From DEPARTMENT OF LABORATORY MEDICINE, DIVISION OF CLINICAL MICROBIOLOGY
Karolinska Institutet, Stockholm, Sweden

GUT DERIVED CHRONIC INFLAMMATION IN HIV-1 AND CHRONIC KIDNEY DISEASE: THE ROLE OF THE MICROBIAL METABOLITE TMAO AND IMMUNOMODULATORY EFFECTS OF VITAMIN D

Catharina Missailidis

Karolinska Institutet

Stockholm 2018

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Eprint AB 2018
© Catharina Missailidis, 2018
ISBN 978-91-7831-272-6

Gut derived chronic inflammation in HIV-1 and chronic kidney disease: the role of the microbial metabolite TMAO and immunomodulatory effects of Vitamin D

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i föreläsningssalen 9Q Månen, Alfred Nobels Allé 8, Karolinska Institutet Huddinge

Fredagen den 23 november 2018, kl 12.30

By

Catharina Missailidis

Principal Supervisor:
Associate Professor Peter Bergman, MD/PhD
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisor(s):
Associate Professor Susanna Brighenti, PhD
Karolinska Institutet
Department of Medicine
Division of Center of Infectious Medicine

Professor Peter Stenvinkel, MD/PhD
Karolinska Institutet
Department of Clinical Science, Intervention and Technology
Division of Renal Medicine

Professor Anders Sönnerborg, MD/PhD
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Opponent:
Associate Professor Susanne Dam Nielsen, MD/PhD
Köpenhamns Universitet
Department of Infectious Diseases, Rigshospitalet
Division of Viro-Immunology Research

Examination Board:
Adjunct Professor Maria Eriksson Svensson MD/PhD
Uppsala Universitet
Department of Medical Sciences
Division of Clinical Diabetology and Metabolism

Associate Professor Guro Gafvelin, PhD
Karolinska Institutet
Department of Clinical Neuroscience
Division of Therapeutic Design

Associate Professor Christer Lidman MD/PhD
Karolinska Institutet
Department of Medicine
Division of Infection and Dermatology

ABSTRACT

Persistent immune activation with premature ageing and increased risk of cardiovascular disease (CVD) are features shared by HIV-1 infected individuals and patients with chronic kidney disease (CKD). Underlying dysbiosis, a failing mucosal barrier function and microbial translocation (MT) are contributing factors in both conditions. Vitamin D supplementation might prove valuable by its modulatory effects on innate and adaptive immunity, including induction of antimicrobial peptides (AMPs), regulation of epithelial barrier integrity and gut microbial composition.

The main objective of this thesis was to assess gut-derived immune activation and investigate the modulatory effects of vitamin D supplementation in HIV-1. Additionally we wanted to explore Trimethylamine-N-Oxid (TMAO), a microbial metabolite with newly ascribed pro-atherosclerotic properties, as a novel link between gut microbiome and inflammaging in CKD and in HIV-1.

In an observational study on HIV-1 infected individuals on antiretroviral treatment (ART) and non-HIV controls, we found that, whereas HIV-1 infected individuals demonstrated increased immune activation in the form of elevated levels of hsCRP and sCD14 with HIV as an independent risk factor, they did not differ from controls in degree of MT measured by LPS.

We next assessed the role of TMAO in cross-sectional and retrospective cohorts of CKD and HIV-1 patients of various disease stages. We found that TMAO levels correlated with increased systemic inflammation measured by hsCRP and was an independent predictor of mortality in CKD. TMAO levels were strongly associated with renal function and normalized after renal transplantation. In HIV-1, we found that TMAO levels were lower in untreated HIV-1 and normalized with treatment, but held no association with markers of immune activation or MT. Higher TMAO-levels in well controlled HIV-1 were not significantly associated with cardiovascular events. Nor was TMAO levels clearly associated with gut microbiome, or degree of dysbiosis.

Finally, in a randomized, double-blind and placebo-controlled trial of daily supplementation with vitamin D and phenylbutyrate in treatment-naïve HIV-1 infected individuals, we found no significant effects on circulating immune activation markers or TMAO and kynurenine/tryptophan ratio. Nor was there a significant treatment effect on the colonic mucosal microbiome.

In conclusion, vitamin D did not associate with biomarkers of immune activation in well controlled HIV-1. Further, we found no support that supplementation with vitamin D and PBA could modulate gut-derived immune activation and dysbiosis in treatment-naïve HIV-1. Our results suggest that TMAO may have a contributory role in immune activation and all-cause mortality in CKD. However, the role of TMAO in cardiovascular pathogenesis in CKD was not specifically addressed. Moreover, elevated TMAO levels were strongly related to kidney functions and the effects should be interpreted with caution. In HIV-1, or data did not support TMAO as a significant link between gut dysbiosis and inflammaging. TMAO levels were disparately confounded by HIV-1, microbial composition and ART, thus limiting its role as a cardiovascular marker in HIV-1.

SAMMANFATTNING PÅ SVENSKA

Kronisk inflammation bidrar till ett för tidigt åldrande med ökad risk för hjärt- och kärl sjukdom, både hos individer med HIV och kronisk njursvikt. En bidragande faktor till denna process är en rubbad (dysbiotisk) tarmflora och sviktande barriärfunktion i tarmväggen, vilket leder till ett inflöde av retande bakteriella produkter från tarmen.

Kosttillskott med vitamin D kan skydda genom att påverka immunförsvaret med ökad frisättningen av antimikrobiella peptider som deltar i tarmväggens försvar mot invasiva patogener. Vitamin D-signalering stärker även tarmväggens barriärfunktion och tycks vidare kunna reglera tamflorans sammansättning.

Syftet med detta doktorandprojekt var dels att studera om vitamin D-ersättning kan påverka tarmrelaterad inflammation och underliggande dysbios hos HIV-infekterade individer. Det andra målet var att studera om den bakteriella produkten Trimethylamine-N Oxid (TMAO) utgör en ny länk mellan tarmflora och tarmdriven inflammation i HIV-1 och kronisk njursvikt. TMAO är en metabolit som skapas av tarmbakterier. Den har kopplats till bildande av ateroskleros och är starkt associerad med ökad risk för hjärt- och kärl sjukdom i stora befolkningsstudier.

I delstudie I studerade vi prevalens av vitamin D-brist, och kopplingen till biomarkörer för tarm-driven inflammation hos HIV-infekterade individer med pågående behandling, i jämförelse med en frisk kontrollgrupp. Vi fann att majoriteten hade lägre vitamin D-nivåer än vad som rekommenderas, men även att vitamin D nivåerna inte kunde sammankopplas med någon biomarkör för tarmrelaterad inflammation. HIV-infekterade individer skiljde sig inte avseende nivåer av LPS, som är en specifik markör för tarmrelaterad inflammation, men hade ökad grad av immunoaktivering mätbar i form av höjda nivåer av högkänsligt CRP (hsCRP) och sCD14 (en markör för monocyt och makrofag aktivering) jämfört med kontroller.

I delstudie II och III studerade vi om cirkulerande TMAO utgör en länk mellan en dysbiotisk tarmflora, systemisk immunoaktivering och medför en ökad risk för mortalitet och hjärt- och kärl sjukdom i patienter med olika stadier av kronisk njursvikt (delstudie II) och HIV-1 (delstudie III). Vi fann att förhöjda TMAO-nivåer korrelerade med ökad grad av systemisk inflammation mätt med hsCRP och var en oberoende prediktor för mortalitet hos njursjuka patienter. Vidare att TMAO nivåer var starkt kopplade till njurfunktion. I HIV fann vi att TMAO-nivåer var låga i obehandlade HIV-infekterade individer och normaliserades efter behandlingsstart, men även att nivåerna inte påverkades av immunstatus, val av behandling eller grad av systemisk inflammation i någon kohort. Vidare sågs ingen tydlig koppling mellan TMAO-nivåer och tarmflora vare sig vid behandlingsstart eller uppföljning. Slutligen, TMAO var inte signifikant kopplat till högre risk för hjärt- och kärl sjukdom hos HIV-infekterade individer med god viral kontroll.

I delstudie IV, genomförde vi en randomiserad, dubbelblind och placebokontrollerad behandlingsstudie med vitamin D och phenylbyturate (PBA) bland obehandlade HIV-

infekterade individer i Etiopien. Vi studerade behandlingseffekt på tarmrelaterad inflammation och tarmflora med ett särskilt fokus på TMAO och tryptofan metabolism. Vi fann att behandlingskombinationen inte gav någon mätbar effekt på grad av inflammatorisk aktivering mätt med sCD14 och antimikrobiell peptid LL-37 trots ökade vitamin D nivåer. Det förelåg ingen skillnad avseende TMAO-nivå eller kynurenine/tryptofan-kvoter. Vidare sågs ingen effekt på tarmfloran vare sig avseende alfadiversitet (skillnad i mångfalden bakterier inom individen) eller betadiversitet (skillnaden i mångfald bakterier mellan individer eller grupper).

Sammanfattningsvis så fann vi låga vitamin D-nivåer i de Svenska och Etiopiska studiegrupperna, men vi fann ingen koppling till tarmdriven inflammation, eller behandlingseffekt avseende tarmflora, inflammatorisk aktivering eller mikrobiella metaboliter, trots normaliserade vitamin D-nivåer. Förhöjda TMAO nivåerna kan utgöra en bidragande risk för utveckling av hjärt- och kärl sjukdom hos patienter med njursvikt, men är samtidigt starkt kopplade till grad av njurfunktion, vilket gör att tolkning måste göras med försiktighet. För HIV-infekterade individer tycks TMAO nivåer påverkas både av HIV, tarmflora, likväl som HIV-behandling och lokal inflammation i tarmen, vilket inte utesluter, men minskar dess användbarhet som en biomarkör för dysbios och risk för hjärt- och kärlsjukdom i HIV.

LIST OF SCIENTIFIC PAPERS

I. **Catharina Missailidis**, Jonas Höijer, Maria Johansson, Lena Ekström, Göran Bratt, Bo Hejdeman, Peter Bergman. *Vitamin D status in well controlled Caucasian HIV patients in relation to inflammatory and metabolic markers – a cross sectional cohort study in Sweden.* Scandinavian Journal of Immunology, 2015, 82, 55-62

II. **Catharina Missailidis**, Jenny Hällqvist, Abdel Rashid Qureshi, Peter Barany, Olof Heimbürger, Bengt Lindholm, Peter Stenvinkel, Peter Bergman.*Serum Trimethylamine-N-Oxide is strongly related to renal function and predicts outcome in chronic kidney disease.* PloS one, 2016, 11.e.0141738

III. **Catharina Missailidis**, Ujjwal Neogi, Peter Stenvinkel, MariusTroseid, Piotr Nowak, Peter Bergman *The microbial metabolite TMAO in association with inflammation and microbial dysregulation in three HIV cohorts at various disease stage.* AIDS, 2018, 32, 1589-1598

IV. **Catharina Missailidis**, Nikolaj Sørensen, Senait Ashenafi, Wondwossen Amogne, Endale Kassa, Amsalu Bekele, Meron Getachew, Nebiat Gebreselassie, Abraham Aseffa, Getachew Aderaye, Jan Andersson, Susanna Brighenti, Peter Bergman. *Vitamin D and phenylbutyrate supplementation does not modulate gut derived immune activation in HIV-1.* In manuscript

CONTENTS

LIST OF ABBREVIATIONS

ART	Antiretroviral treatment
CKD	Chronic kidney disease
CVD	Cardiovascular disease
MT	Microbial translocation
AMPs	Antimicrobial peptides
LPS	Lipopolysaccharide
LBP	Lipopolysaccharide binding protein
sCD14	Soluble cluster of differentiation 14
SCFAs	Short-chain fatty acids
VDR	Vitamin D receptor
VDBP	Vitamin D binding protein
IDO1	indoleamine 2.3-dioxygenase
TMAO	Trimethylamine-N-Oxide
hsCRP	High sensitivity CRP
IL	interleukin
PCR	Polymerase chain reaction
NGS	Next generation sequencing
ELISA	Enzyme linked immunosorbent assay
Rtx	Renal transplantation
RCT	Randomized controlled trial
IBD	Inflammatory bowel disease

1 INTRODUCTION

Despite effective viral control through modern antiretroviral treatment (ART) HIV-1 infected individuals still demonstrate a still demonstrate a persistent immune activation with premature ageing and elevated risk for cardiovascular disease (CVD) [1]. One contributing factor to chronic immune activation is translocation of microbial products from the gut where HIV-1 related loss of lymphoid tissue creates a dysfunctional mucosal barrier and dysregulated gut microbiome that appears to prevail with treatment [2-6]. The observation that dysbiosis (i.e. abnormal changes in the composition of intestinal microbiota) contributes to pathogenesis of disease through intestinal-derived bacterial endotoxin and metabolites is also demonstrated in chronic kidney disease (CKD) [7-9].

When designing interventional trials to improve gut health it is important to address all the relevant players including, innate and adaptive immunity, the intestinal barrier and the microbiome. Vitamin D supplementation may prove a valuable addition in HIV-1 through its important immunomodulatory effects [10, 11], including induction of antimicrobial peptides (AMPs) active in the mucosal defense against pathogenic microorganisms [12-15]. Moreover, vitamin D plays an active part in regulating epithelial mucosal barrier integrity [16, 17], and there is growing evidence that vitamin D signaling is involved in shaping the gut microbiome. [16, 18-20].

However, designing clinical trials aimed at healing the “leaky gut” face problems finding clinically relevant end-points. Established methods of measuring the degree of microbial translocation (MT) are flawed with methodological problems and more importantly, only indirectly linked to the gut. Evaluation of possible effects on CVD requires large prospective studies. Thus, novel and more specific markers that links microbial products with disease pathogenesis and underlying dysregulation of barrier function and microbiome are warranted

In this thesis we explore the immunomodulatory effects of vitamin D on gut-derived immune activation and microbiome in HIV-1 and the role of Trimethylamine-N-Oxide (TMAO), a metabolite produced by gut bacteria with established pro-atherosclerotic properties and a driver of CVD in HIV-1 infected individuals and CKD patients.

2 BACKGROUND

2.1 INFLAMMAGING IN HIV-1 AND CKD

"*Good fences makes good neighbors*" Robert Frost

Inflammaging, defined as an age-related increase in blood inflammatory markers, is a strong risk factor for cardiovascular disease, type 2 diabetes mellitus, CKD, osteopenia as well as dementia (21). Potential mechanisms include; changes in the gut permeability and microbiome (22-24); immune cell dysregulation related to metabolic stress and age-related changes in microRNA transcription (25); and chronic infections.

In HIV-1, inflammaging is characterized by monocyte/ macrophage activation and hypercoagulation caused by virally induced damage to lymphoid tissue and liver function (26). A prominent feature of chronic immune activation is translocation of microbial products, such as lipopolysaccharide (LPS), and flagellin, from a dysregulated gut (27).

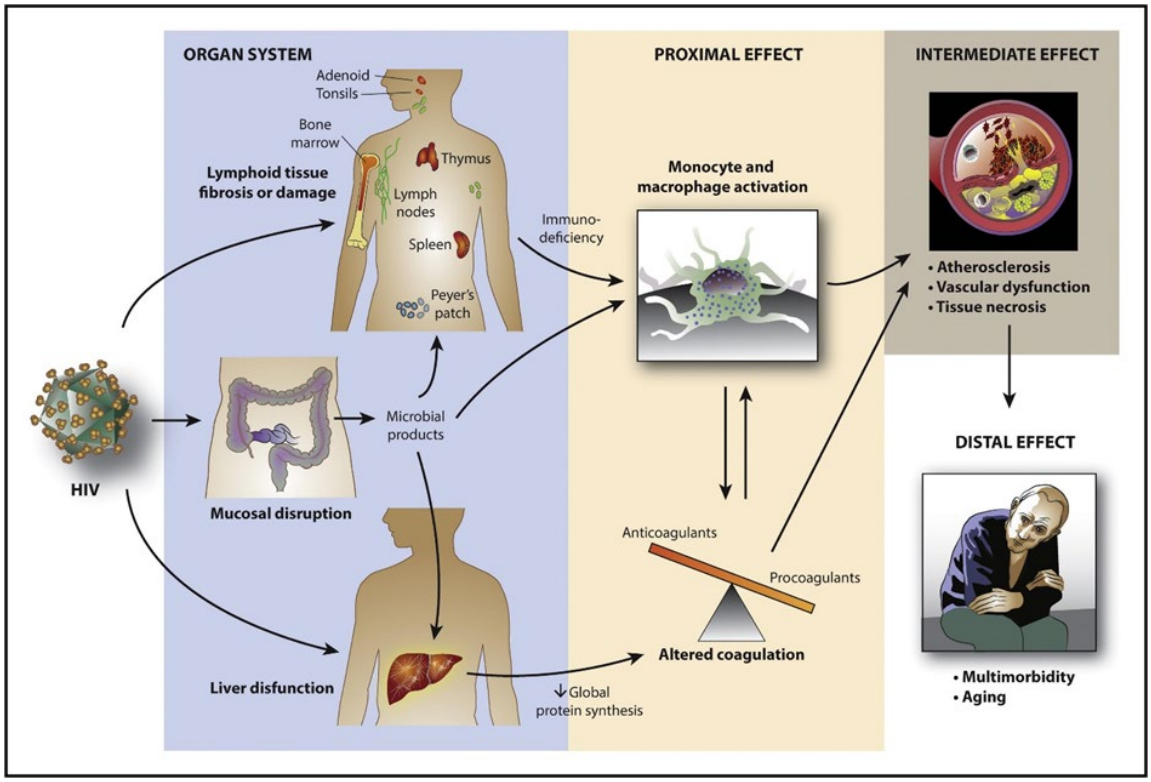


Figure 1. Pathogenesis of inflammation-associated disease in HIV-infected adults. Deeks et al. Immunity. 2013 (155). Reprinted with permission.

In CKD, progressive loss of kidney function is largely caused by factors unrelated to the cause of the initial disease (30), and is strongly associated with inflammaging (31, 32). In recent year focus has been put on the role of the gut microbiome where a growing body of evidence support

the association between dysbiosis, gut-derived inflammation and progression of kidney disease (33).

2.2 GUT BARRIER DYSFUNCTION IN HIV-1 AND CKD

"*Good fences makes good neighbors*" Robert Frost

In a healthy gut, homeostasis is maintained by the interplay between the mucosal intestinal barrier, the innate and adaptive immunity, and the commensal microbiome. The main purpose of the mucosal barrier is to protect the host against infectious microorganisms and to control the exchange of molecules from the intestinal lumen. Its construction involves a layer of tightly bound epithelial cells covered by a mucosal layer that prevents direct contact of microorganism and toxins with the epithelia. A healthy mucosal layer is important for the barrier function and provides the structural framework for epithelial and immune-cells to regulate microbial presence by secreting neutralizing IgA and antimicrobial peptides (AMPs).

Underlying the mucosal barrier is the lamina propria that harbors macrophages and T lymphocytes (T cells) responsible for eliminating microbial pathogens that cross the mucosal barrier, as well as IgA producing B lymphocytes (B cells) and dendritic cells that direct the adaptive immune defense.

The pathology behind gut barrier dysfunction in HIV-1 originates in the high prevalence of activated CD4 T cells expressing HIV-receptors such as CCR5 along the intestinal lining. These cells are highly susceptible to HIV infection and cell death via apoptosis or bystander death through pyroptosis (32, 33). Indeed, HIV infection causes rapid and massive depletion of CD 4 T cells, with a selective reduction of T helper CD4 cells (Th CD4 cells) (26, 34). The mucosal CD 4 cell department may not fully recover despite viral suppression through ART (35), where loss of Th17 and Th22 CD4 cells specifically, has functional consequences for the gut immune barrier. These cells are the main producers of the cytokines interleukin (IL)-17 and IL-22 that are important regulators of epithelial proliferation and AMP production (36, 37). As a consequence, suboptimal CD4 cells reconstitution is associated with loss of functional epithelial barrier integrity and increased degree of MT and systemic immune activation (38, 39)

In CKD, gut barrier dysfunction is believed to be caused by an adoptive mechanism where the colon replaces the failing kidneys as a site for excretion of end-products of purine metabolism, such as urea (40). Elevated levels of urea in plasma leads to overgrowth of colonic bacterial species capable of hydrolyzing urea into ammonia, which changes intestinal pH and promotes structural alterations of the gut mucosal barrier (41).

2.3 MARKERS OF MICROBIAL TRANSLOCATION

Established methods of measuring the degree of microbial translocation (MT) are flawed with methodological problems and many are only indirectly linked to the gut.

Direct markers of MT include pro-inflammatory components of Gram-negative bacteria such as; LPS, a major component of the outer cell membrane; flagellin, a structural part of the flagellum used to propel the bacteria; and bacterial ribosomal (r)RNA/DNA. LPS is the most established marker, and is routinely analyzed by the limulus amoebocyte lysate assay. The assay is troubled by many technical difficulties involving sampling-factors, methodological problems and variation in interpretation (42). Levels of flagellin and flagellin-specific antibodies measured by ELISA may provide a stable assessment of MT, but its use has been relatively limited. Bacterial RNA/DNA is analyzed through PCR of the conserved 16S ribosomal DNA (16SrDNA) region. In recent years numerous studies of the gut microbiome have been published, many with conflicting results that may be related to differences in sampling, storage, nucleic acid isolation, sequencing and analyses. Traditionally Sanger sequencing have been used with restricted specificity and sensitivity, thus limiting its usefulness in the context of MT. However, next generation sequencing (NGS) may improve the diagnostic accuracy.

Several indirect markers are used, such as LPS-binding protein (LBP) and sCD14. LBP is an acute phase protein mainly produced by the liver upon LPS stimuli (43). However, LBP induction can also be triggered by other microbiological structures such as peptidoglycans, which are also found on Gram-positive bacteria (43). CD14 is a recognition receptor found primarily on monocytes and macrophages. Upon activation CD14 is shed, forming soluble (s) CD14, which is considered a marker of monocyte activation. It was initially described as a specific receptor for LPS (44), but the receptor also recognizes other pathogen-associated molecular patterns, such as peptidoglycans (45), and lipoteichoic acid from gram-positive bacteria and lipoproteins (46).

2.4 GUT MICROBIOME

"*A good digestion turneth all to health*" George Herbert.

The human intestinal microbiome is composed of 100 trillion (1014) colony forming units (CFU) of commensal bacteria. The majority belong to four dominating phyla; Firmicutes, Bacteriodetes, Proteobacteria and Actinobacteria, of which Bacteroidetes and Firmicutes together comprise 90% (47, 48). These resident commensals are differentially distributed throughout the intestinal tract depending on bile and pancreatic secretion, peristaltic activity as well as oxygen and nutrient availability (49). Aerobic and facultative anaerobic bacteria from the Firmicutes phylum, predominantly of the Lactobacillales order (Enterococcus, Streptococcus) are prominent in the small intestine as well as Proteobacteria, whereas obligate anaerobic bacterial genus from the Bacteroidetes phylum (Bacteroides, Prevotella), Firmicutes

phylum (Clostridium and Lactobacillus) and Actinobacteria (Bifidobacterium) dominate in the colon.

Composition of the gut microbiome varies with age, diet, geography and socioeconomic factors but may also be influenced by inherited factors. Family members sharing the same household appear to share a similar microbial composition, or enterotype (50).

An important function of commensal bacteria is to protect the host against exogenous pathogens through direct and immune-mediated (indirect) mechanisms of action (51). Direct mechanisms include competition for nutrients but also production of inhibitory substances such as AMPs and bacterial toxins (bacteriocins) by commensal bacteria. Indirect mechanisms involves targeted activation of innate immune defenses and enhanced epithelial production of AMPs. Bacterial activation also regulates IgA production by B cells.

Another important function of the intestinal microbiome is production of short-chain fatty acids (SCFAs) by microbial fermentation of non-digestible carbohydrates in the colon. SCFAs, such as butyrate, function as a direct source of nutrients for colonocytes and are essential for maintaining epithelial barrier functions (52). Moreover, butyrate increases intestinal vitamin D receptor (VDR) expression (53), induces production of AMPs (54) and modulates inflammation in the gut through expansion of regulatory T cells (55, 56).

2.5 DYSBIOSIS IN HIV-1 AND CKD

"*Death sits in the bowels and bad digestion is the root of all evil* " Hippocrates

Several studies have demonstrated reproducible differences in the gut microbiome between HIV-1 infected and non-HIV infected controls. The studies are heterogeneous in terms of sampling method (stool sample, mucosal biopsies or rectal sponge), age, gender, ethnicity and geographic location. Nevertheless, there is consistent report of enrichment of the phylum Proteobacteria and diminished levels of the phylum Bacteroidetes (3, 5, 59, 60) in HIV-1. Proteobacteria and several subtaxa, of the family Enterobacteriaceae (which contains established pathogens such as *Escherichia coli*, *Salmonella and Shigella*) have been associated with activation and depletion of CD4 cells, and elevated levels of circulating LPS and immune activation markers (IL-6, INF-ɣ and sCD14) in HIV-1 infected individuals (2, 5, 61). Microbial translocation of Proteobacteria has also been implicated in the rhesus macaque SIV-model (62).

In CKD the uremic environment affects the intestinal barrier leading to bacterial dysbiosis and immune activation (7, 33). Although CKD related dysbiosis is less characterized than in HIV-1, similar aspects with an increased in relative abundance of Proteobacteria of the family Enterobacteriaceae has been described (9).

However, linking specific members of the microbiome to MT and immune activation is challenging. An alternative approach is to identify disease-specific metabolic pathways and microbial metabolites that contribute to pathogenesis.

In HIV-1, impaired mucosal immunity and MT has been associated with activation of the kynurenine pathway of tryptophan metabolism through the enzyme indoleamine 2.3-dioxygenase 1 (IDO1) in antigen-presenting cells (61, 62). Data show that IDO1 activity alters the balance between Th 17/ Th 22 and regulatory CD4 subsets in the gut and inhibits the hosts ability to maintain protective IL-17-signaling important in gut epithelial health (63). Excessive tryptophan degradation results from IDO1 induction by interferon gamma (INF-ɣ), and other pro-inflammatory molecules such as LPS, but may also be related to gut dysbiosis. Interestingly Vujcovic-Cvijin et al. found that bacteria with the capacity to metabolize tryptophan through the kynurenine pathway were enriched in virally controlled HIV-infected individuals and correlated to kynurenine levels and plasma levels of the inflammatory cytokine IL-6 (5).

In CKD, the microbial metabolites indoxyl sulfate and *p*-cresyl sulfate correlate with progressive kidney failure and have been considered an essential factors in the development of systemic inflammation and CVD. Indoxyl sulfate and *p*-cresyl sulfate are the respective end-products of bacterial fermentation of tryptophan and tyrosine (64).

2.6 TRIMETHYLAMINE-N-OXIDE

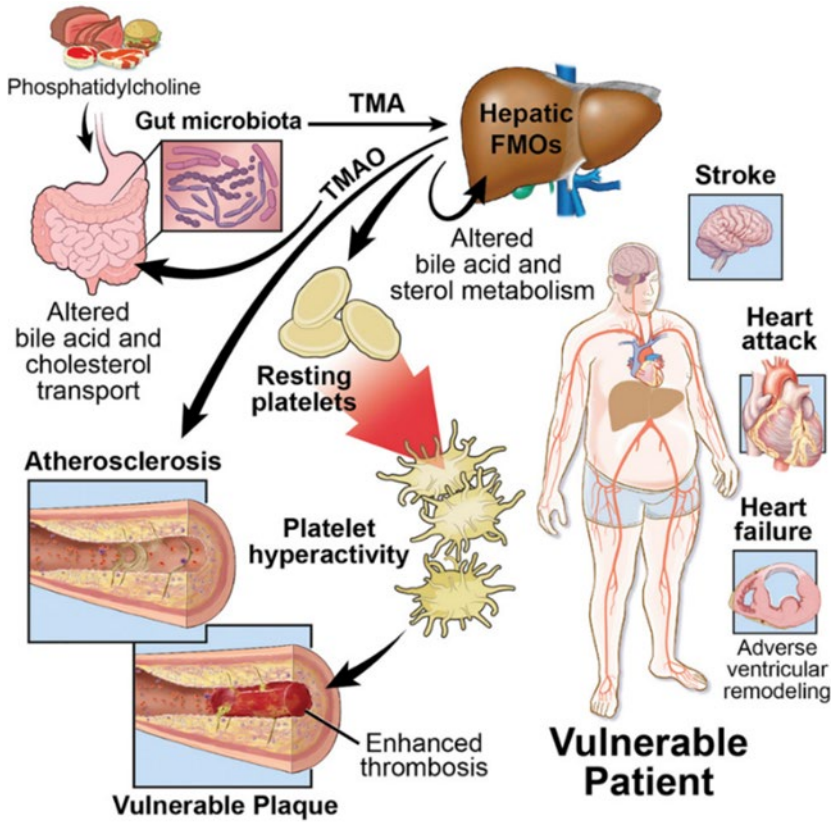


Figure 2. TMAO pathways. Zhu et al. Cell. 2016 (156). Reprinted with permission.

In recent year focus has been on the microbial metabolite Trimethylamine-N-Oxide (TMAO) as a biomarker of CVD development (65-70) and promoter of atherosclerosis (65, 66). The mechanism by which TMAO promotes atherosclerosis is not completely understood but mechanistic studies in murine models have demonstrated an effect on macrophage activation, foam cell formation and altered cholesterol metabolism. More recently, murine and in-vitro studies have linked TMAO to vascular inflammation through activation of nuclear factor-κB signalling and TXNIP-NLRP3 inflammasome activity in endothelial cells (71, 72) and to platelet hyperreactivity (73). Furthermore, TMAO has been described as a uremic toxin contributing to renal failure in CKD (74, 75).

TMAO is the primary metabolite of Trimethylamine (TMA) that is produced by gut bacteria and further converted to TMAO by flavin-containing monooxygenase (FMO3) in the liver (66, 69, 76, 77). Main substrates for TMAO are the essential nutrients choline and carnitine, found in diets rich in fat (dairy products, eggs and red meat), but it can also be converted from betaine. Although plasma levels of TMAO are governed both by diet, FMO3 enzyme activity and renal clearance (70, 78, 79), studies suggest that the gut microbial composition is directly linked to circulating TMAO levels in a healthy population (66, 76, 80, 81). There are two main bacterial pathways generating TMA in man: the CutC/D pathway (82, 83) and the Cnt A/B pathway (84, 85). Notably, genetic components for TMA conversion are detected in the three dominating

phyla; Firmicutes, Proteobacteria and Actinobacteria, but appear to be absent in Bacteroidetes (83, 86).

Studies in HIV-1 and CKD have yielded various results on the relationship between TMAO, immune activation and CVD (67, 70, 87-91).

2.7 VITAMIN D

"*Keep your fact always toward the sunshine and shadows will fall behind you*" Walt Whitman.

Vitamin D is a fat-soluble steroid that is predominantly produced in the skin by exposure to ultraviolet (UVB) sunlight (VD3), with a smaller proportion coming from dietary intake (VD2). VD2 and VD3 are hydroxylated in the liver forming 25-hydroxyvitamin D (25(OH)D3), which is routinely measured as a marker of vitamin D status in plasma. This molecule is further hydroxylated through CYP27B1(1α–hydroxylase) in the kidneys forming active vitamin D (1, 25(OH)D3) that exerts its biological effects through binding to the vitamin D receptor (VDR). VDR is a nuclear hormone receptor and transcription factor found in a variety of tissues. Activation of VDR through vitamin D, or other VDR binding substrates leads to the expression of specific gene products and post-transcriptional mechanisms. Active vitamin D produced in the kidney exerts its effects in an endocrine fashion. In contrast, extra-renal activation of vitamin D occurs "as-needed", in an intracrine, autocrine or paracrine fashion through local CYP27B1 activity. Similar to VDR, CYP27B1 is expressed in a variety of cell-types, including immune- and gut epithelial cells (92).

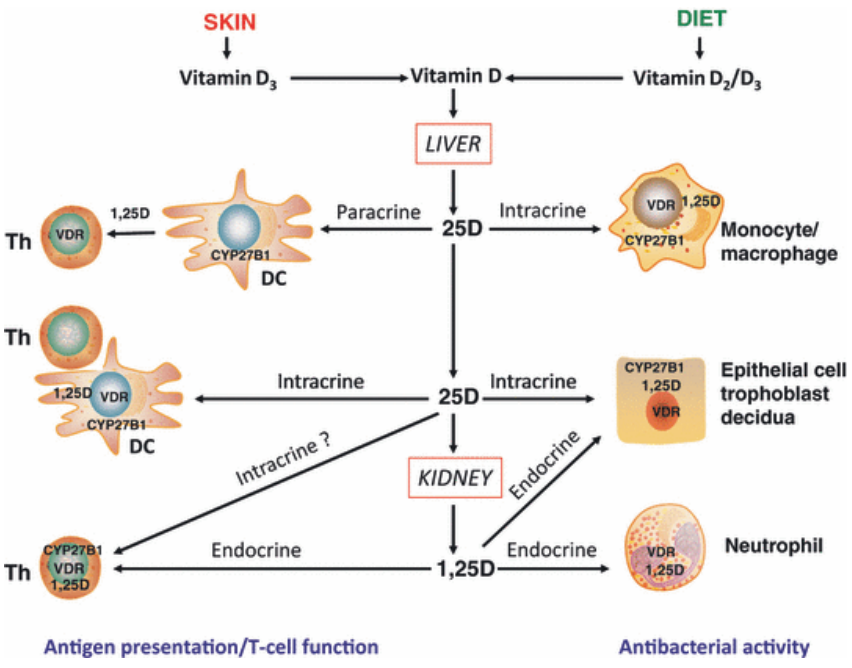


Figure 3. Vitamin D pathways. Hewison et al. Clin Endocrinol (Oxf). 2012 (157). Reprinted with permission.

Although there is no formal definition of what constitutes normal vitamin D levels the consensus at present is to consider vitamin D levels >75 nmol/L as sufficient, whereas vitamin D levels between 50-75 nmol/L are consider sub-optimal and levels <50 nmol/L as deficient (93). Vitamin D insufficiency is associated with increased risk of cardiovascular disease (94-98) and type-1 diabetes (99, 100) in general population and with immune activation and all-cause mortality in HIV-1 (65, 66). In HIV-1, vitamin D insufficiency is highly prevalent (101-108) whereas little is known on the viral effects on VDR expression. Of note, studies have shown reduced mRNA expression of VDR on immune and podocyte cells exposed to HIV *in-vitro* (109, 110). Established risk factors for vitamin D deficiency/insufficiency are; old age (103), dark skin (111), elevated BMI (111) and certain ART in HIV-1 (111-113).

At present little data has been presented on the effects of vitamin D treatment and gut barrier function. In mice, targeted expression of human VDR in intestinal epithelial cells protected against experimental colitis (114). Conversely, reduced expression of VDR correlated with intestinal inflammation and disease activity in patients with inflammatory bowel disease (IBD) (114, 115). Although small in numbers, interventional vitamin D studies in IBD suggest that supplementing Mb Crohn's patients with vitamin D might be beneficial in decreasing local and systemic inflammation (116-118).

2.8 VITAMIN D AND ANTIMICROBIAL PEPTIDE LL-37

One important innate defense mechanism against microbial pathogens in the gut is the synthesis and secretion of AMPs from epithelia and immune cells. There are two major gene families of AMPs expressed in the gut; defensins and cathelicidins. Most AMPs are small cationic molecules that binds to and interact with the negatively charged membranes of microbes, thus killing a broad range of Gram-positive and Gram-negative bacteria, as well as fungi and virus (119). The human cathelicidins, LL-37, may also regulate inflammation, angiogenesis and re-epithelialization essential for wound healing (120). LL-37 expression is regulated by several mechanisms and is either constitutive, or induced by inflammatory signals, ER stress and microbial exposure.

Vitamin D signaling is a strong inducer of the expression of LL-37 and defensins in immune cells and keratinocytes. (121, 122). Another molecule reported to increase LL-37 abundance in the gut is butyrate (54). Importantly butyrate induces expression of LL-37 from gut epithelial cells with VDR as the main transcription factor. Since butyrate is a foul smelling gas and as such unsuitable for interventional studies, we previously investigated the effects of phenylbutyrate (PBA), a synthetic analogue to butyrate used for treatment of urea cycle disorders. Similar to butyrate, PBA induces expression of LL-37 with VDR as the main transcription factor (123). Together, vitamin D and PBA have a synergistic effect on LL-37 expression (124).

2.9 VITAMIN D AND GUT EPITHELIAL BARRIER

The VDR is highly expressed in all cells in the intestine and experimental data have shown that vitamin D signaling is imperative in maintaining epithelial mucosal barrier integrity by promoting epithelial cell differentiation and tight junction formation (17, 125-127). Additionally vitamin D protects against inflammation-induced injury to the intestinal barrier through inhibition of nuclear factor κB (NF-κB) (114, 128).

2.10 VITAMIN D AND THE GUT MICROBIOME

In recent years a growing bulk of evidence points to a significant role of vitamin D in regulating the gut microbiome. The effect is most likely host-mediated via vitamin D activation of innate defense mechanisms.

In murine models, vitamin D deficiency, or genetic deletion of the VDR, results in dysbiosis and aggravated gut inflammation (16, 129, 130). In human the VDR gene has been identified as a host factor influencing the gut microbiome in a genome-wide association analysis (131), further strengthening the connection between vitamin D and the microbiota.

Interventional vitamin D studies support a regulatory effect on microbiota composition where low vitamin D levels associates with a higher abundance of Proteobacteria that is generally considered pro-inflammatory, whereas successful vitamin D supplementation correlates with expansion of members of the Bacteroidetes phylum and decreased abundance of members of the Proteobacteria phylum (19, 20, 132).

3 AIMS

The general aim was to assess gut derived immune activation in HIV-1 and investigate the modulatory effects of vitamin D supplementation on dysbiosis, mucosal barrier function and MT. Additionally we wanted to explore TMAO as a novel link between gut microbiome and inflammaging in CKD and in HIV-1.

Aim I
To investigate the prevalence of vitamin D insufficiency and its associations with markers of MT and immune activation in HIV-1 infected patients on ART to obtain the necessary information to design an interventional study.

Aim II and III
To assess the contribution of TMAO to mortality and cardiovascular risk in CKD and HIV-1 in different disease stages and its association with glomerular filtration rate (GFR), effect of dialysis and renal transplantation (Rtx), as well as associations with degree of MT, immune activation and microbial composition.

Aim IV
To investigate the effects of vitamin D and PBA supplementation on gut-derived immune activation, and microbiome with a special focus on TMAO and tryptophan metabolism in treatment-naïve HIV-1 infected individuals. he laboratory analytical methods and statistical approaches applied in study I-IV have been described in detail in the articles. I will here focus on some of the methodological limitations.

4 METHODOLOGICAL CONSIDERATIONS

For an overview of the study populations see table 1.

4.1 SELECTION OF STUDY POPULATIONS

Paper I: We performed a cross-sectional study of HIV-1 patients recruited at the HIV clinic at Södersjukhuset, Stockholm, Sweden during their regular 6 months HIV-1 control between January-August 2012. Non-HIV controls were recruited from staff at the Karolinska University Hospital, Stockholm, Sweden during September-December 2012.

The cross-sectional approach is ideal to measure prevalence in a group and allows for the study of multiple outcomes. A cross-sectional design can demonstrate simple associations but cannot prove causality. Furthermore, the selection process of subjects may bias the measured effects. In this case recruitment of controls from staff at Karolinska University Hospital was not optimal as they were a) less likely to be smokers, b) more likely to have a lower MSM prevalence, compared with patients at the HIV clinic at Södersjukhuset that specifically caters for the inner-city MSM community. Furthermore, in an effort to match sex and age, the controls were recruited following recruitment of HIV-1 infected individuals, which may have effected comparison of vitamin D levels between the groups.

Paper II: In a cross-sectional study and a retrospective cohort study (ad hoc and based on previously collected material) we assessed CKD patients at different disease stages, recruited at the Karolinska University Hospital, Stockholm, Sweden.

A cohort study is the best method to determine incidence and natural prognosis of a condition in a selected cohort followed over time. The cohort design allows for calculation of relative risk (i.e. what effect a chosen variable may have on the outcome of interest). In contrast to the cross-sectional design, cohort studies can distinguish cause and effect. However, if the selected outcome is rare, cohort design is a less suitable method. Moreover, subject selection and loss of subjects to follow-up might significantly affect the outcome. In retrospective cohort studies, the analyses are performed ad hoc, on already collected material, which prevents control of confounding variables and relevant information might be missing. On the other hand retrospective analysis has the advantage of being less susceptible to selection bias.

In this study sex and age-matched controls to the CKD population, were randomly selected from the population registry in Stockholm as a cross-sectional control group. Although the study populations were well characterized, the retrospective design prevented assessment of variables, such as diet and gut microbiome. Restricted sample availability also limited the number of new laboratory analysis.

Paper III: Here we performed a retrospective cohort study on primary infected and chronic HIV-1 infected individuals recruited at the Karolinska University Hospital in comparison to the cross-sectional HIV cohort collected and described in paper I. Furthermore, HIV populations were compared with cross-sectional non-HIV cohorts consisting of household

members and partners of subjects in the retrospective chronic HIV cohort and the controls from paper I.

Analysis of study populations collected for other purposes has inherent problems, which have been touched upon in the previous paragraph. Comparing different historic study populations adds an extra dimension due to differences in selection, sampling and methodology of previous analyses. In an attempt to overcome unequal representation of HIV versus non-HIV controls we added external controls from paper II, specifically selected to be age and sex-matched with the HIV population from paper I. Similarly as paper II, restricted sample availability also limited the numbers of possible laboratory analyses.

Paper IV: Here we performed a randomized, double-blind and placebo-controlled clinical trial (RCT) in ART-naïve HIV-1 infected individuals recruited at the pre-ART clinic, Department of Internal Medicine, Black Lion University Hospital in Addis Ababa, between 2013 and 2015.

A well-performed RCT provides the ultimate tool to evaluate the effect of a selected exposure. A prospective RCT eliminates confounding variables by randomizing exposure and thus ensuring that confounding variables are present in equal numbers in both groups. However, selection bias may still affect outcome. In this case the study was dominated by women, possibly reflecting gender differences in willingness to screen for HIV-1 and interest in medical controls. Often sicker individuals are less inclined to participate in trials and fail to comply to the treatment protocol with ensuing loss to follow-up. In this study the inclusion criteria excluded individuals with severe HIV-1 related immunosuppression and co-infection with tuberculosis. There was a substantial loss to follow-up from the randomized "intention to treat" (ITT)-population (from 278 to 197 subjects in the modified ITT), primarily related to delayed laboratory reports for viral load and CD4 cell counts that failed to meet inclusion criteria. From the modified ITT cohort, 14 subjects became ineligible for continued participation because of ART start and 16 subjects was lost to follow-up. The final per-protocol cohort consisted of 187 individuals.

4.2 LL-37 ELISA

In paper IV a previously established in-house enzyme-linked immunosorbent assay (ELISA) was used for assessment of plasma-levels of LL-37 (124). In brief, microtiter plates were coated with a monoclonal anti-LL-37 antibody (5 μg/ml) and incubated overnight. Synthetic LL-37 (Innovagen, Lund Sweden) standards and samples were then added and incubated overnight. After washing, biotinylated rabbit anti-LL-37 (1 μg/mL) (Innovagen) was added. After incubation and washing, the samples were incubated with Streptavidin-alkaline phosphatase conjugate (Chemicon, Melbourne, Australia) with four-methylumbelliferyl phosphate as substrate (Molecular Probes, Europe BV, Leiden, the Netherlands). The plates contained subject samples for both time-points with an equal distribution of treatment and placebo plated in duplicates, as well as negative and positive controls.

Despite adequate standards and subject replicates the read-outs had considerable variations between subjects and trial runs, making any analyses other than comparison of intra-subject effect difficult. Possible explanations to this variability could have been reagents, antibodies or human errors.

Methodological and analytical difficulties regarding LPS and sCD14 have been commented upon in section 2.3.

4.3 MICROBIOME ANALYSIS

In recent years numerous studies of the gut microbiome have been published, many with conflicting results that may be related to differences in sampling, storage, nucleic acid isolation, sequencing and analyses. An excellent review of possible pit-falls and considerations has been given by Kim et al. (133). I will here summarize some of the problems with microbiome-analysis.

Some of the confounding variables that needs to be taken into consideration when planning a microbiome study are age, gender, diet and current antibiotics or other medications, of which all may effect gut microbial composition. Furthermore, sampling techniques (fecal stool sample, rectal swab or mucosal biopsies) may produce different results as they essentially represent different microbial niches.

Differences in the storage and handling of samples may result in changes in the microbiome. To avoid changes in the original sample prompt freezing after sampling, or the use of alternative preservative methods is recommended. Moreover, samples may be contaminated by reagents and procedures in the laboratory setting. This may severely impact results, especially if the extracted microbial biomass is low, which allows contaminants to dominate in the sequence read-out. To evaluate the degree of contamination every analysis should include a negative and a positive control.

Analyses of extracted samples through high-throughput sequencing of 16srDNA genes faces other problems, primarily lack of species-specificity and overestimation of the number of taxa. Most sequencing involves universal primers binding to the conserved regions of the 16srDNA gene present in all bacteria. The amplified gene products allow for identification of bacterial taxa through comparison of hypervariable regions of the 16S rDNA gene.

Taxonomic identification is limited by the size of the reference database used and the length of the fragment sequenced. Most next-generation sequencers do not generate full-length sequences but focuses on specific variable regions, which limits the classification process since there simply might not be enough genetic information to assign bacterial species names.

Following gene sequencing the data has to undergo extensive bioinformatic processes to generate interpretable data. To determine which taxa that might associate with a difference in

phenotype multiple corrections are performed and the choice of method in controlling for multiple comparisons might also effect the final interpretation.

In the microbiome analyses in paper III and IV there was no information on diet which might affect the interpretation of data in the HIV-1 infected individuals. In paper III the recruitment of household members and partners as non-HIV controls partly eliminated diet as a variable in comparative analyses, assuming that household members shared their foods. Furthermore, in both paper III and IV inclusion criteria prevented from use of antibiotics. There was, however, no follow-up on the use of antibiotics during the study period in either population.

In paper IV sequence analysis of 16S rDNA on mucosal samples revealed a disproportionate amount of operational taxonomic units (OTUs) belonging to Pseudomonas and Halomonas genera. These bacteria are normally not highly present in the gut and were interpreted as contaminants from the sampling procedure (endoscopic biopsies), as the negative controls showed no contamination. After removal of contamination, there was a substantial loss of data specifically in the treatment group limiting conclusive results.

4.4 STATISTICAL ANALYSES

Choice of statistical method is based on the underlying question one wishes to study but it is also determined by sample size, distribution and variables. A small sample size limits the strengths of statistical analyses and the number of variables that can be addressed in multiple regression analysis. Furthermore, a small sample size is more likely to not meet the assumptions of normal distribution, which is a requirement for parametric tests, whereby choice of analyses is limited to non-parametric tests that inherently have less statistical power. A variable with a high degree of variability and extreme outliers is also subjected to non-parametric testing. The challenges when analyzing the results in study I-IV, were mainly related to small data sets with different populations with non-normal distribution and missing data.

5 RESULTS AND DISCUSSION

Table 1. An overview of study populations

Study design	**Cross-sectional**	**Cross-sectional**	**Cross-sectional**	**Cross-sectional**	**Retrospective cohort**	**Retrospective cohort**	**Retrospective cohort**	**Cross-sectional**	**RCT**
	Chronic HIV	CTRL	CKD 3-4	CRTL	CKD 5	Primary HIV	ART-naïve HIV	CRTL	HIV
Included in paper	I, III	I, III	II	II, III	II	III	III	III	IV
Subjects (Number)	101	30	58	80	116	17	22	9	167
In/exclusion criteria	Subjects (age 30-60) with well controlled HIV-1 on ART with undetectable VL (< 20 copies/mL) for > 6 months.	Sex-matched HIV-1-negative subjects (age 25-67)	Subjects (age 23-79) with CKD stage 3-4	Age and sex-matched subjects (age 22-80) randomly selected by the Statistics Bureau of Sweden	Subjects (age 20-78) with end-stage renal failure (CKD 5) were recruited close to start of dialysis treatment.	Subjects (age 27-53) ART-naïve with primary HIV-1infection.	Subjects (age 19-61) ART-naïve with chronic HIV-1infection.	Age and sex-matched HIV-1-negative subjects (age 28-62) consisting of household members and partners of the patients	Subjects (age 19-62), ART-naïve with confirmed HIV-1 infection
Co-variables	Age, sex, Caucasian origin. MSM HIV-years, ART years, pre-ART CD4-count, pre-ART VL, years with undetectable VL, Use of protease inhibitor/NNRTI	Age, sex, Caucasian origin.	Age, sex, BMI, smoking, diabetes, CVD, SGA-score	Age, sex, BMI, smoking, diabetes, CVD, SGA-score	Age, sex, BMI, smoking, diabetes, CVD, SGA-score	Age, sex, Caucasian origin. MSM, CD4-count, VL, Use of protease inhibitor/NNRTI	Age, sex, Caucasian origin. MSM, CD4-count, VL, Use of protease inhibitor/NNRTI	Age, sex, Caucasian origin.	Age, sex, BMI, CD4-count, VL,
Patients samples analyzed	Plasma Fecal stool-samples	Plasma	Heparin	Heparin	Heparin	Plasma	Plasma Fecal stool-samples	Plasma Fecal stool-samples	Plasma Colon-biopsies

5.1 PAPER I

Based on the hypothesis that vitamin D supplementation may modulate gut immune barrier dysfunction and gut related immune activation, we performed a cross-sectional study of the prevalence of vitamin D insufficiency and its associations with markers of MT and systemic inflammation in HIV-1 infected patients on ART and non-HIV controls, to have the necessary information to design an interventional study. For detailed demographics see table I.

We found that vitamin D levels were similarly distributed and equally low in both HIV-1 patients and non-HIV controls. The majority had insufficient levels (vitamin D ≤ 75nmol/L) (HIV 84%, controls 83%) (**Fig 4a**). HIV-1 infected individuals did not differ in LPS levels but demonstrated increased immune activation in the form of elevated levels of hsCRP and sCD14 (**Fig 4b, c, d**) with HIV as an independent risk factor (**Table 2**). There were no associations between vitamin D and markers for MT and immune activation or dyslipidaemia present in the HIV population.

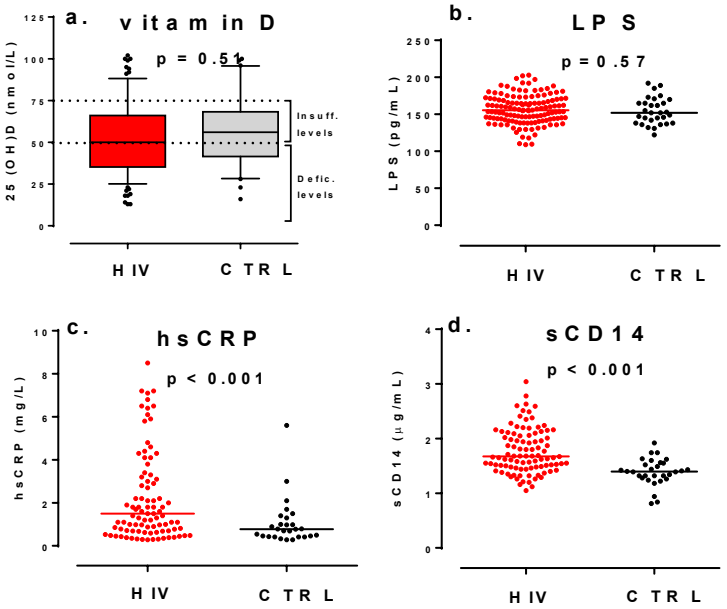


Figure 4.

Comparison of vitamin D (a); LPS (b); hsCRP (c); and sCD14 (d).in HIV-1 and non-HIV controls.

Values represented by median (10th -90th percentile). P-values calculated by Mann-whitney u-test.

Table 2.Regression analyses of predictors for CRP, or sCD14 in HIV patients.

Inflammation marker	Independent variable	Estimate	95 % CI	P-value
hsCRP (model 1)	HIV	1.50	(0.59, 2.40)	0.001
hsCRP (model 2)	HIV	1.46	(0.42, 2.50)	0.006
	Vitamin D	-0.00	(-0.02, 0.01)	0.704
	Age	0.00	(-0.05, 0.05)	0.915
	BMI	0.17	(0.03, 0.30)	0.017
	Smoker	0.47	(-0.46, 1.41)	0.321
sCD14 (model 1)	HIV	0.368	(0.242, 0.493)	<0.001
sCD14 (model 2)	HIV	0.394	(0.247, 0.540)	<0.001
	Vitamin D	0.001	(-0.003, 0.005)	0.573
	Age	-0.004	(-0.012, 0.004)	0.375
	BMI	-0.009	(-0.031, 0.013)	0.431
	Smoker	0.008	(-0.196, 0.212)	0.940

Due to a skewed distribution hsCRP was analyzed by logistic regression. Linear regression was used for sCD14.

Vitamin D insufficiency was highly prevalent in both HIV-1 and non-HIV controls in our material, which is comparable to the results from several vitamin D studies in HIV-1 populations, as well as a population-based Swedish study from the Gothenburg region (116, 134). However, in contrast to many of the previous cross-sectional studies in HIV-1 we found no significant links between vitamin D and degree of measured immune activation. There are many possible explanations for this difference.

First, in the HIV-1 population studies of Legeai et al and Ansemant et al, vitamin D deficiency (here defined as < 25 nmol/L) associated with hsCRP and IL-6. But this association was weakened in multiple regression analyses where vitamin D deficiency mainly associated with smoking, severe immunosuppression and co-infections with hepatitis B and C (102, 108). Since these conditions are known to be associated with a high degree of MT and systemic inflammation the relation between vitamin D, hsCRP and IL-6 remains less clear. In contrast, our HIV-1 study population had well-controlled viremia, a normalized CD4 cell count and patients with hepatitis and other inflammatory or infectious conditions were excluded.

Second, the difference may also be related to race and genetic polymorphisms where the population of Legeai et al. and Ansemant et al., were of mixed ethnicity (102, 108), whereas our study population was strictly Caucasian. The majority of circulating 25(OH)D vitamin is bound to vitamin D binding protein (VDBP) and albumin, and only the unbound fraction of vitamin D is functionally active. A study of Afro-Americans found that genetic polymorphism, not only explained the lower levels of 25(OH)D vitamin frequently observed in populations of African descent, but that it also affected expression of VDBP. Importantly, bioavailable vitamin D levels in the Afro-American population were similar to whites when DBP levels were taken into account, pointing to the inherent difficulties in estimating vitamin D related effects based on 25(OH)D levels alone (135).

Finally and similar to previous studies, we found increased levels of hsCRP and sCD14 in the HIV-1 population despite successful viral suppression through ART (136-138). In contrast we did not find elevated LPS-levels as a marker for MT, suggesting that MT may not play a dominant part as a driver of immune activation in this population. Also, although there is good evidence that MT is a contributory factor to immune activation, few of the common biomarkers are entirely specific for MT, or epithelial disruption in the gut. LPS is believed to be a major trigger of monocyte activation and sCD14 release, but other components such as gram-positive bacteria and inflammatory cytokines can also induce release of sCD14. Of note, few studies have found an association between LPS and sCD14 other than in immunosuppressed HIV-1 populations (139). Taken together evidence suggest that these two markers may reflect different pathologic pathways and sCD14 should not necessarily be regarded as an MT marker. Furthermore, although we did not find any correlation, smoking has been independently associated with immune activation measured by hsCRP and sCD14 in general as well as HIV-1 populations (140-142).

Considering the interest of therapeutic approaches aimed at reversing intestinal damage in HIV-1, it is important to find additional biomarkers that are better at evaluating the restoration

of gut immune barrier function and underlying pathology. Answering to this knowledge-gap, a recently published study addressed whether soluble Dipeptidylpeptidase 4 (sDPP4) levels could be used as a systemic marker of intestinal Th17 levels and gut immune damage during HIV-1 infection. DPP4 (also known as CD26), is a membrane-associated enzyme on CD4 cells with a specifically high expression on Th17 cells. Notably, levels of the soluble form, sDDP4, were decreased in primary and suppressed HIV-1 infection. Further, IL-21 treatment in a non-human primate model restored circulating sDPP4 levels and this increase was associated with restoration of the Th17 cell compartment and reduced inflammation in the gut mucosa (143), thus suggesting that circulating sDPP4 could be used as a surrogate marker for HIV-induced intestinal damage.

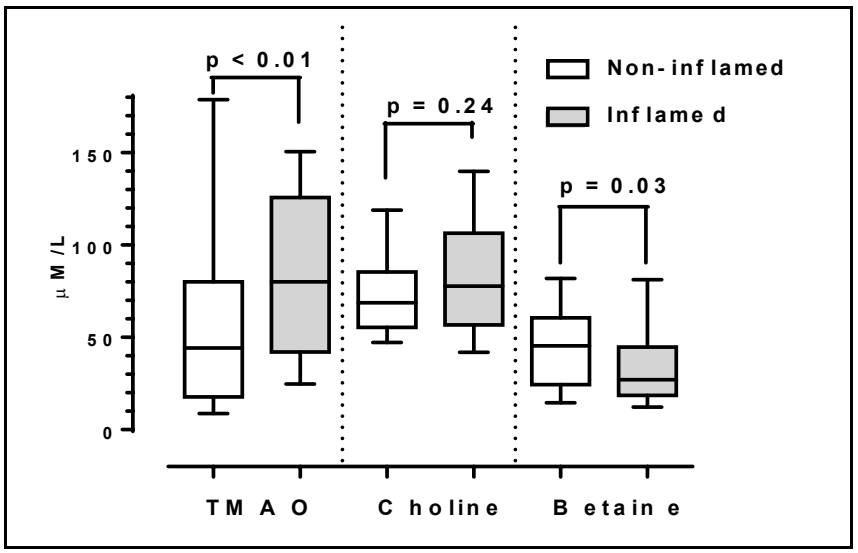
5.2 PAPER II, & III

With the hypothesis that TMAO may constitute a novel link between gut microbiome and inflammaging in CKD and in HIV-1- patients, we assessed contribution of TMAO to mortality and risk of cardiovascular events, in cross-sectional and retrospective cohorts of CKD (paper II) and HIV-1 (paper III)-patients at various disease stages. In addition we studied its association with glomerular filtration rate (GFR), effect of dialysis and renal transplantation (Rtx), as well as associations with degree of MT, immune activation and microbial composition. For demographic data see table I

We found that elevated TMAO associated with increased systemic inflammation measured by hsCRP and was an independent predictor of mortality in CKD 3-5 patients (**Fig 5 a, b and Table 3**). TMAO levels were strongly associated with renal function in CKD-patients and normalized after renal transplantation (**Fig 6**).

In HIV-1, TMAO levels increased significantly after ART-initiation, but never exceeded non-HIV controls (Fig 7). Subjects without an increase in TMAO levels after ART had; lower CD4/CD8 ratio at baseline (BL); a more pronounced gut dysbiosis at follow-up (FU) characterized by loss of Bacteroidetes; and significantly elevated LPS levels at FU (**Fig 8 a-d**). TMAO levels correlated inversely with Bacteroidetes (Rho: -0.62, P=0.002), and positively with Firmicutes (Rho: 0.65, P=0.001), but held no correlation to established TMA-producing genera. TMAO levels were not predicted by ART, immune status or degree of systemic inflammation in any cohort. Elevated TMAO-levels did not predict cardiovascular events in well-controlled HIV-1 (HR 2.76: 95% CI 0.29-26.70, p=0.38)

a.



b.

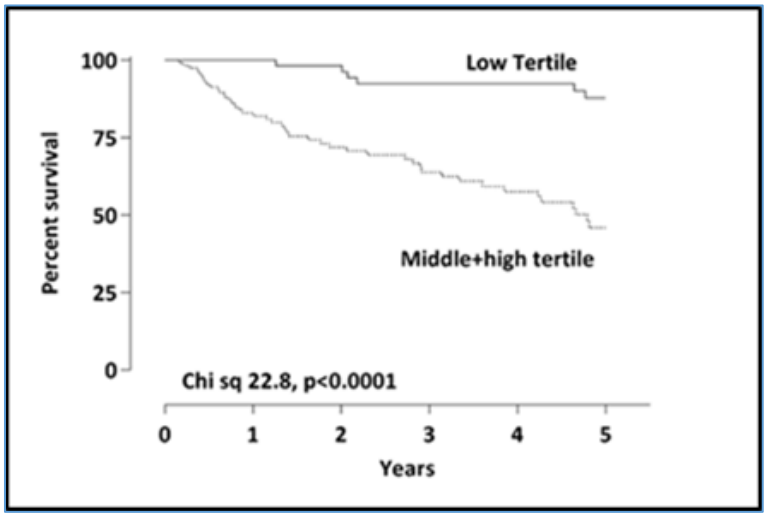


Figure 5.

Elevated TMAO levels in CKD 3-5 patients; associated with an inflamed status (hsCRP ≥10 mg/L) (a); and predicted risk for all-cause mortality in Kaplan-Meier analysis (b).

Boxplots represented by median (10th-90th percentile). P-values calculated by Mann-whitney u-test.

Table 3. Cox proportional hazards analysis of plasma TMAO levels stratified by tertiles in predicting risk of all-cause mortality at 5 years in CKD 3–5 patients.

Variable	HR (95% CI)	*P*-value
Middle (32.2-75.2 μM/L)+ high tertile (>72.2 μM/L)	**6.29 (2.67-14.8)**	**<0.0001**
+gender+age	**6.16 (2.59-14.7)**	**<0.0001**
+gender+age+dm	**8.23 (2.90-23.4)**	**<0.0001**
+gender+age+dm+hsCRP	**6.68 (2.33-19.1)**	**0.0004**
+gender+age+dm+hsCRP+GFR	**4.32 (1.32-14.2)**	**0.016**

Abbreviations: DM, diabetes mellitus; hsCRP, high sensitivity CRP; GFR, glomerular filtration rate

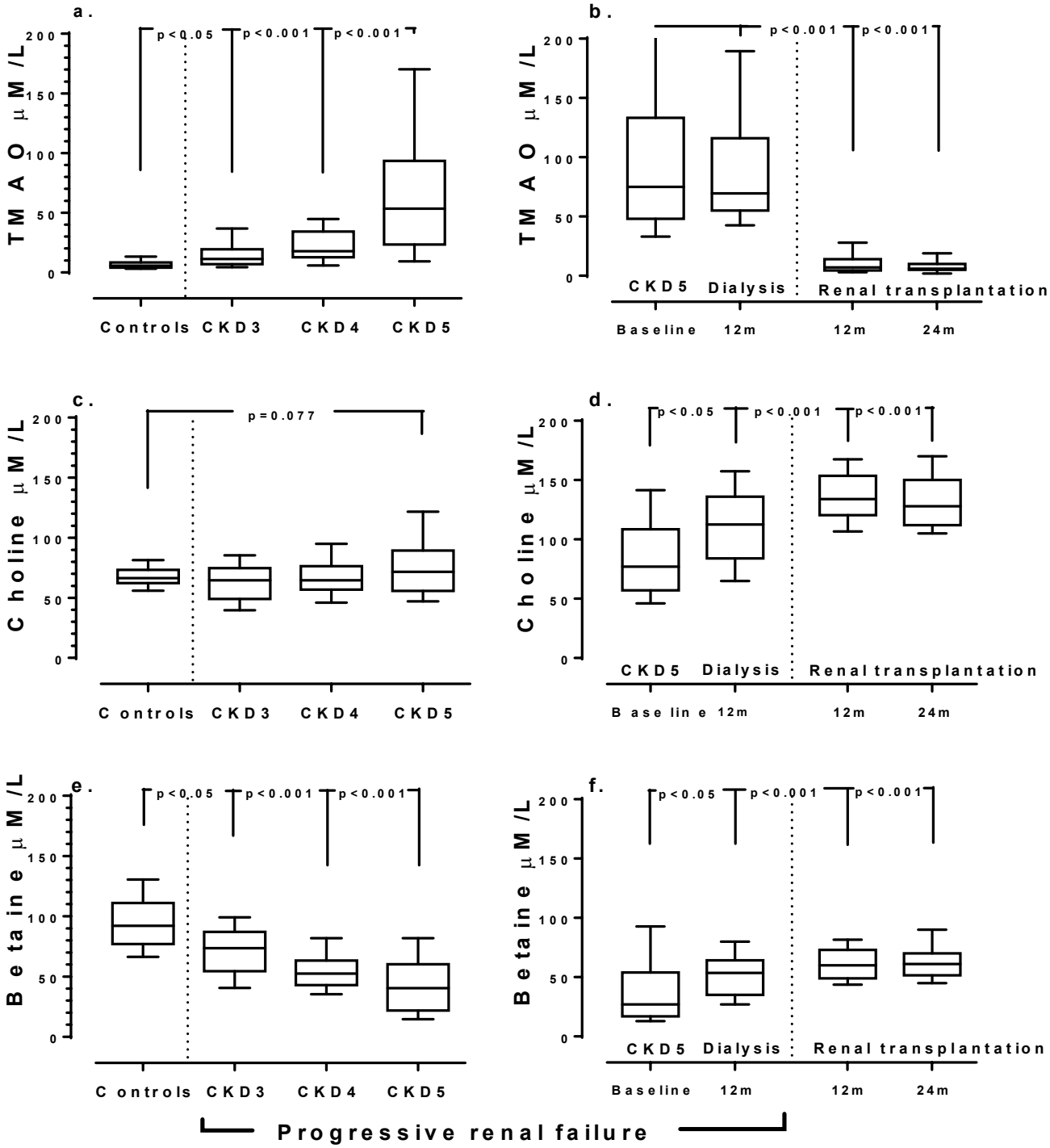


Figure 6.

TMAO levels in CKD stage 3-5, compared to healthy controls (a, c, and e), and end-stage renal disease patients recruited before start of dialysis (baseline), and reassessed 12 months after start of dialysis and/or 12 and 24 months after renal transplantation (b, d and f).

Values expressed as median (10-90th percentile). P-values analyzed by Kruskal-Wallis' on-way ANOVA, followed by Dunn's multiple comparison test.

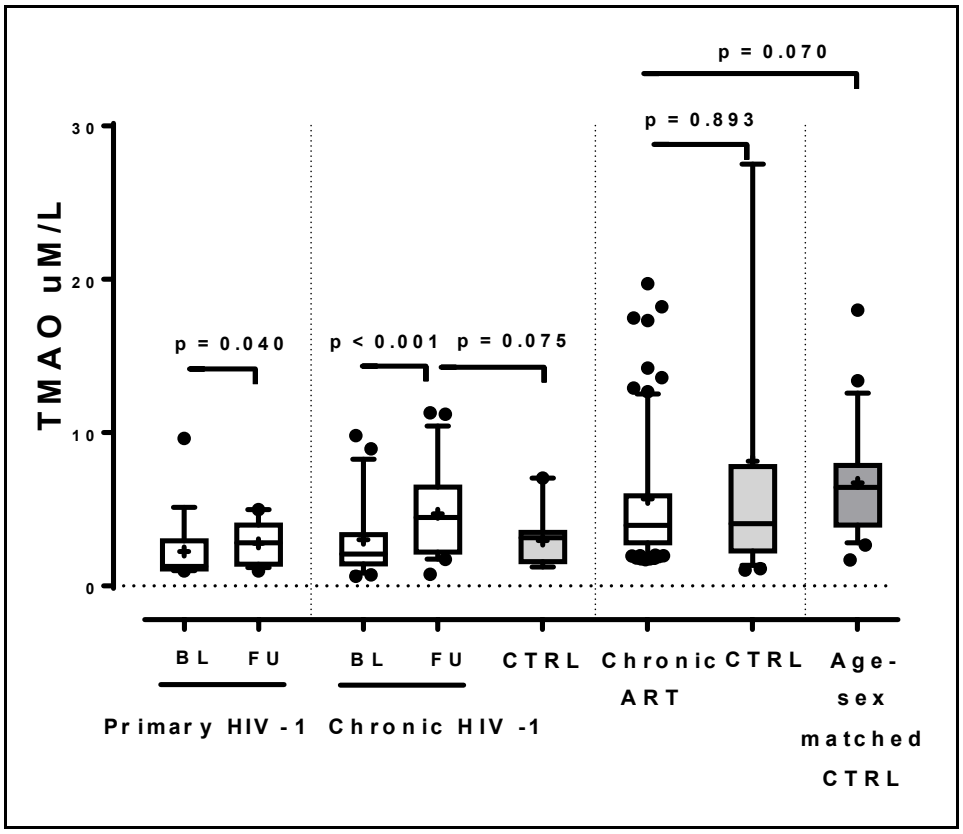


Figure 7.

TMAO levels in; primary infected and chronic HIV-1 infected individuals followed from baseline (BL) to follow-up (FU) after 4-10 months ART treatment; and chronic HIV-1-infected individuals on long-term ART (chronic ART) and healthy controls (CTRL).

P values are generated by Wilcoxon signed-rank (BL-FU) and Mann-Whitney *U* test.

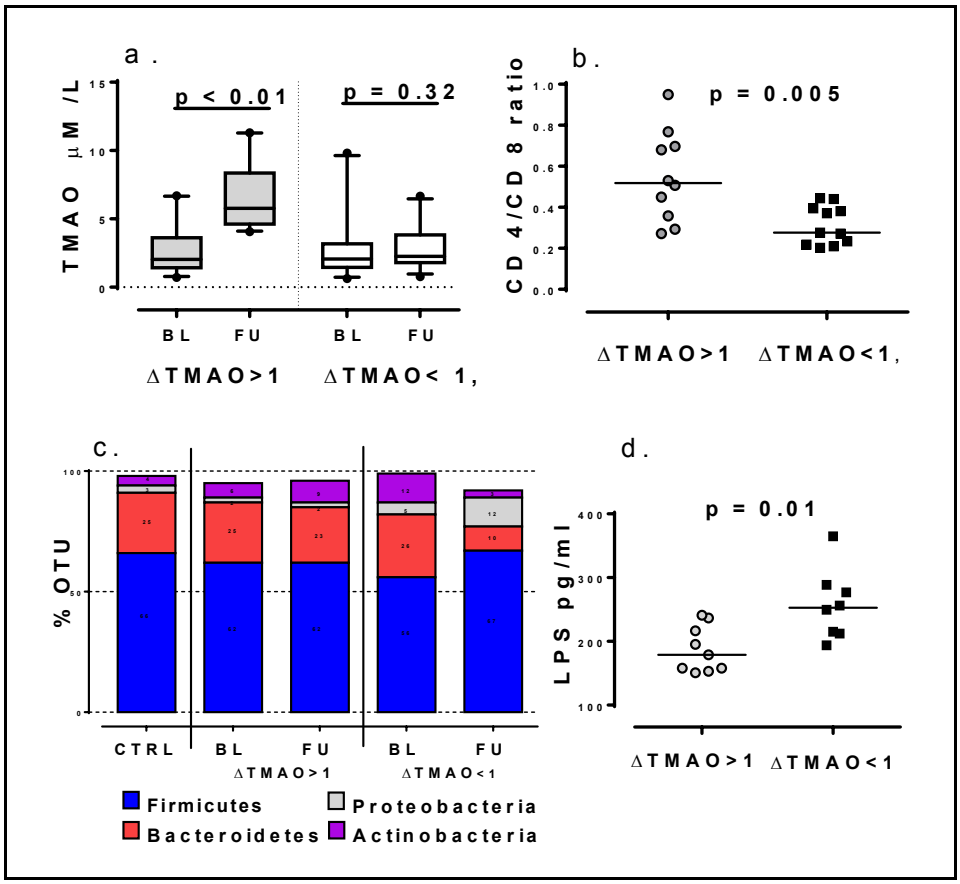


Figure 8.

Differential change in plasma TMAO levels after ART initiation (Δ TMAO: follow-up (FU) – baseline (BL)) (a) in relation to CD4/CD8 ratio at baseline (b), microbial composition (c), and LPS levels at follow-up (d)..

P values are generated by Wilcoxon signed-rank test (a) and Mann-Whitney *U* test (b, d).

In line with our hypothesis we found that TMAO levels did indeed correlate with hsCRP in CKD, but did not observe any association between TMAO and inflammatory biomarkers in HIV-1 regardless of disease stage. In fact, few other studies have found a clear correlation between TMAO and inflammatory markers known to predict cardiovascular risk. In contrast, TMAO was inversely correlated with inflammatory markers in CKD patients on haemodialysis (91). However, a recent study on HIV-1 infected individuals found that TMAO was positively associated with serum sCD14, sCD136 (both markers of monocyte activation and macrophage inflammation) and kynurenine/tryptophan ratio, in multiple regression analyses (144).

Similar to our results, Tang et al. found that TMAO levels increased with CKD and predicted poorer long-term survival (74). Stubbs et al. and Kim et al. found that elevated TMAO was an independent predictor for cardiovascular events and predicted long-term mortality in CKD independent of traditional cardiac risk factors (70, 145). However, other studies suggest a confounding role of TMAO and that the association between TMAO and adverse cardiovascular events may in part be driven by impaired kidney function and poor metabolic control (146). TMAO has since many years been identified as a uremic toxin that accumulate with renal insufficiency. A causal link between TMAO and renal fibrosis, related to its effects on the transforming growth factor-β (TGFβ)-SMAD3 signaling axis, has recently been demonstrated in murine models (73), thus adding another layer of difficulty in interpreting the cause and effect of TMAO in CKD.

In HIV-1, the support for TMAO as a major driver of adverse cardiovascular events is relatively weak (87-90), with the exception of the study of Shan et al. that not only observed a correlation with TMAO and inflammation, but also found that elevated TMAO associated with progression of carotid artery atherosclerosis in HIV-1 (144). In contrast to our study and previous studies of more limited sample size, the results of this study is based on 520 HIV-1 infected individuals followed over 7 years. However, the study did not address renal function as a possible confounder. Nor did they have information regarding clinical CVD events although carotid artery atherosclerosis is a predictor of CVD events. Of note, no study has demonstrated higher TMAO levels in HIV-1 populations compared to non-HIV controls. On the contrary, we found that TMAO levels were decreased in untreated HIV and only normalized with treatment.

Furthermore, contrary to our hypothesis we did not find evidence that gut dysbiosis in CKD and TMAO would lead to elevated TMAO. Previous studies have demonstrated that gut microbiome regulates TMAO levels (73) and that TMA converting enzymes are found in Firmicutes, and Proteobacteria but appear to be missing in Bacteroidetes (82, 83, 86). Studies of the microbiome in CKD have demonstrated increased prevalence of Proteobacteria with TMA converting capacity (9, 147) suggesting that gut microbiota may contribute to the net-impact of circulating TMAO in CKD. There are, however, no studies assessing gut microbiome and TMAO in CKD available at present. Similar to the study by Stubbs et al. (70) we found that TMAO levels normalize with transplantation, which does not support a dominating role of the microbiome in regulating TMAO levels in CKD.

In HIV-1, we failed to find a clear correlation between circulating TMAO and composition of gut microbiota, despite increased prevalence of bacteria from the Proteobacteria and decreased bacteria from the Bacteriodetes phylum after ART treatment that theoretically would suggest an increased prevalence of TMA converting bacteria in the gut. In fact, the lack of TMAO gain after treatment was associated with signs of a more disturbed microbiota and increased MT, suggesting that TMAO levels in HIV-1 might be affected both by HIV-1 infection, ART, as well as microbiome and local gut inflammation

5.3 PAPER IV

To investigate the effects of vitamin D and PBA supplementation on gut-derived immune activation, and microbiome with a special focus on TMAO and tryptophan metabolism in HIV-1, we performed a double-blind, randomized and placebo-controlled trial of daily supplementation with 5000 IU vitamin D and 500 mg PBA in treatment-naïve HIV-1-infected individual in Addis Abeba, Ethiopia,. For demographics see table I.

At inclusion the majority of the subjects were vitamin D insufficient (92%). Vitamin D levels increased significantly after 16 weeks of supplementation, proving high treatment efficacy. However, and in contrast with our hypothesis, treatment failed to produce significant effects on circulating immune activation markers sCD14, LL-37, or levels of TMAO and kynurenine/tryptophan ratio. Nor was there a significant treatment effect on the colonic mucosal microbiome with regard to alpha diversity measured by number of operational taxonomic units and Shannon microbial diversity index, or in beta diversity measured by principal component analyses.

The choice of combining vitamin D and PBA was to increase LL-37 production from epithelial cells and macrophages, as previously described (148), thus strengthening the mucosal defences. However, the combination also infers interpretational difficulties. The role of circulating LL-37 remains to be defined, but has been used by others as a surrogate marker for vitamin D effect on innate immunity. Surprisingly we did not find that supplementation increased LL-37 levels in plasma as previously described (149, 150). It is possible that the lack of effect on LL-37 in our study in part can be explained by methodological problems with an LL-37 assay that showed great inter-individual variations. In addition, it is possible that the combination with PBA, by virtue of its role as an HDAC-inhibitor (151), may have reactivated latent HIV (152), thus confounding the desired outcomes. One may also speculate if un-controlled viremia opposed the modulatory effects of vitamin D and PBA through downregulating of the VDR receptor, as described in studies of human podocytes and T and NK cells exposed to HIV in-vitro (109, 110). In addition, a recent study demonstrated that in-vitro stimulation with butyrate increased IDO1 activity in macrophages (153). Although this has not been studied with PBA, which is a synthetic butyrate analogue, the lack of reduction of the kynurenine/tryptophan-ratio might be related to a similar IDO1-stimulating effect.

Finally, the cohort selection represent HIV-1 infected individuals of relatively good immune status where dysbiosis, and gut derived inflammation may be less advanced, thus making possible treatment-effects too small to detect.

6 OVERALL CONCLUSIONS

Well-controlled Swedish HIV-1 infected individuals were subjected to increased immune activation measured by elevated hsCRP and sCD14 despite many years of effective treatment. They did not, however, display increased levels of MT measured by LPS. The results emphasize the need for new plasma biomarkers that more specifically represent a dysregulated immune gut barrier and/or underlying dysbiosis for evaluation in future treatment strategies.

Assessment of the microbial metabolite TMAO as a novel link between gut microbiome and inflammaging in CKD, supports a contributory role for TMAO levels in immune activation and all-cause mortality. However, the role of TMAO in the cardiovascular pathogenesis in CKD was not specifically addressed. Moreover, elevated TMAO levels were strongly related to kidney functions suggesting that the effects should be interpreted with caution.

In HIV-1, TMAO levels were lower in untreated individuals and normalized with treatment, but held no association with immune status, markers of immune activation or MT. Nor did TMAO levels clearly associate with gut microbiome, or degree of dysbiosis. Although the cardiovascular risks associated with elevated TMAO have been demonstrated in general population, our data does not support TMAO as significant link between gut dysbiosis inflammaging and CVD in HIV.

Assessment of vitamin D in Swedish and Ethiopian HIV-1 cohorts found ample room for vitamin D supplementation, based on present recommendations regarding vitamin D levels. Vitamin D did not, however, correlate to degree of immune activation or MT in any cohort. Nor did supplementation with vitamin D and PBA effect the degree of immune activation, levels of TMAO and kynurenine/tryptophan-ratio or gut microbiome, despite normalized vitamin D levels.

7 FUTURE PERSPECTIVES

Although suppressed HIV-1 infected individuals face a far less risk of severe cardiovascular events and other co-morbidity compared to CKD patients, the two conditions share similar pathognomonic features characterized by inflammaging through a dysregulated gut and underlying dysbiosis. However, although there certainly is mounting evidence linking the gut with systemic immune activation there is a paucity of knowledge regarding underlying microbial network. In fact, the intestine is an entire eco-system inside the human host that possess many qualities of an endocrine organ and our knowledge of what constitutes a healthy gut is still in its infancy.

In order to better address the impact on gut derived inflammaging as a driver of multi-morbidity in HIV and CKD, well-controlled studies on the exact nature of gut dysbiosis, and underlying mechanisms, in relation to diet, lifestyle, and comorbidities, should be performed in diverse ethnic populations. More importantly, prospective cohort studies assessing the microbiome before and during disease development would bring valuable information on disease relevant changes.

Another, and possibly more effective way, is to study the functional pathways of microbial metabolites associated with disease with the intent to tailor treatment that can "drug-the bug" and prevent disease development. In this regard TMAO is one of the best investigated substrates. Importantly, this concept has been proven efficient in a recent murine study demonstrating that inhibition of TMA converting enzymes in the gut reduced TMA and TMAO levels and protected mice from diet-induced atherosclerosis. (154). In CKD there is now a clear indication that TMAO may indeed contribute to CVD, outside of common risk factors, and that directed efforts to limit, or block TMAO mediated effects may prove a valuable treatment option. In contrast TMAO mediated effects does not appear to play a dominating role in HIV-1. In addition, at present there is little data on how dysbiosis may effect TMAO production in humans.

Many other microbial metabolites have been identified as contributors to disease, such as p-cresyl, indoxyl sulfate to name but a few, but there are many candidate substrates that remain to be identified. Methods such as metabolomics can help identify and quantify disease-relevant metabolites to be tested mechanistically in-vitro and in animal models to prove causal effects. In HIV, immune activation and disease progression has been linked with excessive activity of the kynurenine pathway of tryptophan metabolism, driven by inflammation-induced IDO1. Interestingly, evidence now point to a contributory role of gut bacteria with capacity to metabolize tryptophan through the kynurenine pathway, thus presenting a potential interventional target.

Finally, identifying microbial- host receptor signaling system may also prove a valuable way of targeting the dysregulated gut. It is clear that VDR signaling plays a profound role in gut homeostasis and is under both bacterial and host control. VDR is a target for SCFAs, vitamin D, as well as hormonal regulation. In IBD, VDR polymorphisms and down regulation of the

VDR element are linked with disease activity. If VDR downregulation plays an integral part in HIV-1 related effects on gut mucosal barrier function remains to be further investigated. The lack of result observed in our RCT might in part have been the result of conflicting effects of the combination, as well as uncontrolled viremia. Of note, results are underway from a recent RCT on the effect of vitamin D supplementation alone, on immune activation, Th17 cell frequency, gut barrier integrity and the gut microbiome (ClinicalTrials.gov Identifier: NCT03426592). In contrast to our study on ART-naïve individuals, the researcher have only included subjects on suppressive ART, which will make an interesting comparison.

8 ACKNOWLEDGEMENTS

Handledare
Till min huvudhandledare Peter Bergman: Du har hållit mig uppe med din kunskap och personlighet. Du har pushat mig med oförtruligt tålamod, klokhet och gott humör. Du är en enorm positiv kraft och faciliterare som får allt att kännas och bli möjligt.
Till Susanna för all din hjälp och feedback. Din klarsynthet, kunskap, och din språkliga och analytiska färdighet har varit inspirerande.
Till Peter Stenvinkel som med din entusiasm, breda humanistiska approach, enorma kunskap och erfarenhet är en målstjärna för min fortsatta vetenskapliga arbete.
Till Anders Sönnerborg för all din praktiska hjälp och insats när jag bäst behövt det.

Lab och samarbetspartner.
Tack speciellt till Piotr Nowak och mina kollegor Göran Bratt och Bo Hejdeman, för ert fantastiska samarbete runt studierna, som har drivit dom, men även mig framåt. Tack även Marius Trosoid för stimulerande samarbete.
Till Tony Quereshi och Jonas Höijer för utmärkt statistisk rådgivning och stöd.
Tack alla mina olika kollegor på labbet, där ett speciellt tack går till Monica Lindh som har varit helt avgörande för mitt laborerande; till Kajsa min hood-twin (P2 lab, inte Uppsala-hoods) som följt mig från början till slut och till Birgitta Agerberth som gjort mig till en del av AMP gruppen.
Tack till Calle Treutiger, Jonas Axelsson och Francesca Chiodi för era kloka råd och synpunkter vid halvtidskontrollen. Speciellt tack vill jag rikta till Francesca som inspirerade mig och gav mig möjlighet att börja forska när det begav sig.

Klinik
Till Lena Lindborg och Calle Treutiger för ert chefsliga stöd med given tid för forskning och vidareutbildning. Tack även alla mina olika kollegor på infektionskliniken som aldrig klagat över min frånvaro. Jag är nu redo att axla ett större patientansvar och jourbörda.

Familj och vänner.
Till min älskade man Michael, som är min hörnsten som håller mig grundad när tankarna flyger, du ger mig glädje, styrka och modet att pröva nya utmaningar. Kan inte lova att sluta forska men jag lovar dig, ingen mer disputation. Tack även mina barn; Thea, Hanna och Jacob för ert tålamod, har nu ingen ursäkt för att inte axla föräldraansvar i era lagaktiviteter.
Till min underbara mamma och pappa för all er kärlek och ert stöd. Tack även för alla nyfikna frågor runt min forskning som vässat min pedagogiska färdighet och tålamod.
Till Lina, utan din vänskap och stöd i forskningen skulle denna resa ha varit mycket tråkigare och sannolikt även längre p.g.a missade dead-lines. Du har varit som den mentor jag aldrig skaffade.
Slutligen, alla mina vänner som värmer min själ och mitt hjärta, tack för att ni finns och att ni haft tålamod. Ser fram emot att återuppta mitt mer sociala jag.

REFERENCES

1. Allers K, Fehr M, Conrad K, Epple HJ, Schurmann D, Geelhaar-Karsch A, et al. Macrophages accumulate in the gut mucosa of untreated HIV-infected patients. The Journal of infectious diseases. 2014;209(5):739-48.

2. Dillon SM, Lee EJ, Kotter CV, Austin GL, Dong Z, Hecht DK, et al. An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. Mucosal immunology. 2014.

3. Mutlu EA, Keshavarzian A, Losurdo J, Swanson G, Siewe B, Forsyth C, et al. A compositional look at the human gastrointestinal microbiome and immune activation parameters in HIV infected subjects. PLoS pathogens. 2014;10(2):e1003829.

4. Perez-Santiago J, Gianella S, Massanella M, Spina CA, Karris MY, Var SR, et al. Gut Lactobacillales are associated with higher CD4 and less microbial translocation during HIV infection. AIDS (London, England). 2013;27(12):1921-31.

5. Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ, et al. Dysbiosis of the gut microbiota is associated with hiv disease progression and tryptophan catabolism. Science translational medicine. 2013;5(193):193ra91.

6. Nowak P, Troseid M, Avershina E, Barqasho B, Neogi U, Holm K, et al. Gut microbiota diversity predicts immune status in HIV-1 infection. AIDS (London, England). 2015;29(18):2409-18.

7. Vaziri ND, Zhao YY, Pahl MV. Altered intestinal microbial flora and impaired epithelial barrier structure and function in CKD: the nature, mechanisms, consequences and potential treatment. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2015.

8. Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. Journal of the American Society of Nephrology : JASN. 2014;25(4):657-70.

9. Vaziri ND, Wong J, Pahl M, Piceno YM, Yuan J, DeSantis TZ, et al. Chronic kidney disease alters intestinal microbial flora. Kidney international. 2013;83(2):308-15.

10. Cantorna MT, Zhu Y, Froicu M, Wittke A. Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system. The American journal of clinical nutrition. 2004;80(6 Suppl):1717S-20S.

11. Holick MF. Vitamin D deficiency. The New England journal of medicine. 2007;357(3):266-81.

12. Campbell GR, Spector SA. Vitamin D inhibits human immunodeficiency virus type 1 and Mycobacterium tuberculosis infection in macrophages through the induction of autophagy. PLoS pathogens. 2012;8(5):e1002689.

13. Campbell GR, Spector SA. Toll-like receptor 8 ligands activate a vitamin D mediated autophagic response that inhibits human immunodeficiency virus type 1. PLoS pathogens. 2012;8(11):e1003017.

14. Sahay T, Ananthakrishnan AN. Vitamin D deficiency is associated with community-acquired clostridium difficile infection: a case-control study. BMC infectious diseases. 2014;14:661.

15. Martineau AR, Wilkinson KA, Newton SM, Floto RA, Norman AW, Skolimowska K, et al. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. Journal of immunology (Baltimore, Md : 1950). 2007;178(11):7190-8.

16. Assa A, Vong L, Pinnell LJ, Avitzur N, Johnson-Henry KC, Sherman PM. Vitamin D Deficiency Promotes Epithelial Barrier Dysfunction and Intestinal Inflammation. The Journal of infectious diseases. 2014.

17. Zhao H, Zhang H, Wu H, Li H, Liu L, Guo J, et al. Protective role of 1,25(OH)2 vitamin D3 in the mucosal injury and epithelial barrier disruption in DSS-induced acute colitis in mice. BMC gastroenterology. 2012;12:57.

18. Chen J, Waddell A, Lin YD, Cantorna MT. Dysbiosis caused by vitamin D receptor deficiency confers colonization resistance to Citrobacter rodentium through modulation of innate lymphoid cells. Mucosal immunology. 2015;8(3):618-26.

19. Kanhere M, He J, Chassaing B, Ziegler TR, Alvarez JA, Ivie EA, et al. Bolus Weekly Vitamin D3 Supplementation Impacts Gut and Airway Microbiota in Adults With Cystic Fibrosis: A Double-Blind, Randomized, Placebo-Controlled Clinical Trial. The Journal of clinical endocrinology and metabolism. 2018;103(2):564-74.

20. Bashir M, Prietl B, Tauschmann M, Mautner SI, Kump PK, Treiber G, et al. Effects of high doses of vitamin D3 on mucosa-associated gut microbiome vary between regions of the human gastrointestinal tract. European journal of nutrition. 2016;55(4):1479-89.

21. Frasca D, Blomberg BB. Inflammaging decreases adaptive and innate immune responses in mice and humans. Biogerontology. 2016;17(1):7-19.

22. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, et al. Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. Cell host & microbe. 2017;21(4):455-66.e4.

23. Zapata HJ, Quagliarello VJ. The microbiota and microbiome in aging: potential implications in health and age-related diseases. Journal of the American Geriatrics Society. 2015;63(4):776-81.

24. Rampelli S, Candela M, Turroni S, Biagi E, Collino S, Franceschi C, et al. Functional metagenomic profiling of intestinal microbiome in extreme ageing. Aging. 2013;5(12):902-12.

25. Jung HJ, Suh Y. Circulating miRNAs in ageing and ageing-related diseases. Journal of genetics and genomics = Yi chuan xue bao. 2014;41(9):465-72.

26. Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, et al. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. Journal of virology. 2003;77(21):11708-17.

27. Kaspar MB, Sterling RK. Mechanisms of liver disease in patients infected with HIV. BMJ open gastroenterology. 2017;4(1):e000166.

28. Yu HT. Progression of chronic renal failure. Archives of internal medicine. 2003;163(12):1417-29.

29. Amdur RL, Feldman HI, Gupta J, Yang W, Kanetsky P, Shlipak M, et al. Inflammation and Progression of CKD: The CRIC Study. Clinical journal of the American Society of Nephrology : CJASN. 2016;11(9):1546-56.

30. Gupta J, Mitra N, Kanetsky PA, Devaney J, Wing MR, Reilly M, et al. Association between albuminuria, kidney function, and inflammatory biomarker profile in CKD in CRIC. Clinical journal of the American Society of Nephrology : CJASN. 2012;7(12):1938-46.

31. Anders HJ, Andersen K, Stecher B. The intestinal microbiota, a leaky gut, and abnormal immunity in kidney disease. Kidney international. 2013;83(6):1010-6.

32. Steele AK, Lee EJ, Manuzak JA, Dillon SM, Beckham JD, McCarter MD, et al. Microbial exposure alters HIV-1-induced mucosal CD4+ T cell death pathways Ex vivo. Retrovirology. 2014;11:14.

33. Doitsh G, Galloway NL, Geng X, Yang Z, Monroe KM, Zepeda O, et al. Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. Nature. 2014;505(7484):509-14.

34. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. The Journal of experimental medicine. 2004;200(6):749-59.

35. Mehandru S, Poles MA, Tenner-Racz K, Jean-Pierre P, Manuelli V, Lopez P, et al. Lack of mucosal immune reconstitution during prolonged treatment of acute and early HIV-1 infection. PLoS medicine. 2006;3(12):e484.

36. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR, et al. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. Nature. 2015;528(7583):560-4.

37. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nature medicine. 2008;14(3):282-9.

38. Tincati C, Merlini E, Braidotti P, Ancona G, Savi F, Tosi D, et al. Impaired gut junctional complexes feature late-treated individuals with suboptimal CD4+ T-cell recovery upon virologically suppressive combination antiretroviral therapy. AIDS (London, England). 2016;30(7):991-1003.

39. Somsouk M, Estes JD, Deleage C, Dunham RM, Albright R, Inadomi JM, et al. Gut epithelial barrier and systemic inflammation during chronic HIV infection. AIDS (London, England). 2015;29(1):43-51.

40. Hatch M, Vaziri ND. Enhanced enteric excretion of urate in rats with chronic renal failure. Clinical science (London, England : 1979). 1994;86(5):511-6.

41. Felizardo RJ, Castoldi A, Andrade-Oliveira V, Camara NO. The microbiota and chronic kidney diseases: a double-edged sword. Clinical & translational immunology. 2016;5(6):e86.

42. Gnauck A, Lentle RG, Kruger MC. Chasing a ghost?--Issues with the determination of circulating levels of endotoxin in human blood. Critical reviews in clinical laboratory sciences. 2016;53(3):197-215.

43. Schumann RR. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. Biochemical Society transactions. 2011;39(4):989-93.

44. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990;249(4975):1431-3.

45. Rietschel ET, Schletter J, Weidemann B, El-Samalouti V, Mattern T, Zahringer U, et al. Lipopolysaccharide and peptidoglycan: CD14-dependent bacterial inducers of inflammation. Microbial drug resistance (Larchmont, NY). 1998;4(1):37-44.

46. Schmitz G, Orso E. CD14 signalling in lipid rafts: new ligands and co-receptors. Current opinion in lipidology. 2002;13(5):513-21.

47. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647-51.

48. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308(5728):1635-8.

49. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. Nature immunology. 2013;14(7):685-90.

50. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature. 2011;473(7346):174-80.

51. Perez-Lopez A, Behnsen J, Nuccio S-P, Raffatellu M. Mucosal immunity to pathogenic intestinal bacteria. Nature Reviews Immunology. 2016;16:135.

52. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell host & microbe. 2015;17(5):662-71.

53. Sun J. VDR/vitamin D receptor regulates autophagic activity through ATG16L1. Autophagy. 2016;12(6):1057-8.

54. Schauber J, Svanholm C, Termen S, Iffland K, Menzel T, Scheppach W, et al. Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. Gut. 2003;52(5):735-41.

55. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50.

56. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013;341(6145):569-73.

57. Yang L, Poles MA, Fisch GS, Ma Y, Nossa C, Phelan JA, et al. HIV-induced immunosuppression is associated with colonization of the proximal gut by environmental bacteria. AIDS (London, England). 2016;30(1):19-29.

58. Dillon SM, Lee EJ, Kotter CV, Austin GL, Gianella S, Siewe B, et al. Gut dendritic cell activation links an altered colonic microbiome to mucosal and systemic T-cell activation in untreated HIV-1 infection. Mucosal immunology. 2016;9(1):24-37.

59. Dinh DM, Volpe GE, Duffalo C, Bhalchandra S, Tai AK, Kane AV, et al. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. The Journal of infectious diseases. 2015;211(1):19-27.

60. Klase Z, Ortiz A, Deleage C, Mudd JC, Quinones M, Schwartzman E, et al. Dysbiotic bacteria translocate in progressive SIV infection. Mucosal immunology. 2015;8(5):1009-20.

61. Routy JP, Mehraj V, Vyboh K, Cao W, Kema I, Jenabian MA. Clinical Relevance of Kynurenine Pathway in HIV/AIDS: An Immune Checkpoint at the Crossroads of Metabolism and Inflammation. AIDS reviews. 2015;17(2):96-106.

62. Vazquez-Castellanos JF, Serrano-Villar S, Latorre A, Artacho A, Ferrus ML, Madrid N, et al. Altered metabolism of gut microbiota contributes to chronic immune activation in HIV-infected individuals. Mucosal immunology. 2015;8(4):760-72.

63. Favre D, Mold J, Hunt PW, Kanwar B, Loke P, Seu L, et al. Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. Science translational medicine. 2010;2(32):32ra6.

64. Meyer TW, Hostetter TH. Uremic solutes from colon microbes. Kidney international. 2012;81(10):949-54.

65. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57-63.

66. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nature medicine. 2013;19(5):576-85.

67. Troseid M, Ueland T, Hov JR, Svardal A, Gregersen I, Dahl CP, et al. Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure. Journal of internal medicine. 2014.

68. Tang WH, Wang Z, Shrestha K, Borowski AG, Wu Y, Troughton RW, et al. Intestinal microbiota-dependent phosphatidylcholine metabolites, diastolic dysfunction, and adverse clinical outcomes in chronic systolic heart failure. Journal of cardiac failure. 2015;21(2):91-6.

69. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. The New England journal of medicine. 2013;368(17):1575-84.

70. Stubbs JR, House JA, Ocque AJ, Zhang S, Johnson C, Kimber C, et al. Serum Trimethylamine-N-Oxide is Elevated in CKD and Correlates with Coronary Atherosclerosis Burden. Journal of the American Society of Nephrology : JASN. 2015.

71. Seldin MM, Meng Y, Qi H, Zhu W, Wang Z, Hazen SL, et al. Trimethylamine N-Oxide Promotes Vascular Inflammation Through Signaling of Mitogen-Activated Protein Kinase and Nuclear Factor-kappaB. Journal of the American Heart Association. 2016;5(2).

72. Chen ML, Zhu XH, Ran L, Lang HD, Yi L, Mi MT. Trimethylamine-N-Oxide Induces Vascular Inflammation by Activating the NLRP3 Inflammasome Through the SIRT3-SOD2-mtROS Signaling Pathway. Journal of the American Heart Association. 2017;6(9).

73. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. Cell. 2016;165(1):111-24.

74. Tang WH, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, et al. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. Circulation research. 2015;116(3):448-55.

75. Rhee EP, Clish CB, Ghorbani A, Larson MG, Elmariah S, McCabe E, et al. A combined epidemiologic and metabolomic approach improves CKD prediction. Journal of the American Society of Nephrology : JASN. 2013;24(8):1330-8.

76. Miller CA, Corbin KD, da Costa KA, Zhang S, Zhao X, Galanko JA, et al. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. The American journal of clinical nutrition. 2014;100(3):778-86.

77. Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. Cell metabolism. 2013;17(1):49-60.

78. Bell JD, Lee JA, Lee HA, Sadler PJ, Wilkie DR, Woodham RH. Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide. Biochimica et biophysica acta. 1991;1096(2):101-7.

79. Bain MA, Faull R, Fornasini G, Milne RW, Evans AM. Accumulation of trimethylamine and trimethylamine-N-oxide in end-stage renal disease patients undergoing haemodialysis. Nephrology Dialysis Transplantation. 2006;21(5):1300-4.

80. Cho CE, Taesuwan S, Malysheva OV, Bender E, Tulchinsky NF, Yan J, et al. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial. Molecular nutrition & food research. 2017;61(1).

81. Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, et al. Transmission of atherosclerosis susceptibility with gut microbial transplantation. The Journal of biological chemistry. 2015;290(9):5647-60.

82. Martinez-del Campo A, Bodea S, Hamer HA, Marks JA, Haiser HJ, Turnbaugh PJ, et al. Characterization and detection of a widely distributed gene cluster that predicts anaerobic choline utilization by human gut bacteria. mBio. 2015;6(2).

83. Craciun S, Balskus EP. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(52):21307-12.

84. Unemoto T, Hayashi M, Miyaki K, Hayashi M. Formation of trimethylamine from DL-carnitine by Serratia marcescens. Biochimica et biophysica acta. 1966;121(1):220-2.

85. Zhu Y, Jameson E, Crosatti M, Schafer H, Rajakumar K, Bugg TD, et al. Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(11):4268-73.

86. Falony G, Vieira-Silva S, Raes J. Microbiology Meets Big Data: The Case of Gut Microbiota-Derived Trimethylamine. Annual review of microbiology. 2015;69:305-21.

87. Haissman JM, Knudsen A, Hoel H, Kj AA, Kristoffersen US, Berge RK, et al. Microbiota-dependent marker TMAO is elevated in silent ischemia but is not associated with first-time myocardial infarction in HIV infection. Journal of acquired immune deficiency syndromes (1999). 2015.

88. Knudsen A, Christensen TE, Thorsteinsson K, Ghotbi AA, Hasbak P, Lebech AM, et al. Microbiota-Dependent Marker TMAO is Not Associated With Decreased Myocardial Perfusion in Well-Treated HIV-Infected Patients as Assessed by 82Rubidium PET/CT. Journal of acquired immune deficiency syndromes (1999). 2016;72(4):e83-5.

89. Miller PE, Haberlen SA, Brown TT, Margolick JB, DiDonato JA, Hazen SL, et al. Brief Report: Intestinal Microbiota-Produced Trimethylamine-N-Oxide and Its Association With Coronary Stenosis and HIV Serostatus. Journal of acquired immune deficiency syndromes (1999). 2016;72(1):114-8.

90. Srinivasa S, Fitch KV, Lo J, Kadar H, Knight R, Wong K, et al. Plaque burden in HIV-infected patients is associated with serum intestinal microbiota-generated trimethylamine. AIDS (London, England). 2015;29(4):443-52.

91. Kaysen GA, Johansen KL, Chertow GM, Dalrymple LS, Kornak J, Grimes B, et al. Associations of Trimethylamine N-Oxide With Nutritional and Inflammatory Biomarkers and Cardiovascular Outcomes in Patients New to Dialysis. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2015.

92. Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, et al. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. Nature immunology. 2007;8(3):285-93.

93. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism. 2011;96(7):1911-30.

94. Choi AI, Lo JC, Mulligan K, Schnell A, Kalapus SC, Li Y, et al. Association of vitamin D insufficiency with carotid intima-media thickness in HIV-infected persons. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2011;52(7):941-4.

95. Lai H, Gerstenblith G, Fishman EK, Brinker J, Kickler T, Tong W, et al. Vitamin D deficiency is associated with silent coronary artery disease in cardiovascularly asymptomatic African Americans with HIV infection. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;54(12):1747-55.

96. Ginde AA, Scragg R, Schwartz RS, Camargo CA, Jr. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults. Journal of the American Geriatrics Society. 2009;57(9):1595-603.

97. Ross AC, Judd S, Kumari M, Hileman C, Storer N, Labbato D, et al. Vitamin D is linked to carotid intima-media thickness and immune reconstitution in HIV-positive individuals. Antiviral therapy. 2011;16(4):555-63.

98. Wang L, Song Y, Manson JE, Pilz S, Marz W, Michaelsson K, et al. Circulating 25-hydroxy-vitamin D and risk of cardiovascular disease: a meta-analysis of prospective studies. Circulation Cardiovascular quality and outcomes. 2012;5(6):819-29.

99. Gagnon C, Lu ZX, Magliano DJ, Dunstan DW, Shaw JE, Zimmet PZ, et al. Low serum 25-hydroxyvitamin D is associated with increased risk of the development of the metabolic syndrome at five years: results from a national, population-based prospective study (The Australian Diabetes, Obesity and Lifestyle Study: AusDiab). The Journal of clinical endocrinology and metabolism. 2012;97(6):1953-61.

100. Stadlmayr A, Aigner E, Huber-Schonauer U, Niederseer D, Zwerina J, Husar-Memmer E, et al. Relations of vitamin D status, gender and type 2 diabetes in middle-aged Caucasians. Acta diabetologica. 2014.

101. Mehta S, Mugusi FM, Spiegelman D, Villamor E, Finkelstein JL, Hertzmark E, et al. Vitamin D status and its association with morbidity including wasting and opportunistic illnesses in HIV-infected women in Tanzania. AIDS patient care and STDs. 2011;25(10):579-85.

102. Legeai C, Vigouroux C, Souberbielle JC, Bouchaud O, Boufassa F, Bastard JP, et al. Associations between 25-hydroxyvitamin D and immunologic, metabolic, inflammatory markers in treatment-naive HIV-infected persons: the ANRS CO9 <<COPANA>> cohort study. PloS one. 2013;8(9):e74868.

103. Viard JP, Souberbielle JC, Kirk O, Reekie J, Knysz B, Losso M, et al. Vitamin D and clinical disease progression in HIV infection: results from the EuroSIDA study. AIDS (London, England). 2011;25(10):1305-15.

104. Sudfeld CR, Wang M, Aboud S, Giovannucci EL, Mugusi FM, Fawzi WW. Vitamin D and HIV progression among Tanzanian adults initiating antiretroviral therapy. PloS one. 2012;7(6):e40036.

105. Vescini F, Cozzi-Lepri A, Borderi M, Re MC, Maggiolo F, De Luca A, et al. Prevalence of hypovitaminosis D and factors associated with vitamin D deficiency and morbidity among HIV-infected patients enrolled in a large Italian cohort. Journal of acquired immune deficiency syndromes (1999). 2011;58(2):163-72.

106. Havers F, Smeaton L, Gupte N, Detrick B, Bollinger RC, Hakim J, et al. 25-Hydroxyvitamin D Insufficiency and Deficiency is Associated With HIV Disease Progression and Virological Failure Post-Antiretroviral Therapy Initiation in Diverse Multinational Settings. The Journal of infectious diseases. 2014;210(2):244-53.

107. Shepherd L, Souberbielle JC, Bastard JP, Fellahi S, Capeau J, Reekie J, et al. Prognostic Value of Vitamin D Level for All-cause Mortality, and Association With Inflammatory Markers, in HIV-infected Persons. The Journal of infectious diseases. 2014;210(2):234-43.

108. Ansemant T, Mahy S, Piroth C, Ornetti P, Ewing S, Guilland JC, et al. Severe hypovitaminosis D correlates with increased inflammatory markers in HIV infected patients. BMC infectious diseases. 2013;13:7.

109. Aguilar-Jimenez W, Saulle I, Trabattoni D, Vichi F, Lo Caputo S, Mazzotta F, et al. High Expression of Antiviral and Vitamin D Pathway Genes Are a Natural Characteristic of a Small Cohort of HIV-1-Exposed Seronegative Individuals. Frontiers in immunology. 2017;8:136.

110. Chandel N, Ayasolla KS, Lan X, Sultana-Syed M, Chawla A, Lederman R, et al. Epigenetic Modulation of Human Podocyte Vitamin D Receptor in HIV Milieu. Journal of molecular biology. 2015;427(20):3201-15.

111. Dao CN, Patel P, Overton ET, Rhame F, Pals SL, Johnson C, et al. Low vitamin D among HIV-infected adults: prevalence of and risk factors for low vitamin D Levels in a cohort of HIV-infected adults and comparison to prevalence among adults in the US general population. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2011;52(3):396-405.

112. Fox J, Peters B, Prakash M, Arribas J, Hill A, Moecklinghoff C. Improvement in vitamin D deficiency following antiretroviral regime change: Results from the MONET trial. AIDS research and human retroviruses. 2011;27(1):29-34.

113. Schwartz JB, Moore KL, Yin M, Sharma A, Merenstein D, Islam T, et al. Relationship of vitamin D, HIV, HIV treatment, and lipid levels in the Women's Interagency HIV Study of HIV-infected and uninfected women in the United States. Journal of the International Association of Providers of AIDS Care. 2014;13(3):250-9.

114. Liu W, Chen Y, Golan MA, Annunziata ML, Du J, Dougherty U, et al. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. The Journal of clinical investigation. 2013;123(9):3983-96.

115. Garg M, Rosella O, Lubel JS, Gibson PR. Association of circulating vitamin D concentrations with intestinal but not systemic inflammation in inflammatory bowel disease. Inflammatory bowel diseases. 2013;19(12):2634-43.

116. Jorgensen SP, Agnholt J, Glerup H, Lyhne S, Villadsen GE, Hvas CL, et al. Clinical trial: vitamin D3 treatment in Crohn's disease - a randomized double-blind placebo-controlled study. Alimentary pharmacology & therapeutics. 2010;32(3):377-83.

117. Yang L, Weaver V, Smith JP, Bingaman S, Hartman TJ, Cantorna MT. Therapeutic effect of vitamin d supplementation in a pilot study of Crohn's patients. Clinical and translational gastroenterology. 2013;4:e33.

118. Raftery T, Martineau AR, Greiller CL, Ghosh S, McNamara D, Bennett K, et al. Effects of vitamin D supplementation on intestinal permeability, cathelicidin and disease markers in Crohn's disease: Results from a randomised double-blind placebo-controlled study. United European gastroenterology journal. 2015;3(3):294-302.

119. Ostaff MJ, Stange EF, Wehkamp J. Antimicrobial peptides and gut microbiota in homeostasis and pathology. EMBO molecular medicine. 2013;5(10):1465-83.

120. Vandamme D, Landuyt B, Luyten W, Schoofs L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cellular immunology. 2012;280(1):22-35.

121. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. Journal of immunology (Baltimore, Md : 1950). 2004;173(5):2909-12.

122. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2005;19(9):1067-77.

123. Kulkarni NN, Yi Z, Huehnken C, Agerberth B, Gudmundsson GH. Phenylbutyrate induces cathelicidin expression via the vitamin D receptor: Linkage to inflammatory and growth factor cytokines pathways. Molecular immunology. 2015;63(2):530-9.

124. Mily A, Rekha RS, Kamal SM, Akhtar E, Sarker P, Rahim Z, et al. Oral intake of phenylbutyrate with or without vitamin D3 upregulates the cathelicidin LL-37 in human macrophages: a dose finding study for treatment of tuberculosis. BMC pulmonary medicine. 2013;13:23.

125. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, et al. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. American journal of physiology Gastrointestinal and liver physiology. 2008;294(1):G208-16.

126. Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. The Journal of cell biology. 2001;154(2):369-87.

127. Fujita H, Sugimoto K, Inatomi S, Maeda T, Osanai M, Uchiyama Y, et al. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca2+ absorption between enterocytes. Molecular biology of the cell. 2008;19(5):1912-21.

128. Du J, Chen Y, Shi Y, Liu T, Cao Y, Tang Y, et al. 1,25-Dihydroxyvitamin D Protects Intestinal Epithelial Barrier by Regulating the Myosin Light Chain Kinase Signaling Pathway. Inflammatory bowel diseases. 2015;21(11):2495-506.

129. Ooi JH, Li Y, Rogers CJ, Cantorna MT. Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis. The Journal of nutrition. 2013;143(10):1679-86.

130. He L, Liu T, Shi Y, Tian F, Hu H, Deb DK, et al. Gut Epithelial Vitamin D Receptor Regulates Microbiota-Dependent Mucosal Inflammation by Suppressing Intestinal Epithelial Cell Apoptosis. Endocrinology. 2018;159(2):967-79.

131. Wang J, Thingholm LB, Skieceviciene J, Rausch P, Kummen M, Hov JR, et al. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. Nature genetics. 2016;48(11):1396-406.

132. Luthold RV, Fernandes GR, Franco-de-Moraes AC, Folchetti LG, Ferreira SR. Gut microbiota interactions with the immunomodulatory role of vitamin D in normal individuals. Metabolism: clinical and experimental. 2017;69:76-86.

133. Kim D, Hofstaedter CE, Zhao C, Mattei L, Tanes C, Clarke E, et al. Optimizing methods and dodging pitfalls in microbiome research. Microbiome. 2017;5(1):52.

134. Mansueto P, Seidita A, Vitale G, Gangemi S, Iaria C, Cascio A. Vitamin D Deficiency in HIV Infection: Not Only a Bone Disorder. BioMed research international. 2015;2015:735615.

135. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. The New England journal of medicine. 2013;369(21):1991-2000.

136. Hanna DB, Lin J, Post WS, Hodis HN, Xue X, Anastos K, et al. Association of Macrophage Inflammation Biomarkers With Progression of Subclinical Carotid Artery Atherosclerosis in HIV-Infected Women and Men. The Journal of infectious diseases. 2017;215(9):1352-61.

137. Neuhaus J, Jacobs DR, Jr., Baker JV, Calmy A, Duprez D, La Rosa A, et al. Markers of inflammation, coagulation, and renal function are elevated in adults with HIV infection. The Journal of infectious diseases. 2010;201(12):1788-95.

138. McKibben RA, Margolick JB, Grinspoon S, Li X, Palella FJ, Jr., Kingsley LA, et al. Elevated levels of monocyte activation markers are associated with subclinical atherosclerosis in men with and those without HIV infection. The Journal of infectious diseases. 2015;211(8):1219-28.

139. Romero-Sanchez M, Gonzalez-Serna A, Pacheco YM, Ferrando-Martinez S, Machmach K, Garcia-Garcia M, et al. Different biological significance of sCD14 and LPS in HIV-infection: importance of the immunovirology stage and association with HIV-disease progression markers. The Journal of infection. 2012;65(5):431-8.

140. Yanbaeva DG, Dentener MA, Creutzberg EC, Wesseling G, Wouters EF. Systemic effects of smoking. Chest. 2007;131(5):1557-66.

141. Cioe PA, Baker J, Kojic EM, Onen N, Hammer J, Patel P, et al. Elevated Soluble CD14 and Lower D-Dimer Are Associated With Cigarette Smoking and Heavy Episodic Alcohol Use in Persons Living With HIV. Journal of acquired immune deficiency syndromes (1999). 2015;70(4):400-5.

142. Kooij KW, Wit FW, Booiman T, van der Valk M, Schim van der Loeff MF, Kootstra NA, et al. Cigarette Smoking and Inflammation, Monocyte Activation, and Coagulation in HIV-Infected Individuals Receiving Antiretroviral Therapy, Compared With Uninfected Individuals. The Journal of infectious diseases. 2016;214(12):1817-21.

143. Ploquin MJ, Casrouge A, Madec Y, Noel N, Jacquelin B, Huot N, et al. Systemic DPP4 activity is reduced during primary HIV-1 infection and is associated with intestinal RORC(+) CD4(+) cell levels: a surrogate marker candidate of HIV-induced intestinal damage. Journal of the International AIDS Society. 2018;21(7):e25144.

144. Shan Z, Clish CB, Hua S, Scott JM, Hanna DB, Burk RD, et al. Gut Microbial-Related Choline Metabolite Trimethylamine-N-Oxide Is Associated With Progression of Carotid Artery Atherosclerosis in HIV Infection. The Journal of infectious diseases. 2018;218(9):1474-9.

145. Kim RB, Morse BL, Djurdjev O, Tang M, Muirhead N, Barrett B, et al. Advanced chronic kidney disease populations have elevated trimethylamine N-oxide levels associated with increased cardiovascular events. Kidney international. 2016;89(5):1144-52.

146. Mueller DM, Allenspach M, Othman A, Saely CH, Muendlein A, Vonbank A, et al. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. Atherosclerosis. 2015;243(2):638-44.

147. Wang F, Zhang P, Jiang H, Cheng S. Gut bacterial translocation contributes to microinflammation in experimental uremia. Digestive diseases and sciences. 2012;57(11):2856-62.

148. Steinmann J, Halldorsson S, Agerberth B, Gudmundsson GH. Phenylbutyrate induces antimicrobial peptide expression. Antimicrobial agents and chemotherapy. 2009;53(12):5127-33.

149. Lachmann R, Bevan MA, Kim S, Patel N, Hawrylowicz C, Vyakarnam A, et al. A comparative phase 1 clinical trial to identify anti-infective mechanisms of vitamin D in people with HIV infection. AIDS (London, England). 2015;29(10):1127-35.

150. Bhan I, Camargo CA, Jr., Wenger J, Ricciardi C, Ye J, Borregaard N, et al. Circulating levels of 25-hydroxyvitamin D and human cathelicidin in healthy adults. The Journal of allergy and clinical immunology. 2011;127(5):1302-4.e1.

151. Gore SD, Carducci MA. Modifying histones to tame cancer: clinical development of sodium phenylbutyrate and other histone deacetylase inhibitors. Expert opinion on investigational drugs. 2000;9(12):2923-34.

152. Shirakawa K, Chavez L, Hakre S, Calvanese V, Verdin E. Reactivation of latent HIV by histone deacetylase inhibitors. Trends in microbiology. 2013;21(6):277-85.

153. Gurav A, Sivaprakasam S, Bhutia YD, Boettger T, Singh N, Ganapathy V. Slc5a8, a Na+-coupled high-affinity transporter for short-chain fatty acids, is a conditional tumour suppressor in colon that protects against colitis and colon cancer under low-fibre dietary conditions. The Biochemical journal. 2015;469(2):267-78.

154. Wang Z, Roberts AB, Buffa JA, Levison BS, Zhu W, Org E, et al. Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis. Cell. 2015;163(7):1585-95.

155. Deeks SG, Tracy R, Douek DC. Systemic effects of inflammation on health during chronic HIV infection. Immunity. 2013;39(4):633-45.

156. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. Cell. 2016;165(1):111-24.

157. Hewison M. Vitamin D and immune function: an overview. The Proceedings of the Nutrition Society. 2012;71(1):50-61.