endogenous pathways for carnitine and trimethylamine N-oxide (TMAO) formation. Gut microbes metabolize dietary choline, choline-containing compounds, L-carnitine and its intermediate metabolite γ-butyrobetaine (γ-BB) to trimethylamine (TMA). TMA is passively absorbed from the gut to the portal circulation and delivered to the liver, where it is oxidized by flavin-containing monooxygenases (FMO) to TMAO. Trimethyllysine (TML), mainly present in skeletal muscle, is converted to γ-BB before it is transported in plasma to the liver and converted to carnitine.

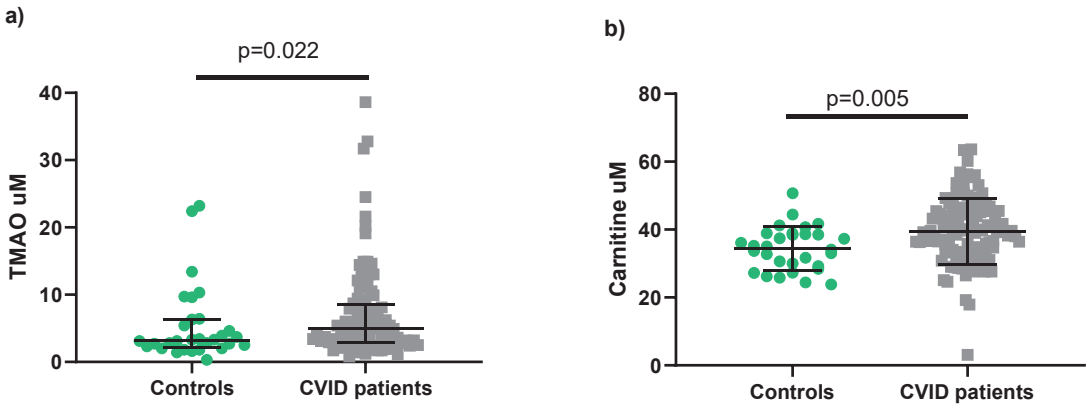


Figure 2: TMAO and Carnitine levels in CVID patients and controls

Plasma levels of trimethylamine N-oxide (TMAO) and carnitine in common variable immunodeficiency (CVID) patients (n=104) and healthy controls (n=30). P-values corrected for age and sex using stepwise linear regression analyses. Each dot represents one individual; bars represent median levels with interquartile range.

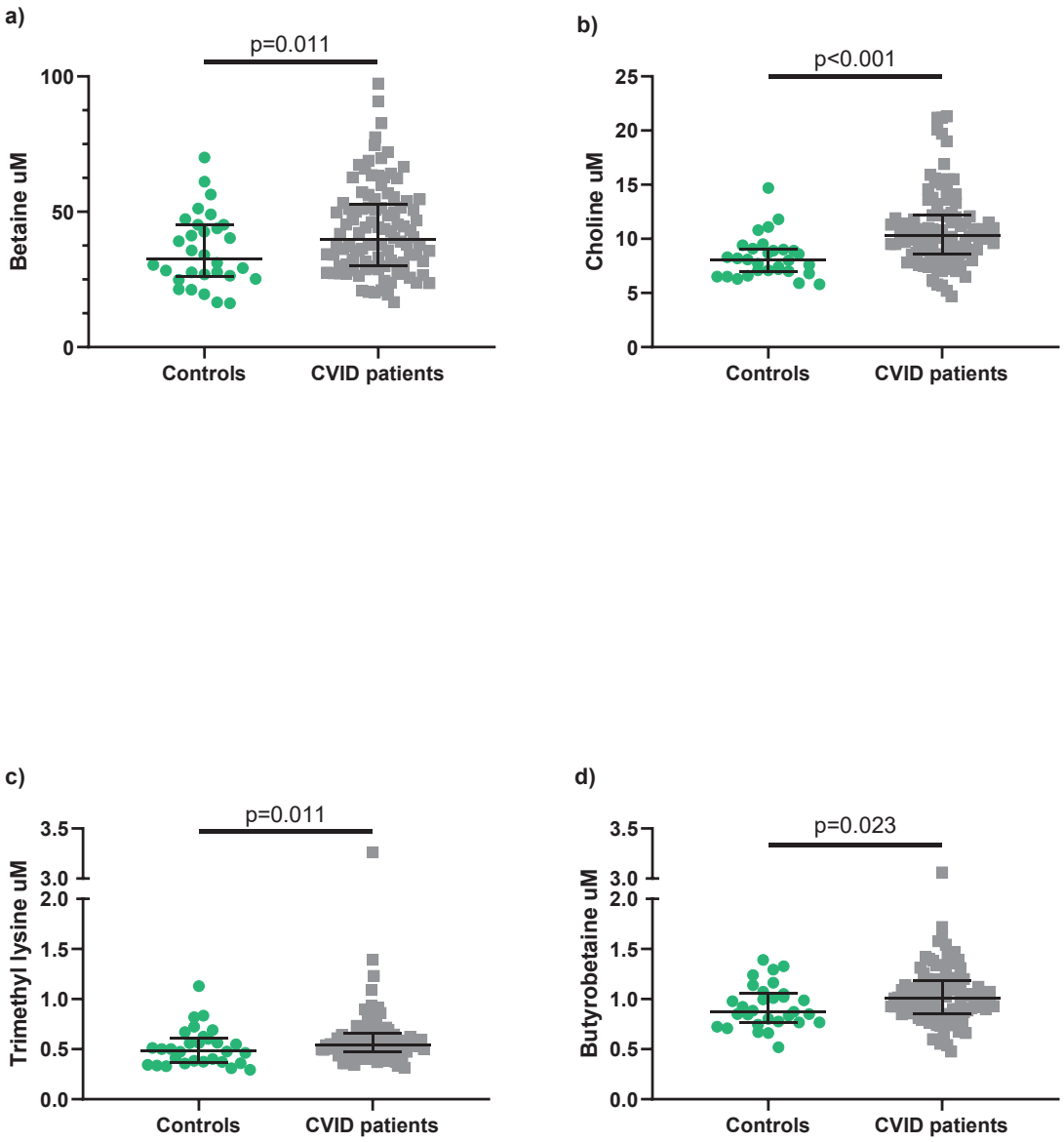


Figure 3: TMAO pathway metabolite levels in CVID patients and controls

Plasma levels of a) betaine, b) choline, c) trimethyl lysine and d) γ-butyrobetaine are all higher in CVID patients (n=104) than in healthy controls (n=30). P-values corrected for age and sex using stepwise linear regression. Each dot represents an individual and bars represent median levels with interquartile range.

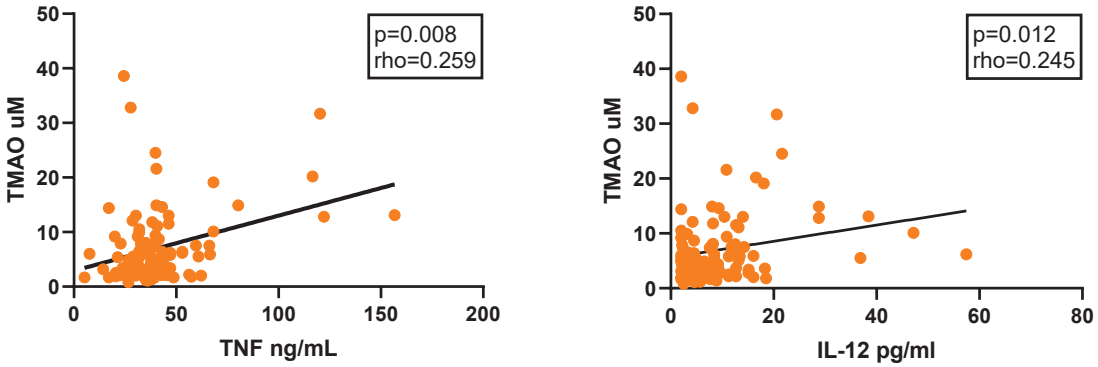


Figure 4: Correlations between TMAO and inflammatory markers

Plasma levels of TMAO correlate positively with both TNF and IL-12 in CVID patients (n=104). Two-tailed p-values were calculated using Spearman's rank correlation.

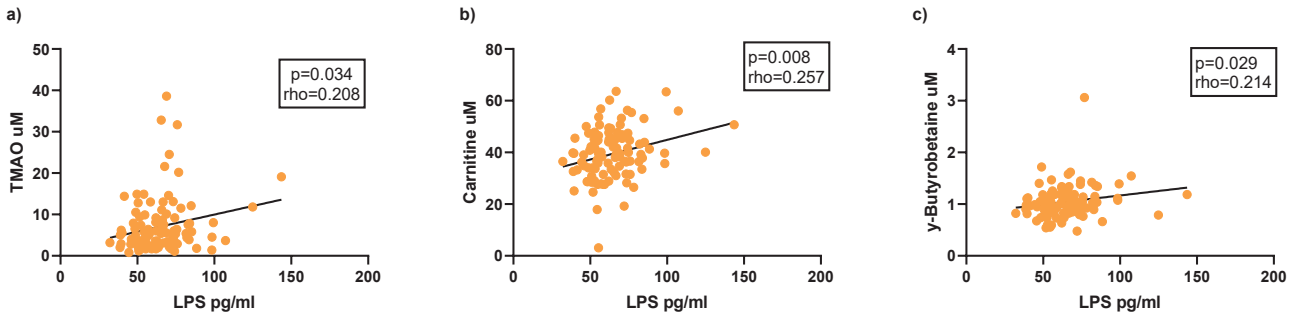


Figure 5: Correlations between carnitine-TMAO metabolites and LPS

Plasma levels of LPS correlate positively with a) TMAO, b) Carnitine and c) γ-Butyrobetaine in CVID patients (n=104). Two-tailed p-values were calculated using Spearman's rank correlation.

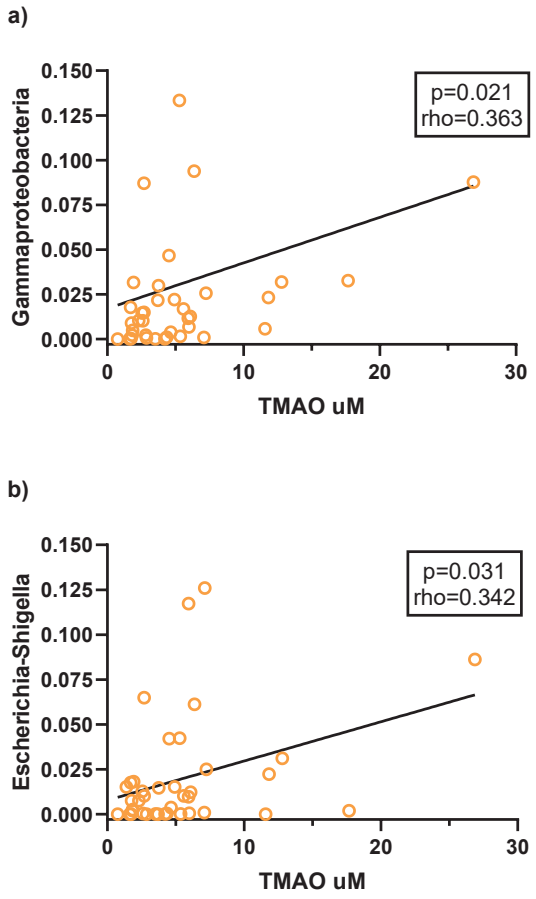


Figure 6: Abundant taxa in CVID gut microbiota correlating with TMAO

Gammaproteobacteria (a) and *Escherichia-Shigella* (b), two taxa found in abundance in the gut microbiota of CVID patients (n=40), correlate positively with plasma levels of TMAO. In panel (a) one outlier is not shown (*Gammaproteobacteria* 0.5, TMAO 1.4 uM). Two-tailed p-values were calculated using Spearman's rank correlation.

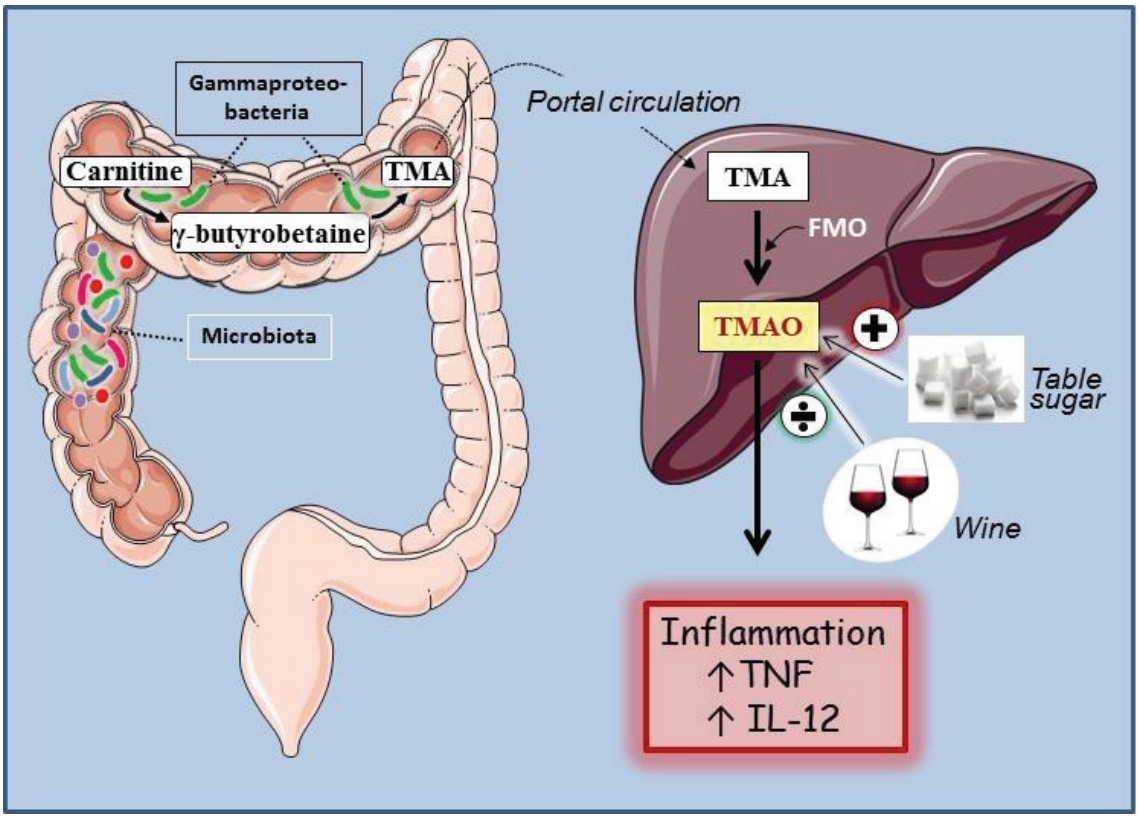


Figure 7: Gut microbiome, carnitine-TMAO pathway, systemic inflammation and dietary factors in CVID patients

CVID patients have an abundance of several Gram-negative taxa such as *Gammaproteobacteria* and *Escherichia-Shigella* in their gut microbiome. These bacteria appear to amplify the conversion of Carnitine to Trimethylamine (TMA) in the gut, which is further oxidized to Trimethylamine N-oxide (TMAO) in the liver. Plasma levels of TMAO are elevated in CVID patients compared to controls, and positively associated with inflammatory markers TNF and IL-12. Dietary factors such as intake of table sugar correlates with higher TMAO levels in plasma, whereas intake of red wine appears to reduce TMAO plasma levels, likely via the gut-microbiota axis.

Tables

Table 1: Background characteristics and non-infectious complications for the main and subset CVID cohort and healthy controls

	Main cohort		Subset cohort	
	CVID n=104	**Controls** n=30	**CVID** n=40	**Controls** n=30
Age in years	46 ± 15	47 ± 13	48 ± 12	50 ± 13
Female	51 (49)	14 (47)	25 (63)	18 (60)
BMI*	24 ± 4	24 ± 3	26 ± 5	25 ± 4
IVIG	19 (18)	-	6 (15)	-
SCIG	76 (73)	-	30 (75)	-
IVIG and SCIG**	7 (7)	-	4 (10)	-
Infection only	25 (24)	-	8 (20)	-
Non-infectious complications	79 (76)	-	32 (80)	-
Splenomegaly	53 (51)	-	16 (40)	-
Enteropathy	27 (26)	-	13 (33)	-
Granulomas	17 (16)	-	6 (15)	-
Organspecific autoimmunity	20 (19)	-	8 (20)	-
Autoimmune Cytopenias	22 (21)	-	8 (20)	-
Lymphoid Hyperplasia	51 (49)	-	23 (56)	-
Nodular regenerative hyperplasia, liver	4 (4)	-	1 (2.5)	-
Lymphocytic interstitial pneumonitis	2 (2)	-	1 (2.5)	-

Continuous data given as mean ± SD and categorical data as n (%). Intravenous immunoglobulins (IVIG). Subcutaneous immunoglobulin (SCIG). *Body Mass Index (BMI) data missing for four of the healthy controls in the subset cohort. **Two CVID patients did not receive any immunoglobulin substitution. Patients divided into subgroups of infection only and various non-infectious complications as previously defined by H. Chapel et al[3].

Table 2: Correlations between dietary intake and TMAO pathway metabolites

	Table sugar		**Wine**	
	p-value	Spearman's rho	p-value	Spearman's rho
Trimethylamine N-Oxide (TMAO)	0.006	0.44	0.023	-0.37
Carnitine	0.257	0.19	0.005	-0.44
Choline	0.005	0.45	0.004	-0.46
Betaine	0.016	0.39	0.014	-0.40
Y-Butyrobetaine	0.045	0.33	0.001	-0.51
Trimethyl lysine	0.053	0.32	0.016	-0.39

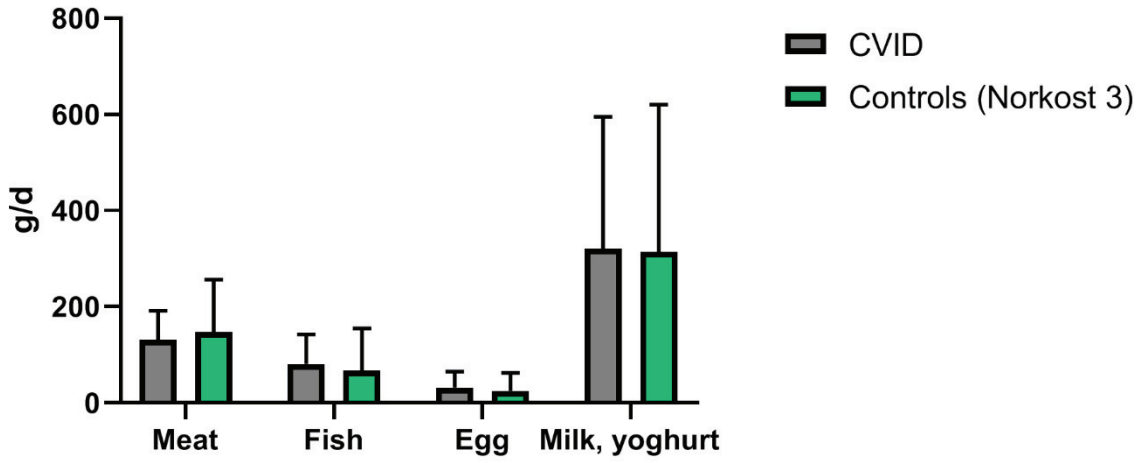
Analyses performed using Spearman's rank-order correlation applied to the CVID subset cohort, where all had plasma levels of the carnitine-TMAO metabolites measured and all but two returned the dietary questionnaire; n=38.

SUPPLEMENTAL MATERIAL

GUT MICROBIOTA-DEPENDENT TRIMETHYLAMINE N-OXIDE LINKED TO INFLAMMATION IN COMMON VARIABLE IMMUNODEFICIENCY

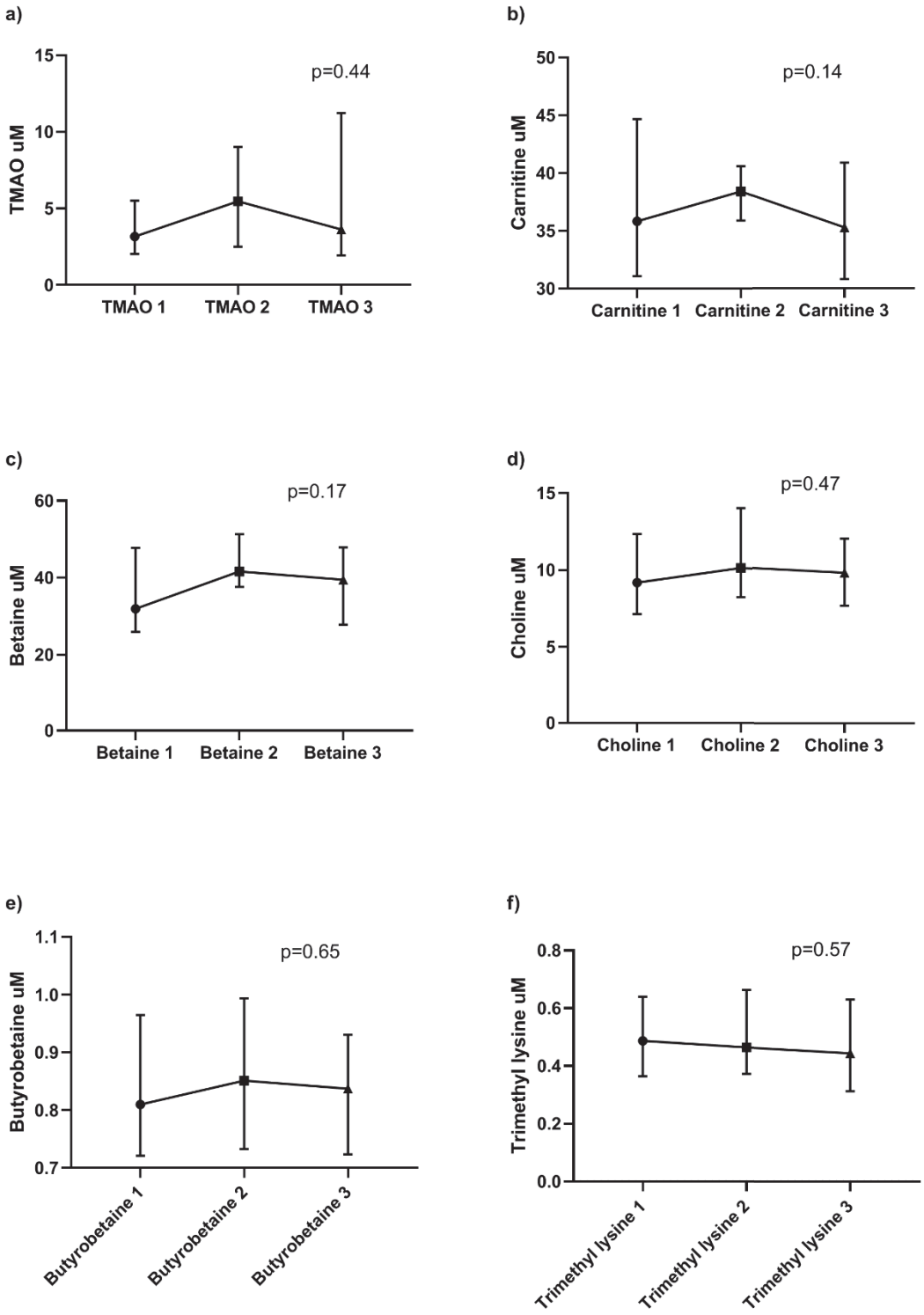
Magnhild E. Macpherson, Johannes E. R. Hov, Thor Ueland, Tuva B. Dahl, Martin Kummen, Kari Otterdal, Kristian Holm, Rolf K. Berge, Bente E. Halvorsen, Pål Aukrust, Børre Fevang, Silje F. Jørgensen

Table of content:



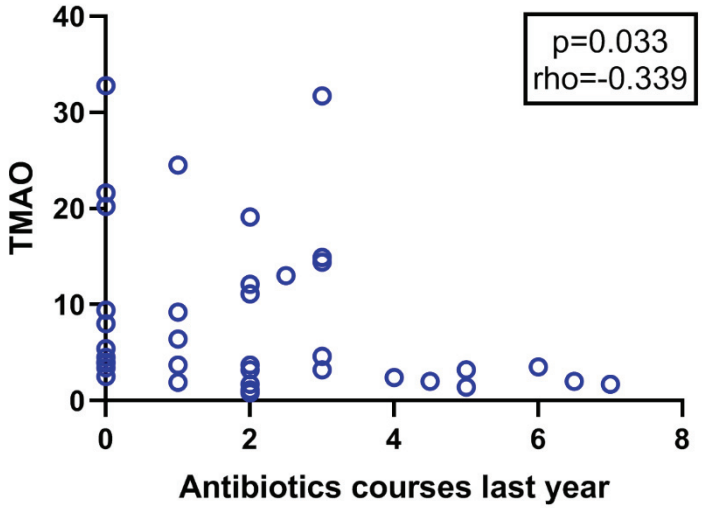
Supplemental Figure S1: Dietary intake amongst CVID patients vs reference population

Daily intake of meat, fish, egg, milk and yoghurt in the CVID patient cohort (n=38) versus the Norwegian reference population (Norkost3, n=1787). Results shown as mean with SD. We found non-significant differences between the groups when applying multiple t-test analysis for meat (p=0.37), fish (p=0.33), egg (p=0.27) and milk/yoghurt (p=0.89) intake. The Norkost3 study used a 24 hour recall questionnaire, aiding accuracy of reported diet, whilst using a high *n* to reduce the seasonal and day-to-day variation.



Supplemental figure S2: Temporal testing of TMAO pathway metabolites

Plasma levels over three time points for a) TMAO, b) carnitine, c) betaine, d) choline, e) butyrobetaine and f) trimethyl lysine in CVID patients (n=16). The patients had no significant change in levels of these metabolites over time, p-values calculated using the Friedman test. Results are shown as median with interquartile range.



Supplemental figure S3: TMAO levels correlating with the use of antibiotics

Plasma TMAO levels in CVID patients (n=40) correlate negatively with the number of antibiotics courses they have used in the last year. Two-tailed p-value calculated using Spearman's rank correlation.

Supplemental Table S1: Metabolites of the TMAO pathway in CVID subgroups by phenotype (n=104)

	Infections only (n=25)	**Non-infectious complications** (n=79)	**p-value**
Trimethylamine N-Oxide (TMAO)	6.2 ± 4.0	7.3 ± 7.3	0.922[a]
Carnitine	40.6 ± 9.6	39.0 ± 9.8	0.502[a]
Choline	10.9 ± 3.9	10.9 ± 3.4	0.857[a]
Betaine	44.7 ± 14.7	42.8 ± 17.0	0.466[a]
Y-Butyrobetaine	1.1 ± 0.2	1.0 ± 0.3	0.221[a]
Trimethyl lysine	0.6 ± 0.2	0.6 ± 0.4	0.724[b]

Continuous data given as mean ± SD. Where datasets were not normally distributed, we log-transformed the data and used parametric analyses where normal distribution was achieved. Where normal distribution was not achieved after log-transformation, we used non-parametric testing; statistical analysis between groups performed using [a]Student's t-test and [b]Mann Whitney test as appropriate.

Supplemental Table S2: Correlations between TMAO and patient characteristics (n=104)

	TMAO	
	p-value	Spearman's rho
Age	<0.001	0.36
Sex	0.015	0.24
BMI	0.802	-0.03
eGFR	0.059	-0.19
ALT	0.789	0.03

Correlations between TMAO and central characteristics of CVID patients, analyses performed using Spearman's rank order correlation with two-tailed p-levels. Age and sex were the two variables reaching significant correlation with TMAO.