Diplomarbeit

The role of methane-producing archaea in the human gut

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Graz, 15.01.2021

Affidavit

I declare that I have authored this thesis independently and without help from others, that I have not used other than the declared sources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz am 15.01.2021 Lukas Michael Gulden eh

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Glossary in alphabetical Order

ACE – Angiotensin-converting enzyme

ANS – Autonomous nervous system

ASV – Amplicon sequence variant

BMI – Body mass index

DNA – Deoxyribonucleic acid

ENS – Enteric nervous system

ESC – European Society of Cardiology

FMT – Faecal microbiome transfer

GERD – Gastro-oesophageal-reflux-disease

GSHA – Graz study on health and aging

HDL – High density lipoprotein

HMG-Co-A-reductase – 3-Hydroxy-3-methylglutaryl-coenzyme-A-reductase

HOMA-IR – Homeostatic model assessment for insulin resistance

LDL – Low density lipoprotein

LDH – Lactate dehydrogenase

LRR – Leucine-rich repeat

MAMP – Microbe associated molecular pattern

McrA-gene – Methyl-coenzyme-M-reductase-subunit-A-gene

M. smithii – Methanobrevibacter smithii

Me. stadtmanae – Methanosphaera stadtmanae

Met. luminyensis – Methanomassiliicoccus luminyensis

mmHg – Millimetre of mercury

NOD – Nucleotide-binding oligomerization domain

NLR – NOD-like-receptor

NLRP3 – NOD-, LRR- and pyrin domain-containing protein 3

PPI – Proton pump inhibitor

PRR – Pattern recognition receptor

qPCR – Quantitative polymerase chain reaction

RAAS – Renin-angiotensin-aldosterone system

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

SCFA – Short chain fatty acid

SRB – Sulphate-reducing bacteria

TLR – Toll-like-receptor

TMA – Trimethylamine

TMAO – Trimethylamine-N-Oxide

WHR – Waist hip ratio

Wnt – Ligand in the Wnt-signalling pathway, that is involved in the mediation of cell proliferation

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Zusammenfassung

Einleitung

Archaeen galten lange als extremophile Mikroorganismen, da diese den Bakterien ähnlichen Prokaryoten in Umgebungen mit hohen Temperaturen, hohem Druck und niedrigem pH-Wert gefunden wurden, welche für die meisten Lebewesen mit dem Leben unvereinbar sind. In den darauffolgenden Jahren stellte sich jedoch heraus, dass Archaeen nicht nur als eigene Domäne neben Bakterien und Eukaryoten ubiquitär in der Natur vorkommen, sondern auch Teil des menschlichen Mikrobioms sind. Seither wurde versucht einen Zusammenhang zwischen Archaeen und der menschlichen Gesundheit, beziehungsweise dem Auftreten von Erkrankungen herzustellen. Bis jetzt fand sich jedoch noch kein einziges pathogenes Archaeon. In dieser Diplomarbeit soll mittels vorhandener Daten, die im Rahmen der Graz Study on Health and Aging (GSHA) erhoben wurden in Kombination mit einer fäkalen Mikrobiom-Analyse festgestellt werden, ob es Zusammenhänge zwischen dem Mikrobiom und bestimmten erhobenen Eigenschaften der StudienteilnehmerInnen gibt. Hierbei wurde ein besonderes Augenmerk auf die Archaeen gelegt.

Methoden

Die erhobenen Daten umfassten unter anderem anthropometrische Daten, Angaben zu vorliegenden Erkrankungen, der Einnahme von Medikamenten und Nahrungsergänzungsmitteln, sowie Blutuntersuchungen, inklusive einem Hormonlabor. Zur Analyse des Mikrobioms wurde jeweils eine universale und eine Archaeen-spezifische PCR und qPCR durchgeführt, welche anschließend statistisch ausgewertet wurden.

Resultate

Es zeigte sich, dass methanogene Archaeen, genauer gesagt *Methanobrevibacter* signifikant mit einem normalen Insulin-Wert assoziiert sind. Bei hohen Insulin-Werten nimmt das Vorkommen von *Methanobrevibacter* stark ab. Zudem fanden sich weitere Assoziationen, die meisten waren jedoch nach einer Adaptierung des p-Werts für multiples Testen nicht mehr signifikant. In ca. 70% der Proben konnten Archaeen nachgewiesen werden, welche hierbei ca. 0.04% der gesamten Mikroorganismen ausmachten.

Schlussfolgerung

Methanogene Archaeen sind in viele komplizierte und kaum verstandene metabolische Vorgänge verstrickt. Eine Assoziation von *Methanobrevibacter* und Insulin wäre aufgrund der möglichen Interaktion mit dem Energiemetabolismus gut möglich. Zur Bestätigung dieser Hypothese, sowie der anderen fraglichen Assoziationen ist eine genauere Untersuchung mit größeren Gruppen notwendig.

Abstract

Introduction

Archaea have long been considered extremophile microorganisms, since these prokaryotes which are similar to bacteria have been found in environments hostile to most lifeforms, where they thrive at high temperatures, under high pressure and low pH-levels. In the years that followed, however, it emerged that archaea not only occur ubiquitously as a separate domain alongside bacteria and eukaryotes but are also part of the human microbiome. Since then, attempts have repeatedly been made to establish a connection between archaea and human health and disease. So far, no pathogenic archaeon has been identified. The object of this diploma thesis is to find a connection between the intestinal microbiome and certain properties of the study participants, using the collected data by the Graz Study on Health and Aging (GSHA) in combination with a faecal microbiome analysis. Special attention was put on the role of archaea.

Methods

The collected data from the GSHA included data on anthropometrics, medical conditions, use of medication and nutritional supplements and blood tests, including a hormone-blood-test. For the microbiome analysis, a universal and an archaea-specific PCR and qPCR were carried out and statistically evaluated.

Results

Methanogenic archaea, especially *Methanobrevibacter* showed a significant association with normal insulin levels. In groups with high insulin levels, the abundance of *Methanobrevibacter* is significantly reduced. In addition, multiple other associations were found, however, most of them were only significant before adjusting the p-level for multiple testing. Archaea were found in up to 70% of all samples and were accountable for about 0.04% of all faecal microorganisms when they were present.

Conclusion

Methanogens are involved in complex and fairly understood metabolic mechanisms. Due to their involvement in the energy metabolism, an association of methanogens and insulin levels seems possible. Nevertheless, more studies with larger group size are necessary to confirm or refute this hypothesis and the other associations in question. In addition, we were only able to detect different methanogenic archaea.

1 Introduction

1.1 The Human Microbiome

The human microbiome is a collective term that describes all microorganisms that are found in and on the human body. There are more microbial cells than human cells in and on the human body (1.3:1) (1). The microbiome is crucial for the resorption, synthesis and degradation of nutrients and vitamins, growth, neuronal development, maintenance of biological barriers and the regulation of the immune system (2–4). It is composed of a variety of different bacteria, archaea, viruses, fungi, and protozoa, while bacteria present the dominating microorganism (5). All surfaces of the body being in contact with the environment, like skin, gastrointestinal tract, urogenital tract, and respiratory tract, are colonized by microorganisms (4). On top of that, each anatomical region has a different microbiome composition due to environmental conditions that are distinct to each body site (6). The gastrointestinal tract is home to the majority of the human microbiome and is mostly composed of strictly anaerobic microorganisms (2,4). However, the gastrointestinal microbiome differs vastly in its composition, largely depending on the anatomical region (oral cavity, oesophagus, stomach, small intestine, large intestine and rectum) (7).

1.1.1 The gastrointestinal microbiome

As mentioned before, the majority of the gastrointestinal microbiome are bacteria, and the observed composition depends largely on the examined anatomic location (4).

The oral microbiome represents one of the most diverse microbiomes, due to dramatic changes in temperature, oxygen, nutrients, and pH, which necessitate the formation of diverse biofilms to endure these conditions (8). On the other hand, many more microorganisms pass the oral cavity as transient members via food intake and aerosols, thereby rendering the oral microbiome one of the least stable microbiomes (8). The oesophageal microbiome consists mostly of *streptococci*, *Prevotella*, *Actinomyces* and *Gemelli* species (4). Interestingly, the gastric microbiome is pretty similar to the oesophageal microbiome in the absence of *Helicobacter pylori* (4). In the presence of *H. pylori* however, the diversity is significantly reduced, with *H. pylori* representing the dominating species in this microbiome (4). The intestinal microbiome of the small intestine then gradually changes from oral to aboral, from mainly aerobic and facultative anaerobic microbes to a microbiome dominated by strict anaerobes in the large intestine (4). Most studies are focused on the gut microbiome since it represents the largest microbiome in humans, using cultivating or DNA-based methods for detection of microorganisms (4).

1.1.2 The intestinal microbiome

The intestinal microbiome is composed of more than 1,000 different species and shows a high interindividual variability (7). This interindividual variability is in part due to the genomic variants of otherwise similar strains, which are host-specific and indicate a strong microbe-host-interaction (1). Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria account for more than 90% of all bacteria in the gut, while Firmicutes and Bacteroidetes are the dominating phyla (1,7). In addition, there are observable differences between the luminal and mucosal microbiome, thus faecal samples might not accurately represent the intestinal microbiome (5,9). The intestinal microbiome encodes more than 150 times more genes than a single human and is highly involved in different metabolic pathways (10). This vast metabolic potential is close to that of the liver, rendering it a separate "metabolic organ", and it contains a wide array of metabolic pathways which are usually not found in the host (4). These metabolic functions have multiple applications, playing an important role in maintaining health and disease, which will be discussed further below. These metabolic functions do not only affect the host, but also other microorganisms, which necessitates adequate communication via metabolites, neurotransmitters, and other signalling molecules (1,7). These interactions enable the microbiome to influence the host and vice versa (1,11). A prime example for this interaction is the microbiota-gut-brain-axis, which is comprised of gut microorganisms, intestinal epithelial cells, the enteric nervous system (ENS) and the central nervous system (CNS) (1). However, the composition and diversity of the microbiome is affected by a multitude of different factors (1,6,7,12–17). Due to these various factors and high interindividual variability, it is difficult to categorize the microbiome of different individuals and establish a connection with a single factor. However, the microbial composition follows a common structure and therefore, some studies proposed the introduction of enterotypes, that are characterized by the dominating genus, namely *Bacteroides*, *Prevotella* and *Ruminococcus* (1,12). These enterotypes are associated with specific diet, for example the *Bacteroides*-enterotype is associated with a high-fat, high-protein diet (western diet), and the *Prevotella*-enterotype is associated with a diet rich in dietary fibre (1). However, to date, there is no consensus on the correct number of enterotypes and whether they are an oversimplification (12).

Interestingly, while the interindividual variability is high, the intraindividual variability is low, indicating, that the individual's microbiome is quite stable over time (1,12).

1.1.3 The microbiome and host metabolism

This section is going to investigate the various tasks and functions of the microbiome which complement and support host metabolism. Due to its vast metabolic potential, the microbiome is highly involved in the metabolism of the host (10). While the main task of the microbiome is the degradation of indigestible food components, it is also heavily involved in the production of various micronutrients and vitamins, regulation and development of the immune system, bile-acid modification and metabolism, degradation of xenobiotics like heterocyclic amines, defence of enteral infections and overall regulation of digestion (1,4,5,7,18). In addition, microorganisms are constantly synthesizing essential vitamins like vitamin K and B, pantothenic acid, riboflavin, thiamine, pyridoxine, nicotinic acid, folic acid, cobalamin, and niacin, thereby providing additional vitamins and nutrients in addition to those consumed (4). However, some metabolic pathways can generate harmful metabolites like hydrogen sulphide or trimethylamine, that can lead to tissue damage and various diseases (2,4,19). The main pathway for food degradation is bacterial fermentation of otherwise indigestible diet components (dietary fibres), especially complex carbohydrates like amylase-resistant starch, non-starch-polysaccharides, oligosaccharides und unabsorbed sugars (raffinose, lactose and stachyose) (4). In addition, some bacteria prefer the fermentation of protein, but this pathway is often associated with the production of potential toxic metabolites like amines, ammonia and branch-chained fatty acids, that are suspected to be involved in the development of colon carcinoma (4). However, even saccharolytic microorganism can switch to other substrates like protein, if there is a shortage of carbohydrates, although this leads to an accumulation of potential harmful metabolites (4,20). Interestingly, most intestinal microorganisms are highly co-dependent, as they require certain substrates provided by other microbes (4). This high co-dependency is one of the many reasons why the isolation and cultivation of the intestinal microbiomes is quite difficult. Overall, extensive host-microbe interaction is necessary, to control and coordinate the metabolic functions and the multitude of different applications of the microbiome in the human body.

1.1.3.1 Bacterial fermentation and short-chain-fatty acid production

As previously mentioned, complex carbohydrates are the primary energy source for most bacteria in the microbiome, and their degradation occurs through bacterial fermentation (20). The fermentation process results in the production of gases like carbon dioxide (CO_2), hydrogen sulphide (H_2S) and hydrogen (H_2), as well as many other metabolites (4). Carbon dioxide is not only produced by bacterial fermentation but also by other processes in the host and is therefore routinely consumed via acetogenesis and methanogenesis (4). In contrast, hydrogen is exclusively produced in bacterial fermentation (4). Since accumulation of hydrogen inhibits the bacterial metabolism, an efficient hydrogen removal is necessary to maintain an ongoing fermentation (21,22). H_2 removal is possible via three pathways: methanogenesis by methanogenic archaea, acetogenesis by acetogenic bacteria and sulphate-reduction by sulphate-reducing bacteria (SRB) (4,21). While methanogenesis and sulphate-reduction are the major routes for H_2 consumption, the dominating routes are decided by sulphate availability (4). An important end-product of saccharolytic fermentation are short-chain-fatty acids (SCFA), namely acetate, propionate, and butyrate in a ratio of 60:20:20 (1,20). However, bacteria cannot synthesize different SCFA at the same time (16). These SCFA are of various use for the host, since they are essential for colonocytes (12), exhibit anti-inflammatory and anti-oxidative properties (2) and play an important role in the microbe-gut-brain-axis (1). Since both propionate and butyrate are mostly metabolized in the intestinal epithelia and the liver, only a small fraction is found in the systemic circulation, where acetate represents the majority of SCFA (1). Butyrate is of special interest since it is the primary energy-source for colonocytes and thus crucial for their lipid- and membrane-synthesis, as well as detoxification-processes and ion-absorption, which in turn are essential for an adequate function of the intestinal barrier (19). In addition, butyrate inhibits the growth of colorectal cancer cells and induces apoptosis in damaged cells via Wnt activity (23,24). Interestingly, butyrate is already completely absorbed in the small intestine, even though it is vital for colonocytes, thereby the only source of butyrate in the large intestine is bacterial fermentation (20). Furthermore, the production of SCFA lowers pH-levels, thus inhibiting the growth of potential pathogens like *Escherichia coli* and *Enterobacteriaceae* that need higher pH-levels to thrive (16). Primary butyrate-producers are *Faecalibacterium prausnitzii*, *Lachnospiraceae*, *Eubacterium hallii*, *Clostridium indolis* and *Anaerostipes caccae* from the Firmicutes phylum, as well as Bacteroidetes-members (4). Interestingly, the fermentation process is a series of multiple fermentation reactions, in which the metabolites of one reaction act as the substrate for another reaction (4).

1.1.3.2 Bile acid metabolism

Bile acids are synthesized in the liver, stored in the gall bladder, and secreted in the duodenum where they primarily act as detergents, helping in the breakdown and resorption of lipids (18). However, bile acids were recently shown to have multiple applications, like acting as signalling molecules, altering genome expression, or influencing the systemic lipid-metabolism (18). In addition, the microbiome is also affected by bile acids, and some strains harbour the ability to modify bile acids and influence their synthesis (18). Bile acids are synthesized from cholesterol in liver hepatocytes, the main bile acids are the primary bile acids cholic acid and chenodeoxycholic acid (18). These primary bile acids are then conjugated with taurine or glycine into a hydrophilic state and stored in the gall bladder (18). After their secretion in the duodenum, microorganisms with bile-salt-hydrolases (BSH) start to deconjugate bile acids and modify them, thereby producing deconjugated and secondary bile acids, further enhancing the bile acid pool (18). Up to 95% of the bile acids are efficiently reabsorbed in the terminal ileum via active transporters and transported back to the liver, where they are again stored in the gall bladder, entrapped in the enterohepatic circulation (18). However, the resorption of conjugated bile acids via these transporters is better than that of deconjugated ones (1). The other 5% are passively absorbed in the large intestine. Bile acids have an antimicrobial effect, that is achieved via the interference with bacterial membranes, DNA damage and oxidative stress (18). Thereby, microorganisms that possess bile-salt-hydrolases, are more resistant to the exposure of bile acids (18). Furthermore, these microbes maintain the ratio of unconjugated to conjugated, as well as secondary to primary bile acids (18). These different bile acids are ligands of different magnitude to a multitude of receptors, namely farnesoid-x-receptor (FXR), vitamin-D-receptor (VDR) and TGR5 (18). While FXR primarily binds unconjugated bile acids, TGR5 preferably binds conjugated bile acids (18). Microorganisms can therefore influence which receptors are activated, by changing the ratio of the conjugated and unconjugated bile acids (18). FXR controls the de-novo-synthesis of bile acids via a negative feedback mechanism, inhibits lipogenesis and induces lipolysis, thus reducing triglyceride- and cholesterol-levels (18). In addition, activation of FXR has an anti-inflammatory effect via interleukin 8, and inducible nitric oxide synthase (18). In the gut-brain-axis, FGR also plays a role since it can inhibit hypothalamic neurons via fibroblast-growth-factor-γ (1). TGR5 has also anti-inflammatory effects and seems to improve glucose-regulation and weight-gain via an enhance synthesis of thyroxine (1,18). Both FXR and TGR5 inhibit the intestinal secretion of Cl^-, thereby controlling the faecal viscosity and preventing diarrhoea (18). In addition,

both receptors are found in intestinal epithelia, and immune cells, indicating their role in the regulation of immune tolerance (18). Via these mechanisms, bile acids control the growth of microorganisms in the gut and are therefore responsible for the prevention of small-intestinal bowel overgrowth (SIBO) (18). In conclusion, bile acids and microorganisms are influencing each other and the host heavily; therefore, it is necessary that a delicate balance is maintained. If this balance is broken, for example by the administration of broad spectrum antibiotics, which affect bacteria involved in the synthesis of secondary bile acids, pathogens such as *Clostridium difficile*, which are usually inhibited in growth by secondary bile acids, can multiply and cause an infection (18). In addition, reduced bile-salt-hydrolase-activity is associated with weight gain, thereby indicating, that changes in bile acid profiles, mediated by the microbiome, can influence host metabolism (18).

1.1.3.3 Hydrogen Sulphide metabolism

Hydrogen sulphide (H_2S) is a dose-dependent, lipophilic toxic gas, that is endogenously produced in the colon, as well as by bacterial fermentation of sulphur containing amino acids and sulphate-reduction (4,25). However, both pathways need sulphur, which is usually provided in form of the two amino acids methionine and cysteine (19). Sulphate reduction is performed by sulphate-reducing bacteria (SRB) under the consumption of hydrogen and sulphur (4,19). The majority of sulphate-reducing bacteria belong to the phylum Proteobacteria and the dominating species in humans is *Desulfovibrio* (19). Sulphate reduction via sulphate reducing bacteria is one of the three pathways in the elimination of hydrogen, and the decision whether methanogenesis or sulphate-reduction are dominating depends largely on the availability of sulphur (4,21).

Even millimolar concentrations of H_2S can disrupt colonocyte metabolism, especially butyrate oxidation, which is crucial for several cellular functions and the maintenance of the intestinal barrier (19,25). In lower concentrations H_2S acts as a gasotransmitter; due to its high lipophilicity it can easily penetrate cell membranes and induce multiple effects, like relaxation of smooth muscles, inhibition of intestinal motility, increasement of the secretion of Cl^-, as well as having direct and indirect antioxidative effects (25). However, with increasing concentration hydrogen sulphide is toxic, due to its inhibition of the electron transport chain via binding to cytochrome-c-oxidase, thereby disrupting the ATP and energy-production (25). In addition, H_2S in higher dosages inflicts direct DNA damage and disturbs the balance of apoptosis, proliferation, and differentiation, thus acting as a carcinogen (25). Since H_2S has a high reduction potential and antioxidative effects, it reduces

oxidative stress and protects microbes from oxygen, thereby maintaining anaerobic conditions in the colon (19). However, increasing hydrogen sulphide levels inhibit the metabolism of most bacteria and can disrupt archaeal methanogenesis (19). Therefore, H_2S-production and elimination need to be tightly regulated to maintain homeostasis. Faecal components have a large capacity for binding hydrogen sulphide, and thus large amounts of intestinal H_2S are eliminated by this mechanism (25). Intestinal epithelia are also capable of eliminating H_2S via rhodanase and thiol methyltransferase (TMT), which oxidizes H_2S to thiosulphate and sulphate (19,25).
Interestingly, these detoxification mechanisms for H_2S are impaired in ulcerative colitis and colon cancer, thereby increasing H_2S concentrations in these medical conditions (19,25). In addition, sulphate reducing bacteria levels are elevated in patients with ulcerative colitis, but not in patients with colon cancer (19). Consequently, it has been suggested, that high hydrogen sulphide levels, due to an increased production or impaired detoxification might cause ulcerative colitis and colon cancer (19). This is supported by the fact that 5-aminosalcylate (5-ASA), a drug commonly used in the treatment of ulcerative colitis, inhibits sulphidogenesis and the growth of sulphate-reducing bacteria, thereby reducing H_2S levels (19). Concerning colon cancer, H_2S might be involved in the multistep mutation that leads to the formation of cancer cells, due to its genotoxic potential (19).

1.1.4 Microbiome-Host-Interactions

Microorganisms are strongly involved in metabolic functions of the host, development, as well as health and disease (4). To ensure the maintenance of homeostasis, it is crucial that both host and microbiome can communicate with each other; this is realized through various pathways (1,11). The immune system is also heavily involved in these interactions, since it is responsible to limit the growth of pathogenic microbes while tolerating beneficial ones. Firstly, microbes and host can interact via proteins, metabolites, small molecules, and nucleic acids (11). These are recognized by host cells and microbes, via receptors or influence their cellular functions. One method for bacteria to interact with host proteins is via “molecular mimicry” (11). This means that bacteria mimic the structure or sequence of host proteins, thereby enabling these bacteria to interact with them and influence cellular functions (11). In addition, both microbiome and host interact via the production and recognition of signalling molecules and metabolites, that can be produced on purpose to induce a certain reaction.

1.1.4.1 The gut-brain-axis

The brain is highly influenced by the microbiome, either via circulating metabolites, or by interacting with the enteric nervous system (ENS) and autonomous nervous system (ANS), however this interaction is bidirectional (1). The enteric nervous system is comprised of two neuronal networks that reside in the gastrointestinal tract, namely submucosal and the myenteric plexus, which coordinate intestinal motility and fluid movements (1). Interestingly, the enteric nervous system's development is modulated by the microbiome (via PRR like Toll-like-receptor 2 and 4), and it reacts to viral RNA and bacterial lipopolysaccharides (1). The enteric nervous system is in turn modulated in its function by the autonomous nervous system, especially by the vagal nerve (X^{th} cranial nerve). The autonomous nervous system controls most digestive functions, like bile secretion, luminal osmolarity, mucus secretion, fluid secretion, as well as the mucosal immune reaction (1). However, the microbiome can synthesize neuromodulatory metabolites, like serotonin, histamine, gamma-amino-butyric-acid (GABA) and catecholamines that modulate both the enteric and the autonomous nervous system (1). In addition, these neuromodulators also have a direct effect on microbes, for example inducing genes responsible for virulence, migration, and growth (1). Besides the ENS and ANS, the gastrointestinal tract harbours enteroendocrine cells, which secrete digestive hormones like GLP-1 and PYY in the presence of nutrients. However, these cells are also activated by short-chain-fatty acids (SCFA) and lipopolysaccharides, both provided by microorganisms. Since bacterial fermentation is practically always running, it is continuously producing SCFA. Therefore, it has been suggested that microbial SCFA-production might be responsible for basal secretion of GLP-1 and PYY, thus influencing hunger and weight gain (1). Furthermore, short-chain-fatty acids have multiple other applications via the inhibition of intracellular histone deacetylation or activation of vagal receptors (1). SCFA enhance the intestinal barrier by an increase in mucus secretion and formation of tight junctions, as well as the blood-brain-barrier. In addition, they are involved in the regulation of the immune system and the microglia via regulatory T-cells and influencing neutrophilic chemotaxis (1). Hippocampal and striatal neurons are also modulated by SCFAs, thus influencing cognition and learning (1). Another important communication device are bile acids, which has been discussed in the chapter on **bile acid metabolism** (1,18). Bile acids can directly influence brain function via the farnesoid-x-receptor/fibroblast-growth-factor-pathway, inhibiting hypothalamic neurons and improving glucose-tolerance (1). Overall, both brain and microbiome can influence each other by various means, maintaining homeostasis.

1.1.4.2 The immune system and the microbiome

The human body hosts more than 10^{14} bacterial cells (2), meaning that about half of all cells in a person are of microbial origin (1), it is therefore vital for the body to control the growth and spread of beneficial or harmful microorganisms (9). This task is performed by epithelial cells in collaboration with the immune system, which is further classified in the innate and adaptive immune system. About 70% of all lymphocytes are found in the intestine-associated lymphatic tissue of the gut, highlighting the importance of the immune system in the regulation of the microbiome (10). The innate immune system recognizes microbe-associated-molecular-patterns (MAMP) of commensal and pathogenic microorganisms, like lipopolysaccharides or peptidoglycans, via membrane bound pattern-recognition-receptors (PRR), most notably Toll-like-Receptors (TLR) and NOD-Like-Receptors (NLR) (8). After the recognition of MAMPs, innate immune cells and intestinal epithelia secrete pro-inflammatory cytokines and anti-microbial proteins (AMPs), which interact with the cell wall of the affected microbes, thereby disturbing membrane integrity and initiating the complement cascade (8,26). However, it is vital that the immune system is able to distinguish between beneficial microorganisms and potential pathogenic ones, in order to enable the beneficial organisms to live in symbiosis with the host and, at the same time to avoid a permanent or exacerbating inflammatory reaction (8,26). Experiments on germ-free mice showed that their immune system is impaired in the absence of microorganisms, and that the microbiome is necessary for immunomodulation (26,27). Therefore, multiple mechanisms to ensure tolerance exist (7). An example of this is the double mucus layer in the intestine, the luminal layer of which is loosely adherent and contains most microbes, while the underlying highly adherent layer is germ-free and serves as a physical barrier (7). The adaptive immune system is primarily responsible for the differentiation between commensals and pathogens, as well as the regulated inflammation (8,9). Here, microbial antigens are recognized and presented by antigen-presenting cells (for example monocyte derived dendritic cells) after phagocytosis, which then activate T- and B-cells to initiate further phagocytosis and antibody-production via secretion of proinflammatory cytokines (8,28). The interaction between microbiome and immune system is crucial for the formation and priming of a competent immune system (7). There is even the hypothesis that the adaptive immune system is an evolutionary response driven by the presence of microbiota themselves (8).

1.1.5 Factors influencing the intestinal microbiome

The shape and diversity of the microbiome depend on various factors of different magnitude; these factors include: age, diet, host genetics, lifestyle, sex, environment (for example pets), physical exercise, medication, use of anti-, pre- or probiotics, stress and social activity (1,13,16). Some of these factors even play a crucial role in the development of the microbiome during pregnancy (1). Since the microbiome is heavily involved in neuronal development and cognition, changes in its composition in early childhood can have lasting, unforeseen effects (1). Overall, the microbiome is part of many crucial processes and functions, that shape the hosts metabolism and in turn the microbiome itself. However, it is difficult to say which factors are decisive for changes in the microbial composition since they can mutually influence or consequently cause one another. Lifestyle for example includes choices in diet, physical exercise, and other habits, while lifestyle itself is influenced by age, geography, and social-economic status.

1.1.5.1 Host genetics and the microbiome

Since interindividual differences shape the microbiome, one of the most evident and unique factors is the host genome (4,9,13). Host genetics have a major impact on the composition of the microbiome, that is equal to the impact of diet (13). To date it is suspected that the effect of host genetics shapes the microbiome over a long time and is therefore best observed in older populations. So far, little is known about how exactly host genetics influence the microbiome and most research is centred on the heritability of the maternal microbiome (6). However, studies showed that the microbiome of monozygotic twins is more similar than that of dizygotic twins (6,7,15,21). In addition, some bacteria like *Christensenellaceae* turned out to be highly heritable and associated with diverse co-occurrence networks including other microorganisms like archaea (7,15,29). Interestingly, *Christensenellaceae* are primarily observed in lean individuals, with a high microbial diversity and can, in combination with co-occurrence networks, negate the pro-obese effects from obese individual’s microbiomes, as was shown on germ-free mice (29). Nevertheless, the question arises, whether these co-occurrence networks, are the result of independent inheritance of each taxon or the correlation of other taxa with the inheritance of a few key taxa (7).

Overall, some taxa are highly heritable and can induce effects on the microbiome and its host (6,29). In addition, the microbiome is extremely resilient; for example, when probiotics are used, transient colonization occurs in only 60% of cases (30). This chance of colonization depends on interindividual factors, the strains used and, above all, on the prevailing microbiome(30). However, the impact of host genetics needs to be further explored.

1.1.5.2 Diet

Changes in diet can lead to changes in diversity and composition, that can be observed as early as 24 hours after introducing a new diet (7). Interestingly, these changes only seem to be significant, if the ratio of macro-nutrients (carbohydrates, fat, and protein) is altered (14), and they persist only for as long as the new diet is maintained (7). This is mainly due to the fact that carbohydrates are the primary source of energy for most microorganisms and their proportion in the ingested food thus decisively shapes the microbiome (14). While long-term-diet and host genetics have a major impact on the microbiome, age and lifestyle are presumed to be the dominating factors that shape the composition of the microbiome (13). However, since both age and lifestyle, as well as other factors like geography and ethnicity influence the choice of diet to a large extent, the impact of maintaining a diet might be of higher magnitude than expected (16). Geographic and ethnic differences in the microbiome can be explained to be to some part due to a different choice of diet (4,16). “Western”-diets are high in fat and carbohydrates and low in fibre, thereby promoting the growth of Firmicutes and reducing the amount of Bacteroidetes like *Prevotella*, that are responsible for fibre-degradation (4). In contrast, diets high in fibre, low in fat and carbohydrates do the opposite (4). However, it should be noted that changes on the phylum level are difficult to interpret since they include a multitude of different species, which in turn have fundamentally different metabolic functions. Changes in the microbiome with increasing age can also be partially attributed to the changing diet (13). The microbiome of a new-born is low in diversity and specialized on breast milk; with the introduction of solid foods diversity increases and peaks in adulthood (10,16). However, with increasing age, the choice of diet is limited, due to a decline in dentition, reduced salivatory function, digestion, prolonged intestinal transit time and impairment of the taste-buds, resulting in a loss of appetite (10,16). This is reflected in a decrease in diversity, which is particularly pronounced in residential care homes (10,13). Overall, a complex and diverse diet, results in a diverse microbiome, that is more resilient against perturbations (1,12,16). Interestingly, the microbial genome can probably be extended via horizontal gene transfer from other microorganisms in ingested food, thereby enabling the microbiome to degrade food components that would otherwise be indigestible (4). Different bacterial strains and species prefer distinct metabolic pathways for energy production, for example *Bacteroides*, *Clostridium*, *Propionibacterium*, *Fusobacterium* and *Lactobacillus* are highly involved in proteolysis, and fermentation of amino acids (4). This is reflected in an increase of *Bacteroides* in protein-rich diets (12).

In conclusion, diet has a major impact on the microbiome, however, the microbiome is indeed capable of regulating appetite, thus it may also take part in the choice of diet (1).

1.1.5.3 The gut microbiome over the course of a lifetime

Current research strongly debates whether microbial colonization begins during pregnancy *in utero*, since microorganisms were isolated from meconium, amniotic fluid, placenta, and the umbilical cord, thereby indicating that colonization potentially could already begin in utero (4,13,16). By swallowing amniotic fluid, microorganisms could then colonize the foetal gastrointestinal tract (16). It is possible, that this first colonization is vital for the protection against early infections, such as necrotizing enterocolitis, which mainly affects premature children (16). In addition, colonization could develop gradually during the foetal period and can be disrupted, resulting in an altered composition of the microbiome (16). However, the intestinal microbiome changes after birth and resembles the microbiome the infant encounters during birth, which is, depending on the mode of delivery, the vaginal microbiome (vaginal delivery) or the skin microbiome (caesarean section) (4,16). Interestingly, children delivered vaginally show higher populations of Bacteroidetes than Firmicutes, when compared to children delivered via caesarean section in the first weeks after birth (16). The microbiome is initially mostly composed of aerobes and facultative anaerobes, which gradually reduce oxygen, thereby creating an environment, suitable for strictly anaerobic gut bacteria (4). After birth, the microbiome is primarily shaped by diet, which ideally consists of breast milk (13,16). Breast milk is not sterile, and contains different strains of Bifidobacteria and indigestible oligosaccharides, which serve as prebiotics for Bifidobacteria, and thus further enhancing their growth and colonization (4,16). This results in a microbiome with low diversity, that is formed by this diet, and specialized in the degradation of its by-products (16). Furthermore, a metagenomic analysis of faecal samples from un-weaned new-borns has shown that their microbiome harbours many genes, involved in the synthesis of folate or the degradation of oligosaccharides, lactose, and mucin (17). In addition, formula fed infants prove to have a different microbiome composition than breast-fed infants (4). The microbiome is then again subject to change after the introduction of solid foods in the diet (4). At the age of three years, the microbiome closely resembles that of an adult, however, the transition appears to happen slowly over time (7,16). With increasing age, the diversity is declining and shifts in the composition can be observed, however, this is strongly related to certain living conditions, such as living in residential care homes (12). In addition, the Firmicutes-Bacteroidetes-ratio also changes over the course of a lifetime:

beginning low at 0.4 at infancy, rising to 10.9 in adulthood, and declining back to 0.6 with increasing age (31). The microbiome shifts with increasing age to a proinflammatory phenotype, due to a reduced abundance of Firmicutes, Bifidobacteria, *Faecalibacterium prausnitzii* and an increase in Proteobacteria such as *E. coli*, resulting in increasing DNA-damage, an impaired immune response and reduced synthesis of vitamin B12 (7,12). Overall, diversity increases with age until adulthood and starts to decline with advancing age, due to a variety of different factors (16). These factors include changes in diet, immune tolerance, mobility, physical appearance, and gastrointestinal diseases (10). Changes in diet can be the result of a decline in dentition, digestion, and prolonged intestinal transit time, cumulating in a loss of appetite, limiting the choice of diet to a less diverse spectrum (10,16). With higher age the immune system undergoes a process called immunosenescence, which results in T-cell dysfunction and dysregulation of the adaptive immune system (10,12). This in term leads to permanent low-grade inflammation due to an imbalance of pro-and anti-inflammatory cytokines, which consecutively affects the intestinal microbiome (10). However, since the microbiome can induce and regulate the tolerance of the immune system through various metabolites, it can thereby contribute to the regulation or exacerbation of immunosenescence, for example by controlling the permeability of the intestinal epithelial barrier (12). With increasing intestinal permeability, translocation of microorganisms and antigens can rise, resulting in a syndrome called "leaky gut- syndrome" (10). In turn, the increased bacterial translocation leads to a low-grade systemic inflammation (10). The resulting inflammation impairs the adaptive immune system and damages macro-molecules and cell structures through metabolites and end-products (12). However, the primary cause is unclear, since the mechanisms that eventually lead to dysbiosis and immunosenescence, are bidirectional and can influence or exacerbate each other (10). Overall, the microbiome in older populations shows a reduced diversity and loss of diversity-associated taxa, like *Prevotella*, *Bifidobacterium* and *Lactobacillus*, while *Bacteroides* increase in abundance (10,12). This is further reflected in a decrease in genes for short-chain-fatty-acid production, saccharolysis and an increase in proteolytic potential (10,12). In addition, the phylum of Proteobacteria, which includes many opportunistic bacteria increases in abundance (10). This results in a microbiome with a pro-inflammatory phenotype, that is marked by instability and low diversity (1,7). The loss of short-chain-fatty-acid producers like *Faecalibacterium prausnitzii* and *Ruminococcus* leads to decreased SCFA-levels (12). However, SCFA are essential in the metabolism of colonocytes and the microbiome's communication with the host (1,5,7). In addition, it has been suggested, that the loss of few

key species can cause an extinction-cascade, destabilizing the microbiome and finally leading to its collapse (6).
In this regard, a diverse microbiome might be more resilient and could protect the microbiome from collapsing (6). Furthermore, frailty is especially associated with a decrease in diversity and diversity associated taxa, that are mainly involved in the digestion of dietary fibre, like *F. prausnitzii* (12).

1.1.5.4 Antibiotics

Up to date, antibiotics are routinely used and widespread in medical procedures and treatments, as well as in the environment. In general, antibiotics cannot distinguish between beneficial and harmful bacteria. Therefore, the use of antibiotics leads to a decreasing microbial diversity and beneficials (10,16). This is especially important concerning children, since antibiotic treatment in early childhood (<6 months), is associated with crohn's disease and obesity (6). In addition, to an immediate perturbation, antibiotics can have a long-lasting effect on microbial composition and the development of the central nervous system and the enteric system, both of which are mediated by the intestinal microbiome (1,6). Furthermore, antibiotics can promote the growth and spread of *Clostridium difficile*. *C. difficile* is usually inhibited by secondary bile acids, produced by intestinal bacteria (18). Interestingly, these beneficial bacteria are depleted after antibiotic treatment, and thereby the growth of *C. difficile* is no longer inhibited and can now spread freely, leading to an infection (18). By altering the microbiome's composition, bile acid metabolism and bile acid pool composition are also affected, thereby influencing host-lipid metabolism (18). Overall, the depletion of beneficial microorganisms leads to an impaired systemic-allergic reaction, even reducing the efficacy of influenza vaccines, due to a compromised immune reaction (10). Furthermore, it is hypothesized, that the cause of vitamin K-deficiency might be the depletion of beneficials by antibiotic treatment and not malnutrition (4). The important role of the microbiome in immune regulation and induction, which is crucial in chemotherapy, can also be impaired by antibiotics (7,26). For example, antibiotic treatment in chemotherapy is associated with a reduced progression free survival and reduced overall survival (26). Therefore, antibiotics should be used with care and only with a valid indication.

1.1.6 Microbiome in Health and Disease

Due to its strong involvement in host functions, vast metabolic potential and continuous interaction with the host immune system, microorganisms have a major impact in human health and disease (4,6). This section aims to summarize certain microbiome features, which are associated with certain diseases and the underlying pathophysiology, as far as they are known. Changes in the microbiome that lead to an altered immune response and an enhanced inflammation are summarized in the term “dysbiosis” (10). Dysbiosis is associated with multiple medical conditions and diseases, however, it is unclear whether dysbiosis is caused by these diseases or predisposes the host to these diseases (10).

1.1.6.1 Obesity

Obesity has reached epidemic proportions in western populations and is highly associated with a multitude of other metabolic conditions and a major reason for increasing mortality (32). Specific changes in composition and function of the microbiome are observed in obesity (5), such as a decrease in microbial diversity and richness (5–7,16,18,33,34). These changes are so specific, that they are more predictive for obesity than the host genome (35). There are two pathways for the microbiome to influence energy balance: First by increasing total energy extraction from food and second by influencing host genes, which regulate energy storing and expenditure (5). Some studies observed a change in the abundance of the phyla Firmicutes and Bacteroidetes with a high-calorie diet (34). While Firmicutes have increased in abundance in obese individuals, Bacteroidetes decline, thereby increasing the Firmicutes-Bacteroidetes-ratio (2,34). However, existing data is inconclusive since these changes were not consistently observed in other studies (32,33). Other factors like diet, use of antibiotics and age could be causative of these inconsistent changes in the mentioned studies (5). In addition, the microbiome in obesity is characterized by an increase in energy production and energy-harvesting processes, as well as an increase in pro-inflammatory microbes (7,18,36). Bile acids metabolism is also involved in obesity due to its implications in lipid- and glucose-metabolism, thus it is not surprising that bile acid levels are elevated in obesity (2,18). Faecal microbiome transfer (FMT) in mice and humans has recently shown that the microbiome is extraordinarily responsible for the development of obesity and that it is transferable (5). It was observed in studies on mice, that transplantation of an “obese” microbiome in lean mice results in an obese phenotype (5). So far, this has also been observed in at least one human case, since the FMT led to obesity in a patient after receiving the microbiome of an obese donor (5). Up to now, most centres that perform FMT require

donors to be non-obese for transplantation (37). In addition, to date the only indication for faecal microbiome transfer is recurring infection with *C. difficile* (5). Another interesting observation is that the microbiome has changed in its composition in mice after a gastric bypass regardless of the diet or weight loss (5). Thereby indicating, that gastric bypass not only has a mechanical effect but also changes the microbiome into a more favourable profile, thus promoting weight loss (5). Overall, the microbiome is strongly involved in the development and upkeep of obesity, respective its prevention, thus treatments targeting the microbiome might be beneficial. One therapeutic approach is a change in diet, but even a low-fat, low-carbohydrate diet for one year was not capable to restore the microbiome back into a "normal" composition (5). However, data coverage is still inconsistent and much more research in this field is needed.

1.1.6.2 Inflammatory Bowel Disease

Inflammatory bowel disease is a term that encompasses both ulcerative colitis and crohn's disease. These diseases are characterized by a gastrointestinal inflammation of different degrees and typical locations. Although the exact pathophysiologic mechanisms are unclear, it is assumed that the microbiome plays a major role in inflammatory bowel disease. Changes in the microbiome of IBD patients and dysbiosis are frequently observed, however, it is unclear whether these changes are caused by the disease or cause the disease (10). Ulcerative colitis, which is limited to the colon and rectum, is highly associated with the presence of sulphate reducing bacteria and the production of hydrogen sulphide (25). Since hydrogen sulphide inhibits butyrate oxidation in colonocytes, it leads to an impairment of the intestinal barrier and potential intestinal cell damage, due to its toxic effects (25). Due to the impaired barrier function, the immune response to microbial antigens and microbes is altered and results in inflammation, thereby leading to dysbiosis and further inflammation (10). Overall, butyrate levels are significantly lower in patients with ulcerative colitis (19). However, not all studies reported an increase in sulphate reducing bacteria in patients with ulcerative colitis, but these studies did not take treatment with 5-ASA in account (19). 5-ASA is a drug commonly used for treatment in ulcerative colitis, that also inhibits sulphidogenesis and growth of sulphate reducing bacteria, thereby non-elevated levels of sulphate reducing bacteria could be attributed to the use of 5-ASA (19). In addition, most patients with ulcerative colitis have diets high in protein, which contain large amounts of sulphur, thereby "feeding" sulphate reducing bacteria and promoting H_2S-poduction (19). Another indication that sulphate reducing bacteria play an important role, can be found in biopsies of pouches

from ulcerative colitis and familial adenomatous polyposis (FAP) patients (19). In patients with ulcerative colitis, sulphate reducing bacteria levels are significantly increased in pouch biopsies, when compared to familial adenomatous polyposis patients (19). In addition, hydrogen sulphide concentration and severity of pouchitis have a positive correlation in ulcerative colitis (19).

In summary, the microbiome is involved in inflammatory bowel disease, but it cannot be said with certainty whether the microbiome is also responsible for its causation. However, it has been suggested that increasing H_2S levels might be contributed by an insufficient detoxification due to genetic polymorphism, especially in the rhodanese gene (19).

1.1.6.3 Neurodegenerative Diseases

Since the microbiome and the nervous system can communicate and influence each other, it is highly probable that intestinal microbes are also involved in neurodegenerative diseases such as Parkinson's disease or Alzheimer's disease. These diseases are characterized by the loss of certain brain neurons, due to multiples causes. However, recent studies have associated proinflammatory bacteria like *Escherichia coli* and *Shigella* with an increased peripheric inflammation and amyloidosis of the brain (10). Oxidative stress is one of the major driving factors in neurodegenerative diseases and stroke (3). Usually, intestinal microorganisms are inducing oxidative stress at the intestinal barrier, that in turn influences the microbiome and intestinal barrier (3). While oxidative stress is physiological and part of most cellular functions, an excess can have neurotoxic effects and cause biomolecular damage, thus finally resulting in mutation, impairment, or cell death (3). Due to its high demand of oxygen, the brain is especially exposed to oxidative stress, which can be influenced via neurotransmitters as well as oxidative and antioxidative metabolites produced by microbiota (3). In addition, with increasing age, microglia, the immune cells in the nervous system, become increasingly aggressive and proinflammatory, thus promoting neurodegeneration (1). However, short-chain-fatty acids produced by microbiota can reduce the aggressiveness of microglia, which can help in the prevention of these diseases (1).

1.1.6.4 Atherosclerosis

Trimethylamine is a microbial metabolite which is produced in the microbial degradation of choline, lecithin, and L-carnitine, which are found in red meat, eggs, and dairy products (2,13). Trimethylamine is then oxidized in the liver to Trimethylamine-N-Oxide, which is highly pro-atherosclerotic (2). TMAO impairs lipid-metabolism, platelet-function, and immune response, which in turns leads to an accumulation of cholesterol, foam-cell formation, and inadequate platelet activation, thus facilitating the formation of atherosclerotic plaques (2). In addition, TMAO inhibits bile acid synthesis, thereby influencing the bile acid pool, lipid-metabolism, and the microbiome (18). Interestingly, intestinal microorganisms can also be found in atherosclerotic plaques, indicating that their translocation might also be part of the formation or exacerbation of atherosclerosis (2).

1.1.6.5 Colorectal Cancer

Colorectal cancer is caused by an accumulation of mutations in a multistep process (19). These mutations can be attributed to a multitude of pro-carcinogenic factors, of which some are caused by the microbiome (19). The degradation of non-resorbed bile acids in the colon by Clostridia for example is associated with colorectal cancer (18). However, one of the major carcinogenic metabolites produced by microbiota is hydrogen sulphide. H_2S is highly genotoxic, and hyperproliferative due to an inhibition of apoptosis and impairment differentiation (25). The role of H_2S in the promotion of colorectal cancer is further evidenced by an increase in abundance of Fusobacteria which are hydrogen sulphide producers and have a pro-inflammatory effect (6,19). In addition, rhodanase which is a major enzyme in the detoxification process of hydrogen sulphide is reduced in patients with colon cancer (25). *Enterococcus faecalis* is another microorganism, with genotoxic effects, that is suspected in the promotion of colon cancer (6). Overall, colorectal cancer is associated with a reduced microbial diversity and dysbiosis (10,16). Interestingly, the microbiome of colon cancer is also different, depending on the affected side (left colon or right colon), microbiota in right sided colon cancer are associated with highly invasive and inflammatory biofilms, that have pro-carcinogenic effects (7). Dysbiosis could also be a factor contributing to the development of colon cancer, since dysbiosis could disrupt the production of short-chain-fatty acids like butyrate, that are known to inhibit growth and spread of colorectal cancer (23,24). In addition, the microbiome is crucial for the effects of chemotherapy and immune therapy in cancer due to its interaction with the host immune system (26).

1.2 Archaea

Archaea are unicellular organisms, that form a third domain of life besides bacteria and eukaryotes (27,38). For a long time, they were considered to be extremophiles, since they are able to thrive under extreme conditions, for example high pressure and temperatures up to 113°C (39,40). However, due to the advancements in the detection of microorganisms in the recent years, they were also detected in large numbers in marine and seafloor sediments, plants, animals and even humans and are now recognized to be widespread in the environment (40). Stool samples have shown that archaea make up about 0.8% of the faecal microbiome in the European population (41). Besides the intestinal microbiome, archaea are also part of the microbiome of the human skin, oral cavity and even the respiratory tract (42). Currently there are four major groups of archaea: the Euryarchaeota phylum, the TACK superphylum (Thaumarchaeota, Aigarchaeota, Crenarchaeota, and Korarchaeota), the Asgard superphylum (Heimdallarchaeota, Thorarchaeota, Odinarchaeota) and the DPANN superphylum (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota and Nanohaloarchaea) (Fig. 1) (27,43). To date the Euryarchaeota phylum is the most extensively studied phylum, since it includes the methanogens, which are the most abundant and widespread archaea in humans (27).

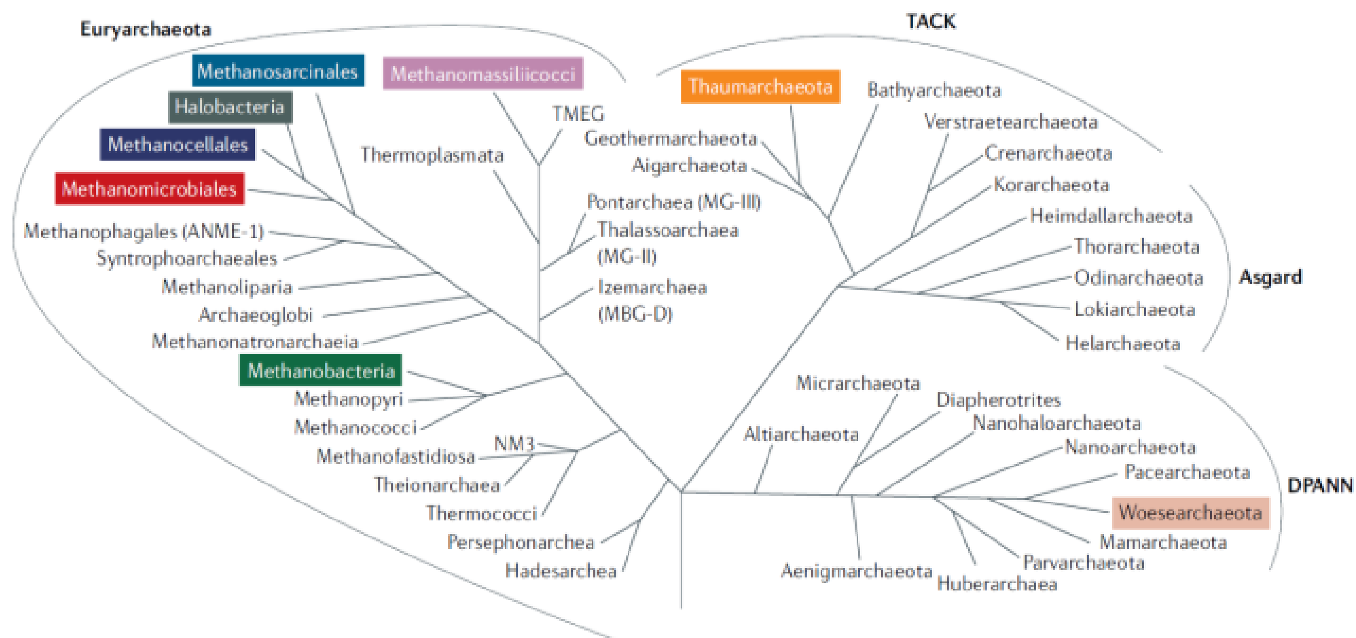

Figure 1 ***Archaeal diversity****. Shown is a schematic of the current tree of Archaea based on the most recent phylogenomic analyses; host- associated clades are highlighted, including Methanosarcinales, Methanomassiliicocci, Thaumarchaeota, Halobacteria, Methanocellales, Methanomicrobiales, Methanobacteria and Woesearchaeota.*

Reference: *The host- associated archaeome, G. Borrel, J. Brugère, S. Gribaldo, R. Schmitz & C. Moissl-Eichinger, Nature Reviews Microbiology volume 18, pages 622–636 (2020)*

Although archaea have a lot in common with bacteria and eukaryotes, they have some unique properties that distinguish them from other lifeforms. The similarities with bacteria include properties such as the presence of one circular chromosome, the lack of introns, similar post-transcriptional modifications, and a highly variable morphology (40). Whereas archaea share similar molecular mechanisms for DNA-replication, RNA-transcription, and protein translation, as well as the presence of histones for DNA-packaging with eukaryotes (40). However, some unique features of archaea are, that their cell wall is not composed of peptidoglycan (murein) like in bacteria, but of pseudopeptidoglycan (pseudomurein) (Fig. 2) (44). Also, there are significant differences between Pseudomurein and murein, regarding the composition and linkage of their components, which results in a high resistance of archaea versus most known cell-wall-antibiotics (44). It is suspected that this thereby acquired antibiotic resistance is also linked to the increasing diversity of archaea in older populations, as it is assumed that the antibiotic treatments which are accumulated over the course of a lifetime are selectively promoting archaea due to this resistance (40). Interestingly, in some archaeal species, the phospholipid bilayer of the cell membrane is replaced by a monolayer composed of tetraether lipids that enhances stability and rigidity, which are necessary to survive in extreme conditions (Fig. 2) (8). Another unique feature is the composition of the archaeal phospholipid cell membrane, which is, in contrast to other organisms not composed of fatty acid-glycerol-esters, but of isoprenoid-glycerol-ethers (8,44,45).

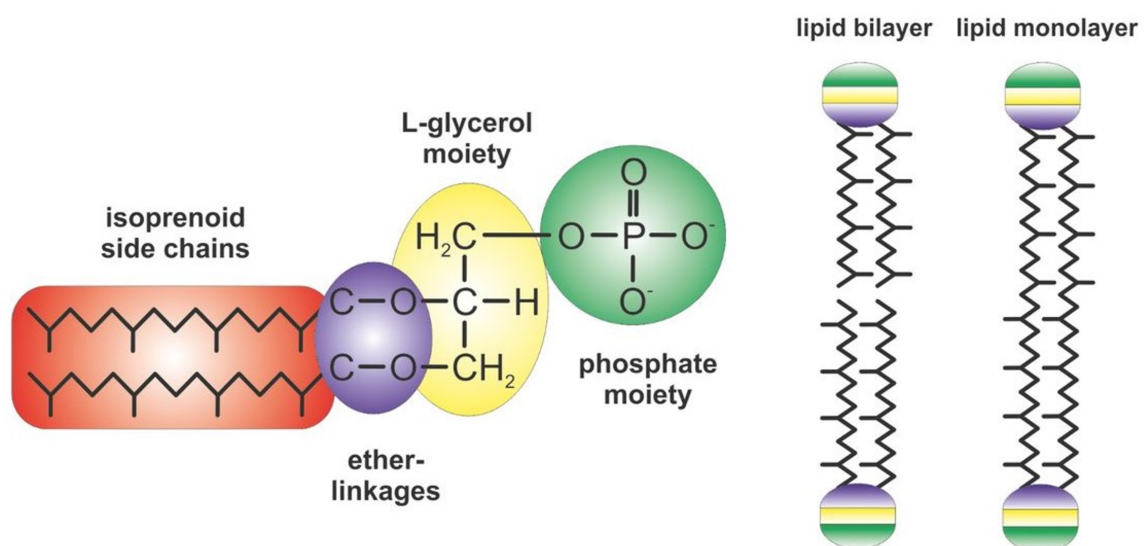

Figure 2 ***Archaeal cell membrane.*** *The composition and structure of archaea's cell membrane is unique. In detail, the glycerol-ether lipids are composed of L-glycerols and isoprenoid side chains. In addition, in several archaea the cell membrane forming phospholipid bilayer is replaced by a monolayer in order to enhance stability and rigidity of the membrane.*

Reference*: Modified figure from Bang, Corinna and Schmitz, Ruth A, archaea associated with human surfaces: not to be underestimated, FEMS Microbiology Reviews, 2015, 39, 631-648, by permission of Oxford University Press*

These isoprenoids are synthesised by using the HMG-CoA reductase, which is the reason why some statins, such as Lovastatin can interfere with the archaeal membrane synthesis (44,46). However, the inhibition of the archaeal HMG-CoA-reductase has only been shown to work in vitro with Lovastatin and Mevastatin (47,48), but not with other statins like Atorvastatin or Simvastatin (44). Besides, it is currently unclear, whether the statin levels being necessary for inhibition can be achieved *in vivo* in the gut. Archaea typically possess only a single cytoplasmic membrane; however, some interacting archaea do possess a double membrane which is possibly energized to support the interconnection with other organisms (27,45). Since the visualization of this second membrane is extremely difficult, there is the assumption that significantly more archaeal species have such a membrane and, due to the methodological difficulties, it just could not yet be observed very often (27).

Since archaea and eukaryotes are sharing many key characteristics, it has been suggested that eukaryotes emerged from within the archaea, which, nevertheless remains quite controversial (40). Recently, strong evidence was found, that eukaryotes might have arisen from a symbiosis of archaea and bacteria (8). Due to the similarities regarding archaeal and eukaryotic rRNA, it has been a challenge in the past to perform a PCR without amplifying the host rRNA in a sample. It was not until 2011, that adequate universal primers for bacterial and archaeal 16S rRNA which do not amplify the host rRNA-genes became available (46). To date there are no known pathogenic archaea, although they impact the human health and play a role in some diseases like periodontal disease, diverticulitis, inflammatory bowel disease and irritable bowel disease (36,46,49). Archaea are also the only known organisms to perform methanogenesis and thereby produce methane for energy gain (44). Another characteristic is their long doubling time in comparison to most bacteria, which can range from several hours to days, depending on the environment (40,50,51). Another interesting fact is that some hyperthermophilic archaea are one of the fastest (swimming) organisms if measured in body lengths per second (bps), with speeds up to 500 bps and they show a unique swimming pattern (52). Depending on their environment, they exhibit a slow "zigzag" movement or a fast movement in straight lines, with nearly no change in direction. This mode of swimming differs fundamentally from the one observed in most bacteria and is probably related to their mostly hostile environment (52).

1.2.1 The human archaeome

The human archaeome is defined as the archaeal component of the human-associated microbial community (43).The archaeome is distinct for each location in and on the human body in its composition and abundance (42,43). While the gastrointestinal archaeome is mostly composed of methanogenic archaea, as well as some halophilic archaea (namely *Halofax massiliensis* and *Halofax assiliense*), the archaeome of the skin is dominated by Thaumarcheota, which probably can metabolize urea for ammonia oxidation (42,43,53). The presence of Thaumarcheota on the skin could also be linked to age and skin physiology, however more research in this direction is needed (43). In contrast, the archaeome of the lung is largely inhabited by Woesearcheota from the DPANN superphylum (42). Interestingly, the archaeome of the nose and upper respiratory tract is a mixture of the archaeome of the skin and gastrointestinal tract (42,43).

The nasal archaeome also shows the highest diversity compared to the other body sites (42). However, the impact of non-methanogenic or halophilic archaea on the microbiome in the gut are currently unclear, it is subject of discussion, whether Halobacteriales are only transient members of the gastrointestinal tract and associated with the consumption of salt-containing food (43).

In addition, the role of Woesearcheota is unclear, since they have so far mostly been found on human-associated areas like doorknobs, dust in the international space station and environmental samples (42).

In contrast, the gastrointestinal archaeome, being dominated by methanogens has been extensively studied via faecal samples and biopsies (42,43,53). So far, it is known, that these methanogens are responsible for the production of 0.35 litres of methane per day per person, which accounts for about 0.2% of all anthropogenic emitted methane (43). However, methane production varies between different geographic locations and ethnicities (43). In addition, the archaeal composition of the gastrointestinal archaeome varies among the gastrointestinal tract, and it is even exhibiting some clustering, for example in the ileum and left colon, indicating a strong mucosal association of archaea (42). Furthermore, the difference of the archaeome of left and right colon could have an impact on diseases and therapies. For example, the use of EGFR-inhibitors in palliative chemotherapy shows a different response in right- or left-sided colon carcinoma (54). However, whether this mechanism is mediated by the archaeome remains to be explored.

1.2.2 Human methanogens and Methanogenesis

The most extensively studied archaea are the methanogens, since they are the most widespread among the archaea in humans and are believed to have a major impact on human health and well-being (36,40,42,46). They are characterized by their ability to produce methane, which is unique, as archaea are the only organisms that are capable of methanogenesis (44). These methanogens are strictly anaerobic and are further categorized based on their preferred substrate for methanogenesis in hydrogenotrophic, acetotrophic and methylotrophic methanogens. Most methanogens are hydrogenotrophs, which utilize hydrogen and carbon-dioxide to produce methane ($4\ H_2 + CO_2 \rightarrow 2\ H_2O + CH_4$) (22,36,42,44). To date there are seven orders of methanogens: the first order of Methanobacteriales, the second order of Methanococcales, the third order of Methanomicrobiales, the fourth order of Methanosarcinales, the fifth order of Methanopyrales, the sixth order of Methanocellales and the recently added seventh order of Methanomassiliicoccales (36,40,46). The order of the Methanosarcinales is outstanding as they are the only acetotrophic archaea among the methanogens (40). The majority of the hydrogen and other substrates being essential for archaea, originate from the anaerobic degradation of organic matter by fermentative bacteria, which places archaea in a terminal position of the food chain (22,36,40,42). Since methanogens reduce the present amount of hydrogen, they streamline the bacterial fermentation by the bacteria which benefit from a lower hydrogen-partial pressure, as an accumulation of hydrogen inhibits bacterial NADH-dehydrogenases, thereby reducing their total ATP yield (21,40). Some bacteria like Ruminococcaceae for example can double their ATP yield, when they occur together with methanogenic archaea (22). Therefore, fermentative bacteria can thrive and produce more hydrogen when methanogens are present, which consecutively thrive and reduce the hydrogen-partial pressure. As both methanogens and fermentative bacteria benefit from each other they often form a syntrophic relationship (21,22,36). In addition to Ruminococcaceae, there are some other bacteria like Veillonellaceae and Bifidobacteriaceae with which archaea live in syntrophy (22). Interestingly, archaea form this relationship with Bifidobacteriaceae, although they produce no hydrogen in contrast to Ruminococcaceae and Veillonellaceae (22). In ruminants this optimized fermentation leads to a total caloric energy loss of the host for about 6%, whereas it is speculated that this streamlined process could increase the caloric intake in humans by the enhanced production of short chain fatty acids through fermentative bacteria (46). However, archaea are not the only microorganisms to consume hydrogen, besides methanogenic archaea, there are also acetogenic bacteria and sulphate-reducing

bacteria (21). Therefore, archaea, acetogenic and sulphate-reducing bacteria are in competition for hydrogen, which is reflected in the inverse correlation of acetogenesis by acetogens and methanogenesis by methanogens (22). Although sulphate-reducing bacteria and methanogens compete for hydrogen, they are known to grow syntrophically on lactate (22). Since sulphate-reducing bacteria play a critical role in diseases like colon cancer, ulcerative colitis, and irritable bowel syndrome by the production of hydrogen sulphite (H_2S), their interaction and competition with methanogens is of vital importance, which will be discussed in another section (19,55).

To date, only eight different species of methanogenic archaea have been isolated and cultivated from the human body: *Methanobrevibacter smithii, Methanobrevibacter oralis, Methanosphaera stadtmanae, Methanobrevibacter arbophilus, Methanobrevibacter massiliensis, Methanomethylophilus alvus, Methanomassiliicoccus intestinalis and Methanomassiliicoccus luminyensis* (42,56). Although it has been shown, that *M. smithii* is present in up to 95% of population (57), only about a third of the population produces measurable quantities of breath-methane (21,36). Since it is the most abundant methanogen it has been reported to comprise up to 10% of the anaerobes in the human colon (46). Following the most abundant *M. smithii* is *Me. stadtmanae* and lastly *Met. luminyensis* (36).

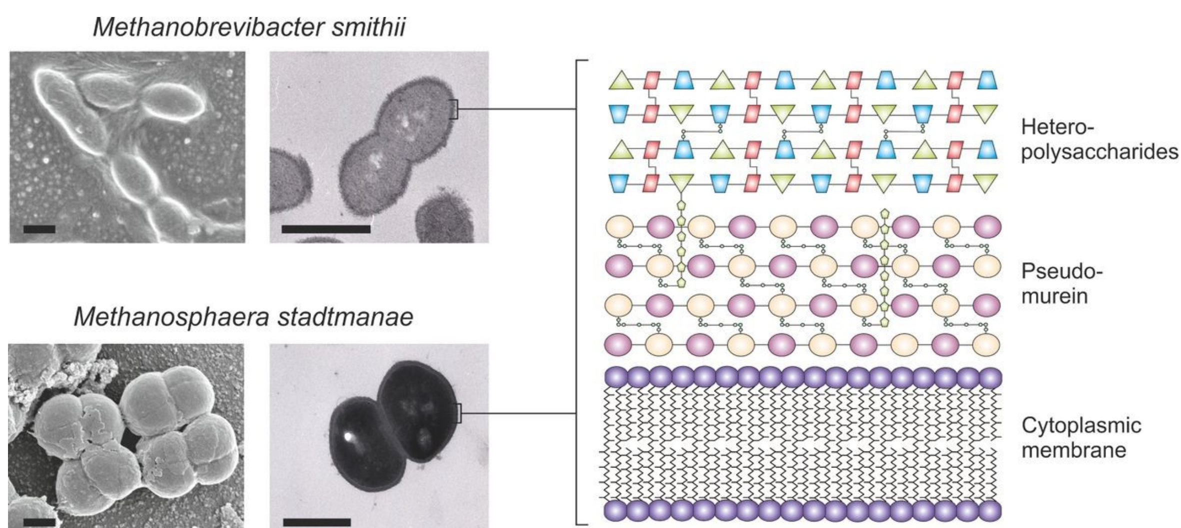

Figure 3 ***Cell envelope of M. smithii and Me. stadtmanae****. Scanning electron (left) and transmission electron microscopy (right) photographs of M. smithii and Me. stadtmanae are depicted in order to highlight the cell wall components. A detailed structure of the cell wall components including cytoplasmic membrane, pseudomurein and heteropolysaccharides is illustrated. Scanning electron microscopy has been performed in collaboration with M. Spinner and S. N. Gorb (Zoological Institute, University of Kiel). Transmission electron microscopy was done by T. Goldmann and T. Gutsmann (Clinical and Experimental Pathology, Biophysics, Research Center Borstel). The scale bar is 500 nm for each graphic.*

Reference: *Bang, Corinna and Schmitz, Ruth A, archaea associated with human surfaces: not to be underestimated, FEMS Microbiology Reviews, 2015, 39, 631-648, by permission of Oxford University Press*

While *M. smithii* uses hydrogen and carbon dioxide as substrate for methanogenesis, *Me. stadtmanae* uses hydrogen and methanol (36,46). *Met. luminyensis* is also dependant on methanol and hydrogen, but in contrast to *Me. stadtmanae* it completely lacks the genes involved in the CO_2-reduction-pathway, which renders *Met. luminyensis* unable to use CO_2 as substrate (40). However, *Met. luminyensis* has the genetic potential to encode the amino acid pyrrolysine, which is part of a methyltransferase necessary in methylotrophic methanogenesis from methylamines (40). This enables, *Met. luminyensis* to utilize methylamines like trimethylamine (TMA) as substrate (36,40). In a recent study on gut colonization with methanogenic archaea in mice, it was shown that high abundances of *M. smithii* can reduce trimethylamine-levels, which leads to the conclusion, that *M. smithii* is also capable of using TMA as substrate (58). Nevertheless *M. smithii*, *Me. stadtmanae* and *Met. luminyensis* all need hydrogen to perform methanogenesis (36).

1.2.3 Archaeal Diversity over the Lifetime

Over the course of a lifetime the archaeome undergoes drastic changes, as new-borns are usually not populated with archaea, whereas elderly show the highest diversity in their archaeome (22). It is presumed that the colonization with archaea occurs via vertical transmission after birth through breastfeeding since archaea are present in milk from healthy women and can even be cultured from it (35,59). This is also mirrored in the same prevalence of archaea in children and their mother (21). After the colonization there is a continuous increase in archaeal diversity with age (22). So, methanogens are less likely to be found in faecal samples from children than from adults (65% vs. 89%) and they are also less abundant in children (22). Interestingly, the host genome also appears to affect the microbiome, as has been shown in some studies (21,36,46). For example, monozygotic twins have a higher concordance regarding the prevalence of methanogens than dizygotic twins (21). It is therefore suggested that the presence of methanogens is highly heritable (21,36,46). This can also be the reason for the high stability in the archaeome in older populations, which are stable even after extreme dietary changes (36). Another reason for the correlation of archaea with age could be the higher prevalence of chronic constipation in older populations (60). Since archaea have a relatively long doubling time (50,51), they are significantly more dependent on the intestinal transit time than most bacteria and could thereby profit from longer transit times, that are usually present in constipation (46).

1.2.4 Gene transfer and important Genes

Through gene analysis of methanogens, it was revealed that they possess genes to encode adhesin-like-proteins (ALP), which are in homology to bacterial adhesins and part of the adaption to the environment of the gut (21,27,46). These adhesin-like-proteins in combination with genes for surface epitopes, which are similar to sialic acids on host cells (21), suggest that methanogens are not only living in the lumen but also in the mucosa of the gut (42) where they are capable of interacting with host cells and the immune system (36,46). Many of archaeal genes for adhesin-like-proteins, glycosyltransferases and even for core mechanisms in methanogenesis are obtained and exchanged via horizontal gene transfer between other archaea and bacteria (21,46). It should be mentioned that this horizontal gene transfer has contributed significantly to the fact that archaea have been able to adapt to the environment of the gut, which is why it is practiced so extensively by archaea (46).

1.2.5 Detection

To date there exist three different methods for detecting methanogens in humans: breath-methane measurement, cultivation, and molecular detection via PCR; the simplest of which is the detection by measuring the breath methane levels of an individuum (61). However, not all people harbouring methanogen archaea produce *per se* measurable amounts of methane, which is also reflected in the high discrepancy between methane producers (30-50%) and the occurrence of methanogens in PCR-related studies (95%) (21,57,61). In addition, this method only proves that methanogens are present in the gastrointestinal tract, but it is not possible to determine exactly which methanogens are present. Therefore, a more precise analysis using PCR or cultivation is necessary. However, cultivation of archaea is quite difficult and time consuming, due to their long doubling times and strict anaerobic nature, while PCR on the other hand can be carried out much faster (50). The PCR is usually performed with primers targeting the Methyl-coenzyme-M-reductase-subunit-A-gene (mcrA-gene) or the 16S rRNA (21,36,46). The mcrA-gene is responsible for the encoding of the Methyl-Coenzyme-M-reductase, a protein which is essential for the methanogenesis pathway (36). Therefore, by targeting the mcrA-gene, only the signals of methanogens are amplified. By targeting the 16S rRNA gene, it is possible to detect the signals of other non-methanogenic archaea as well, thereby this is a more universal approach for the detection of archaea.

1.2.6 Under-representation of archaea

Most microbiome studies are centred on bacteria, and archaea are therefore often underrepresented and less studied than bacteria. One of the main reasons for this is that until now no pathogenic archaeon has been discovered, which in turn has led to the fact that they have been largely ignored (42). Further reasons for the under-representation of archaea are sometimes low abundances of archaeal DNA in samples, primer mismatches caused by "universal primers", improper DNA extraction methods and the incompleteness of the 16S rRNA gene reference databases (62). This can lead to the removal of archaeal signatures in datasets even if they are present. Interestingly, in a study examining the presence of Methanobacterium in the human gut, it was only detected in samples from the ileum and the left colon, further implying the association of archaea and the intestinal mucosa, which in turn could hinder detection in faecal samples (42).

1.2.7 Archaea in Health and Disease

This section focuses on archaeal interaction in human health and disease. As mentioned before, so far, no sign of pathogenicity has been shown for archaea yet, but there is strong evidence that they play a major part in the development and prevention of certain diseases, which is explained further below (40,46). Therefore, this section summarizes what is currently known about the involvement of archaea in diseases like periodontitis, diverticulosis/diverticulitis, inflammatory bowel disease, irritable bowel disease, atherosclerosis, and obesity. Also, for a better understanding of the underlying mechanisms, the interaction between archaea and the human immune system is detailed below.

1.2.7.1 Archaeal pathogenicity

Since no pathogenic archaeon has yet been identified, several hypotheses have been developed to try to explain this fact. Firstly, archaea do not compete with eukaryotes for co-factors, because they use different co-factors than eukaryotes, and as such, have no advantage of pathogenicity to acquire said co-factors. However, archaea could take an advantage by acquiring metabolites instead of vitamins (43). Secondly, archaea have unique viruses and thus cannot acquire virulence factors from bacterial or eukaryotic viruses (43). In addition, the abundance of archaeal lytic viruses could prevent the preservation and transmission of virulence factors in the mobile gene pool. However, our current knowledge of archaeal viruses, especially those of host-associated archaea is sparse and highly incomplete (43). Thirdly, the emergence of pathogenicity is a rare event, and has occurred

only in a small fraction of bacteria and eukaryotes, but not yet in archaea. In addition, only few archaeal lineages are engaged in associations with eukaryotic hosts, thereby narrowing the chances for archaea to develop virulence in a certain time frame (43). And lastly, archaea are adapted to chronic energy stress, and it is presumed, that this adaption which enables them to dominate and thrive in extreme environments, is incompatible with rapid adaptability necessary for most pathogens (43).

Whether and which of these hypotheses can be held responsible for the absence of pathogenic archaea cannot be conclusively clarified, but it is highly probable that the reason lies in a combination of the mentioned hypotheses, including the fact that most host-associated archaea depend on a supply of hydrogen by bacteria, thereby rendering them unable to act as an independent pathogen. (43).

1.2.7.2 Archaea and the human immune system

Since archaea have been revealed to be an important part of the human microbiome, attempts were made to establish a connection to human health and disease (36). As discussed before, microbiota are recognized by the host's immune system and epithelial cells. Since only a few archaeal species in humans have been cultivated so far, only *M. smithii* and *Me. stadtmanae* were examined for their immunogenic potential (8). Interestingly *M. smithii* and *Me. stadtmanae* do not provoke an inflammatory response in host epithelial cells, indicating that both species are tolerated by human epithelia, which is typical for commensal bacteria (8). However, both the innate and the adaptive immune system show an inflammatory response to these two species, while the reaction to *Me. stadtmanae* is significantly more severe (8). This indicates a higher immunogenic potential of *Me. stadtmanae*, further highlighting its potential role in human disease (8) and shows that *M. smithii* is much more tolerated by the host immune system. In summary, archaea like other commensals are recognized by the immune system and can trigger an immune response (8). It was long unknown, which archaeal MAMPs and host PRRs were responsible for the immune response, as most TLR (TLR-2, -3, -4, -5, -7, -8, -9) and NLR (NOD-1, -2) studied did not appear to be responsible for the recognition of archaea (8). However, recent research has shown, that RNA from *Me. stadtmanae* is a potent immune stimulator, triggering the NLRP3 inflammasome, via TLR-8 and to lesser extent TLR-7 (43,63).

1.2.8 Archaea in Disease

To date, methanogenic archaea were found in different medical conditions and diseases. Especially with the widespread use of PCR and DNA sequencing, more archaea are found in and on the human body. Due to their interaction with other microbes and the host, archaea might also be highly involved in the development or prevention of disease. This section highlights the involvement of archaea in several diseases and their implications for human health.

1.2.8.1 Periodontitis

Periodontitis is an inflammation of the tissue supporting the teeth, which is suspected to be caused by the loss of tolerance to local residing microbes, resulting in an excessive inflammatory response (55). The colonization of dental plaque and the formation of biofilms harbouring pathogenic bacteria as a result of neglected oral hygiene is the driving factor of this disease (64). This reaction is not caused by a single species, but by a conglomerate, organized in a biofilm (46). As already described above, archaea possess glycosylated surfaces and adhesive proteins, thereby contributing to the formation of biofilms (49). While methanogens have been found in human subgingival plaques as early as 1987, *Methanobrevibacter oralis* is the most abundant one in the oral (46). *M. oralis* highly correlates with the prevalence of periodontitis, especially in severe cases with root canal infection and has a higher positive-predictive value unreached by any other bacteria involved in periodontitis (8). Animal studies also have shown, that *M. oralis* promotes the growth of sulphate-reducing-bacteria through syntrophic interactions (36). This interaction is quite interesting as both methanogens and sulphate-reducing bacteria are hydrogenotrophic and should therefore be competing for substrate with each other (21). There is not only a significant difference in the abundance of *M. oralis* between healthy subject and patients suffering from periodontitis, but also between different sites within the same patient (46). Interestingly the antibiotic metronidazole, that is commonly used in this disease is one of the few antibiotics that also target archaea (*M. oralis*), thereby hinting a possible contribution of this archaeon in periodontitis (46). However, it is not possible to conclude a direct involvement of archaea in the development of periodontal disease, but it is safe to say that they are promoting the further growth of pathogenic bacteria and are therefore indirectly contributing to this disease (8,55). It should also be noted that the oral microbiome is one of the most diverse microbiomes, due to its diverse range of conditions (changes in temperature, oxygen, nutrients and pH) and it is also subject to a variety of changes as a result of the high

flux of transient microorganisms, thereby rendering it one of the least stable microbiomes in the human body (8).

1.2.8.2 Diverticulosis and chronic constipation

Chronic constipation is thought to be one of the major risk factors in the development of diverticulosis caused by an increase in intraluminal pressure (65). Interestingly methanogenic archaea are strongly associated with the prevalence of constipation and diverticulosis, since methane production is significantly increased in patients suffering from these conditions (61). This could be explained by the fact, that methane plays a role in the development of chronic constipation as a gasotransmitter, by increasing segmental muscle contraction (61), decreasing the overall intestinal motility (8) and thereby slowing the intestinal transit time down by 59% (46). Due to the constipation resulting in a prolonged intestinal transit time, the growth of archaea with their long doubling time could be promoted by this condition (46). This could lead to a vicious cycle, as methanogens would produce more methane, promoting constipation, which in turn promotes further growth of methanogens (46). However, it is difficult to determine whether the high abundance of methanogens within this disease is the cause or the result of constipation. In addition, methane is suspected to increase the intraluminal pressure, either by its function as a gasotransmitter or its expansion in the intestine (61). Therefore, archaea seem to be involved in the progression of this disease (36,46,61). However, adequately planned PCR-based studies are necessary, since to date the correlation between methanogens and diverticulosis has only been examined using methods of cultivation and the measurement of breath-methane. Unfortunately, both methods are hardly suitable to find a valid connection between diverticulosis and methanogens since they cannot map all present methanogens (8). Although breath methane correlates positively with the presence of methanogens in faecal samples, this analysis is inferior to the PCR in terms of its informative value and to date most of the studies on constipation, diverticulosis and methanogens are based solely on measurements of breath methane and can therefore not be representative (61,66). We should also bear in mind, that not all people harbouring methanogens automatically produce measurable amounts of methane (8).

1.2.8.3 Inflammatory Bowel Disease

Different studies presented contradictory evidence regarding the presence of methanogenic archaea in inflammatory bowel disease (8). For example, studies examining the breath-methane levels of IBD patients and healthy controls found out, that methane producers were significantly reduced in the IBD group (36). This resulted in the assumption that methanogens might be reduced in IBD due to diarrhoea and shortened intestinal transit times ("colonic purging") (36). In contrast, PCR-based studies reported an increased abundance of methanogens, especially *Me. stadtmanae* (8). However, these contradictions could be explained by the use of different and insufficient detection methods, including breath methane, cultivation and PCR using mcrA- or 16S rRNA-targeted primers, since each method has its advantages and drawbacks regarding the results (8). Furthermore, it is now known that methanogenic archaea are preferentially associated with the intestinal mucosa (40,42) and thus could be less affected by the consequences of diarrhoea. Fast intestinal transit, however, can disrupt bacterial fermentation and thus reduce archaeal methane production, which could explain the results of the mentioned study. In a recent study using specific primer pairs for *M. smithii* and *Me. stadtmanae*, comparing IBD-patients with healthy controls, it was shown that the abundance of *Me. stadtmanae* is significantly increased in IBD, whereas *M. smithii's* abundance did not differ between both groups (8,40). Since *Me. stadtmanae* is known to induce an extremely severe inflammatory reaction, its participation in the course of this disease is most likely (8). This is further evidenced by recent findings of higher circulating *Me. stadtmanae*-specific IgG-antibodies in IBD patients (8,40). Interestingly one study even found halophilic archaea in biopsies from IBD patients (8,46), however, their involvement in this disease and the presence in the physiological microbiome has not yet been verified.

1.2.8.4 Irritable Bowel Syndrome

Irritable bowel syndrome is a common, gastrointestinal disorder characterized by altered bowel habits, abdominal pain, and an absence of organic cause (67). This disorder can further be divided in a constipation- or diarrhoea dominant form (36). Between these groups there are differences in the number of methane producers, which can also be attributed to differing intestinal transit times from the results of the beforementioned studies on IBD and constipation (36). In concordance with these results, the number of methane producers is increased in IBS with constipation-dominant symptoms and reduced in IBS with diarrhoea-dominant phenotype (36). Since IBS is not an immunologically triggered disease (67) and

given the results of the studies mentioned above, the differences observed, appear to be more a result of the disorder than its cause. However, methanogens could aggravate symptoms like constipation by methanogenesis and this knowledge could be used to differentiate the two subgroups by the presence of methanogens (36).

1.2.8.5 Atherosclerosis

Methanogens might also play a role in the prevention of atherosclerosis since some species are capable of using methylamines like trimethylamine (TMA) as a substrate for methanogenesis (40). For example, *Met. luminyensis* seems to be completely dependent on methylamines like TMA as substrate, since it lacks the genes necessary for CO_2-reduction/Methyl-oxidation (40,68). Trimethylamine (TMA) is exclusively produced by gut bacteria from substrates like L-carnithine, choline and lecithin, which are common in red meat, eggs, and dairy products (2,40). It is then oxidized in hepatocytes to trimethylamine-N-Oxide (TMAO), which has multiple negative effects on the human body: it is highly proinflammatory, and it affects the lipid-metabolism, therefore causing an accumulation of cholesterol and it has a direct impact on the thrombocyte-function (2). TMAO is therefore considered to highly promote the development of cardiovascular diseases like atherosclerosis (58). In addition, an accumulation of TMA can also lead to Fish-odour-syndrome, which has no direct impact on health, but leads to harsh social and psychological consequences (40,58). Since TMA is an exclusively microbiota derived product, its levels and consequences can be reduced by broad-spectrum antibiotics (58). This is however no permanent solution and not suitable for disease prevention. Another solution could be the degradation of TMA through methanogens like *Met. luminyensis*. In a recent study on mice, it was shown that high abundances of *M. smithii* or *Met. luminyensis* reduce TMAO-levels significantly, thereby proving that both species are capable to degrade TMA (58). However, these effects were only observable for a short period of time, decreasing with the abundance of the transplanted methanogens (58). These positive effects of methanogens in the prevention of cardiovascular disease might lead to the development of an "archaebiotic" (27,40).

1.2.8.6 Obesity

Since methanogenic archaea streamline the process of bacterial fermentation in the human gut, they promote a higher energy uptake by the degradation of otherwise indigestible nutrients (36,69). In ruminants, however, archaea lead to an energy loss of about 6% (43,46). Studies were able to show an increase of short chain fatty acids like butyrate in the presence of methanogens, which are vital for colonic epithelial cell in their metabolism (19,36). Methanogens are therefore suspected to promote obesity by an increased energy uptake. In concordance with this assumption high abundances of methanogens were found in obese individuals (36,46,69), however current studies showed that obesity correlates with a reduced abundance of methanogens (34,35). New evidence seems to prove that *M. smithii* is highly associated with lean individuals or a body-mass-index below 25 kg/m^2 (15,46,70). In addition, some studies reported an increased abundance in anorexia (36), while other reported a decrease in the same condition (35). In line with what is already known about the microbiome in obesity, one study described a decreasing archaeal richness with an increasing BMI (34). In the same study, they showed, that *M. smithii* was more abundant in individuals with a higher richness, therefore negatively correlating with the BMI (34). Overall, the results are contradictory, and it is therefore difficult to make a clear statement, however, more recent studies tend to show an association with methanogenic archaea and lean, healthy individuals (15,35,70).

1.2.9 Conclusion

In summary, methanogenic archaea are involved in the development and prevention of disease, but to what extent is still unknown. Some archaea like *M. oralis* and *Me. stadtmanae* are highly associated with certain diseases, for example periodontitis and inflammatory bowel disease (36,40), while other archaea are associated with healthy individuals (35). In addition, the role of *Me. stadtmanae* in IBD is also supported by its high immunogenic potential (8). In contrast, *M. smithii* is highly tolerated by the immune system and has proved to be a ubiquitous gut inhabitant that is vital in its metabolic function as a hydrogen consumer to stabilize the ecosystem of the gut microbiome (8). Due to its symbiosis with gut bacteria, *M. smithii* promotes the production of short-chain-fatty acids like butyrate, which are vital for colonic epithelial cells and proved to induce apoptosis in colorectal cancer cells, thereby indicating its beneficial role (23–25). Although no pathogenic archaeon has been discovered so far, which may be due to a variety of factors, as mentioned in the chapter on archaeal pathogenicity, it is highly probable that they are involved in processes of disease development and health maintenance. Overall, more research is needed to further establish solid links between archaea and health or disease.

1.3 Hypothesis

Although archaea are ubiquitous, their exact role in human health and disease is still poorly understood. The goal of this study is to examine the role of archaea in the body and identify potential links in health or disease. For this purpose, various anthropological markers and medical data, which were collected within the Gaz study on health and aging (GSHA, https://www.medunigraz.at/grazstudy/), are used to objectify the health status. The collected data was then matched with the faecal microbiome to reveal possible correlations. In addition, our focus is not only on the role of methanogens, but also to identify and link non-methanogenic archaea, such as Halobacteriales to human health and disease. Therefore, special primer pairs were used, which had proven in previous studies that they are capable of displaying an exceptionally high archaeal diversity (62).

We hypothesise, that archaea are an important part of the microbiome and involved in metabolic pathways that contribute to the maintenance of human health and disease.

2 Material and Methods

2.1 Recruitment

Only inhabitants of the city of Graz with an age of 45 years and above were recruited for this study, regardless of any existing medical conditions. In this process 103 volunteers were recruited (n=103). Most volunteers were female (n=66) and about a third was male (n=37). However, we included only subjects, which provided faecal samples, and thus nine subjects were excluded. Therefore, our study population now consisted of 58 women and 36 men. The anthropometric characteristics of our study population can be found in table_1. The complete metadata can be found in table 15-18 in the supplements.

Table 1 ***Median anthropometric characteristics from all subjects which provided faecal samples***

Sex	Age	Height	Weight	BMI-Level	Neck circumference	Waist circumference	Hip circumference	WHR	Thigh circumference	Temperature	Mean systolic RR	Mean diastolic RR
Overall (n=94)	68 ±7.83	166 ±7.7	75.6 ±11.51	26.32 ±3.73	37 ±3.03	96 ±10.92	102 ±6.77	0.91 ±0.07	55 ±4.67	36.7 ±0.22	138 ±16.22	85 ±7.48
Female (n=58)	67 ±7.84	162 ±4.47	70.1 ±11.12	26.32 ±4.48	35 ±1.98	93 ±11.93	103 ±8.25	0.89 ±0.06	58 ±5.05	36.6 ±0.24	136.17 ±16.02	83.25 ±7.54
Male (n=36)	70 ±7.58	176.8 ±5.13	80.5 ±10.03	25.99 ±2.54	40 ±1.9	99 ±8.65	101 ±3.94	0.95 ±0.06	53.25 ±3.5	36.7 ±0.19	141.75 ±16.23	85.83 ±7.15

2.2 Selection of samples and DNA extraction

Stool samples were obtained from 94 out of the 103 volunteers (n=94). Faecal microbiome DNA extraction was performed by the Core Facility Molecular Biology (Medical University of Graz, ZMF). Briefly, total DNA was isolated using the Magna Pure LC DNA III Isolation Kit (Bacteria, Fungi) (Roche, Mannheim, Germany) (71). Total DNA was eluted in 100 µL elution buffer and stored at −20 °C until analysis.

2.3 PCR and sequencing

The extracted DNA was then further analysed by an universal and an archaea-targeted PCR.

2.3.1 Archaea targeted PCR

For the archaea-targeted PCR, a nested approach was chosen to increase the specificity for archaea (42,62) and to avoid the formation of primer dimers caused by the tag, attached to the primers (72). The nested archaea-targeted PCR was performed with the primer pair 344F-ACGGGGYGCAGCAGGCGCGA and 1041R-GGCCATGCACCWCCTCTC in the first PCR, followed by the second PCR with the more universal primers 519F-CAGCMGCCGCGGTAA and 806R-GGACTACVSGGGTATCTAAT. This primer combination was chosen since it can detect a higher spectrum of the archaeome than most other primer combinations (62). The archaea-specific primers were selected, based on their archaeal specificity *in-silico*, their low amplification of eukaryotic DNA and an amplicon length of 150-300 base pairs which is suitable for Next-Generation-Sequencing (62).

For the first PCR, each reaction was performed in a final volume of 20µl containing: 2µl TAKARA Ex Taq® buffer with MgCl2 (10 X; Takara Bio Inc., Tokyo, Japan), 1µl forward primer 344F and 1µl reverse primer 1041R 500 nM, 1.6µl dNTP mix 200 µM, 0.1µl TAKARA Ex Taq® Polymerase 0.5 U, 12.30µl water, and 2µl DNA template (1-30 ng/µl). The first PCR reaction was performed in 30 cycles at 60°C annealing temperature. Five µl from the obtained PCR-product were then transferred into the subsequent second PCR containing the following mixture in a final volume of 25µl: 2,5µl TAKARA Ex Taq® buffer with MgCl2 (10 X; Takara Bio Inc., Tokyo, Japan), 1.25µl forward primer 519F and 1.25µl reverse primer 806R 500 nM, 2µl dNTP mix 200 µM, 0.1µl TAKARA Ex Taq® Polymerase 0.5 U and 12.90µl water. The second PCR reaction was performed in 30 cycles at 63°C annealing temperature. Negative controls were also included for each PCR. The same procedure was described by Pausan et al (62).

2.3.2 Archaea targeted qPCR

Quantitative PCR for the archaeal dataset was performed in triplicates for each sample with the primer pair A806F-ATTAGATACCCSBGTAGTCC and A958R-YCCGGCGTTGAM TCCAATT. The reaction mix contained SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA), 300 nM of each of the aforementioned Primers, genomic DNA template and water (62). The qPCR was performed using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The qPCR conditions are listed in table 2.

2.3.3 Universally targeted PCR

The universal PCR was performed by the Core Facility Molecular Biology from the medical university of Graz. The PCR reaction was performed using the target specific primers 515F-GTGYCAGCMGCCGCGGTAA and 926R-CCGYCAATTYMTTTRAGTTT for 30 cycles at 55 °C annealing temperature as described by Klymiuk et al (73).

2.3.4 Universally targeted qPCR

Quantitative PCR for the universal dataset was performed in triplicates, using the primer pair Bac-331F-TCCTACGGGAGGCAGCAGT and Bac-797R-GGACTACCAGGGTATCTA ATCCTGTT. The reaction mix contained SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA), 300 nM of each of the aforementioned Primers, genomic DNA template and water (62). The qPCR was performed using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The qPCR conditions are listed in table 2.

Table 2 ***qPCR primer and conditions***

Target and Primers		qPCR conditions				Standard curves	
Target gene	Primer Pair	Annealing temperature	No. of cycles	qPCR Standards (*E. Coli*)		Efficiency	R^2
16S rRNA Archaea	331F - 797R	54°C	40	EC-2	2.68E+09	68,7%	0.979
				EC-3	2.68E+08		
				EC-4	2.68E+07		
16S rRNA Bacteria	806F-957R	54°C	40	EC-5	2.68E+06	119.60%	0.971
				EC-6	2.68E+05		
				EC-7	2.68E+04		
				EC-8	2.68E+03		
				EC-9	2.68E+02		
				EC-10	2.68E+01		

2.3.5 Calculating absolute bacterial and archaeal abundances

Following the completed qPCR, the median was determined for each triplet and the sample closest to the median was selected for analysis. Crossing values for qPCR were determined using the regression method within the Bio-Rad CFX Manager software version 3.1. Absolute copy numbers of bacterial and archaeal 16S rRNA genes per µl were calculated using the crossing point values and the reaction efficiencies based on standard curves obtained from defined DNA samples (62).

2.4 Statistical analysis and bioinformatics

The sequenced data was then further processed by using the in-house Galaxy set-up. Fastq data analysis was performed using QIIME2 (74), as described previously by Mahnert et al (75). DADA2 algorithm was used for denoising and generation of amplicon sequence variants (ASVs) (76). Taxonomic classification (77) was based on SILVA v132 (78) and the resulting feature table and taxonomy file were used for further analysis. Subsequent statistical analysis was performed using Calypso v.8.72 for PCR results and IBM SPSS Statistics 25 for qPCR results. For the taxonomic analysis in Calypso, samples were rarefied to a minimal read depth of 100. In addition, alpha-diversity was calculated using the Shannon-index. Furthermore, the identification of discriminating taxa between the groups was performed with a linear discriminant effect size (LEfSe) analysis. In addition, we adjusted the p-level for significance for multiple testing, using the Benjamin-Hochberg-method. The adjusted significance levels are listed in table 14.

In the Archaea-targeted PCR and the Archaea-qPCR one sample was found to have no archaeal or bacterial reads (GSHA-74) and 30 samples had no archaeal reads in the PCR.

2.5 Metadata and group formation

Metadata was generated out of the existing data provided by the Graz Study on Health and Aging (GSHA). We focussed on the anthropometric data, medical history, current medication, and blood tests, which included hormone tests. Medical history and current medication were self-reported by each participant, which led in some cases to contradicting or incomplete information. 94 out of 103 test subjects provided stool samples for microbiome analysis, therefore only those 94 participants were included in this study. Groups were then formed out of the provided data for a more detailed analysis. These groups were then analysed on the microbiome level to find potential links between the intestinal microbiome composition, with special focus on the human archaeome and several conditions. More detailed information on the grouping can be found in the following section. In addition, groups with less than five subjects were excluded from analysis or merged with other groups, thus the following categories were excluded from analysis due to low sample size: diabetes (n=4), migraine (n=3), GERD (n=3).

The blood tests levels were sorted into categories based on the reference values provided by the LKH Graz and the endocrinological laboratory under the direction of Univ.-Prof. Dr.med.univ. Barbara Obermayer-Pietsch. Again, groups containing less than 5 participants were excluded. Some categories, such as sodium, potassium, calcium, chloride and fT4 formed only one group, which is why they were also excluded from the analysis.

2.5.1. Anthropometrics

Table 3 ***Anthropometric metadata***

Sample	Age in years	Sex	Height in cm	Weight in kg	BMI-Level in kg/m2	Neck circumference in cm	Waist circumference in cm	Hip circumference in cm	WHR	Thigh circumference in cm	Temperature in °C	Mean systolic RR in mmHg	Mean diastolic RR in mmHg	Time to graduation in years
GSHA-1	56	male	175.50	79.00	25.65	40.00	90.00	101.00	0.89	53.00	36.80	150.00	90.00	16.00
GSHA-2	77	female	172.00	76.00	25.69	34.00	96.00	107.00	0.90	61.00	36.50	134.00	82.50	11.00
GSHA-3	54	female	165.00	63.00	23.14	33.00	77.00	96.00	0.80	55.00	37.10	114.50	73.00	13.00
GSHA-4	78	male	173.00	63.00	21.05	39.00	81.00	93.00	0.87	49.00	36.40	170.67	85.33	12.00
GSHA-5	72	male	178.00	85.00	26.83	41.00	93.00	100.00	0.93	56.00	36.90	132.00	85.00	12.00
GSHA-6	68	male	178.00	76.00	23.99	40.00	91.00	99.00	0.92	55.00	36.80	139.50	82.50	11.00
GSHA-7	74	female	161.00	56.00	21.60	31.00	82.00	99.00	0.83	52.00	36.80	135.33	83.00	12.00
GSHA-8	78	male	176.60	69.00	22.12	37.00	85.00	101.00	0.84	51.00	36.50	134.00	82.33	13.00
GSHA-9	76	male	172.00	88.00	29.75	43.00	112.00	109.00	1.03	55.00	37.00	124.00	72.67	5.00
GSHA-10	77	female	164.00	69.00	25.65	36.00	85.00	110.00	0.77	60.00	36.60	114.67	78.33	8.00
GSHA-11	72	male	187.00	121.90	34.86	46.00	115.00	118.00	0.97	86.00	36.60	156.00	87.00	12.00
GSHA-12	66	female	166.60	75.60	27.24	36.00	97.00	108.00	0.90	60.00	37.00	166.33	83.00	11.00
GSHA-13	82	female	159.00	65.00	25.71	36.00	89.00	99.00	0.90	52.00	36.60	128.00	71.50	11.00
GSHA-14	81	female	152.00	72.00	31.16	37.00	114.00	103.00	1.11	47.00	36.40	179.50	90.00	8.00
GSHA-15	83	male	179.60	95.10	29.48	43.00	116.00	108.00	1.07	51.00	36.70	120.00	70.50	18.00
GSHA-16	57	male	171.00	113.00	38.64	44.00	128.00	115.00	1.11	59.00	36.80	135.50	95.50	11.00
GSHA-17	54	female	161.50	78.00	29.91	35.00	99.00	109.00	0.91	62.00	37.10	121.00	78.00	11.00
GSHA-18	67	female	159.00	92.00	36.39	38.00	120.00	125.00	0.96	66.00	36.60	137.50	87.50	8.00
GSHA-19	70	male	166.00	67.00	24.31	39.00	92.00	101.00	0.91	54.00	36.90	168.67	85.67	19.00
GSHA-20	59	male	183.30	83.00	24.70	41.00	101.00	100.00	1.01	55.00	36.30	122.33	83.33	25.00
GSHA-21	78	female	159.50	51.00	20.05	33.00	80.00	92.00	0.87	47.00	36.40	162.33	90.33	20.00
GSHA-22	55	female	173.00	91.00	30.41	35.00	100.00	118.00	0.85	65.00	36.70	116.00	85.00	16.00
GSHA-23	73	male	175.00	68.00	22.20	38.00	87.00	97.00	0.90	49.00	36.50	170.33	106.00	19.00
GSHA-24	62	female	163.50	55.00	20.57	33.00	72.00	94.00	0.77	53.50	36.40	154.50	87.00	12.00
GSHA-25	55	male	177.90	94.30	29.80	41.00	105.00	110.00	0.95	55.00	36.70	148.33	92.67	11.00
GSHA-26	64	female	162.20	51.40	19.54	32.50	73.60	90.00	0.82	47.00	36.50	111.67	78.67	14.00
GSHA-27	48	female	154.00	46.40	19.56	30.00	66.60	89.00	0.75	48.00	37.00	140.33	94.00	16.00
GSHA-28	76	female	165.30	64.00	23.42	33.50	86.00	100.00	0.86	53.50	36.20	139.00	82.50	10.00
GSHA-29	75	female	159.20	61.40	24.23	35.70	91.00	100.00	0.91	53.00	36.50	166.00	83.50	11.00
GSHA-30	72	female	NA	NA	NA	NA	NA	NA	NA	NA	NA	132.33	81.00	10.00
GSHA-31	51	male	174.50	72.00	23.65	36.50	99.00	100.00	0.99	50.00	37.10	146.50	92.00	12.00
GSHA-32	79	female	164.50	74.00	27.35	38.00	98.50	106.00	0.93	54.50	36.30	128.50	70.50	10.00
GSHA-33	59	female	157.00	55.00	22.31	32.00	73.00	95.00	0.77	51.00	36.80	111.00	74.50	16.00
GSHA-34	54	male	172.50	74.00	24.87	38.00	87.00	97.00	0.90	52.00	36.90	119.67	76.67	12.00
GSHA-35	73	male	164.60	73.10	26.98	39.50	105.00	100.00	1.05	50.50	37.10	146.50	93.00	24.00
GSHA-36	54	female	160.50	76.90	29.85	37.00	100.00	110.00	0.91	66.00	36.60	106.33	72.33	15.00
GSHA-37	69	male	172.00	72.00	24.34	40.00	90.00	97.00	0.93	50.00	36.70	155.33	91.00	19.00
GSHA-38	76	female	173.90	70.10	23.18	35.50	88.00	102.00	0.86	56.00	36.80	137.00	88.33	10.00
GSHA-39	68	female	165.60	81.20	29.61	34.50	107.00	103.00	1.04	59.00	36.80	178.33	100.33	17.00
GSHA-40	55	female	152.50	71.00	30.53	34.00	93.00	104.00	0.89	60.00	37.20	140.00	98.50	11.00
GSHA-41	46	female	170.40	66.70	22.97	31.50	81.00	104.00	0.78	59.00	37.00	131.67	92.67	20.00
GSHA-42	48	female	160.90	57.90	22.36	33.50	84.00	96.00	0.88	54.00	36.90	116.00	80.50	12.00
GSHA-43	77	male	171.00	85.00	29.07	41.00	110.00	101.00	1.09	52.00	36.20	123.50	77.00	13.00
GSHA-44	54	male	182.40	87.00	26.15	40.00	100.00	105.00	0.95	57.00	36.60	152.33	100.00	13.00
GSHA-45	66	female	153.00	66.00	28.19	37.00	98.50	92.50	1.06	51.00	37.10	168.00	93.50	11.00
GSHA-46	64	female	157.50	49.90	20.12	32.00	77.00	91.00	0.85	50.00	36.30	122.50	81.50	32.00
GSHA-47	70	male	177.40	85.80	27.26	43.00	99.50	103.00	0.97	57.50	36.80	178.00	88.00	13.00
GSHA-48	69	female	161.60	65.00	24.89	32.50	91.50	101.00	0.91	56.00	36.50	154.00	93.50	15.00
GSHA-49	71	female	165.00	66.50	24.43	35.00	99.00	103.00	0.96	54.00	36.40	140.67	80.33	13.00
GSHA-50	74	female	166.00	98.50	35.75	39.00	114.00	124.00	0.92	66.00	36.10	147.00	74.50	13.00
GSHA-51	61	female	161.00	75.80	29.24	35.00	92.00	108.00	0.85	62.00	36.40	132.00	73.50	12.00
GSHA-52	66	female	158.30	63.90	25.50	33.50	81.00	103.00	0.79	60.00	36.40	129.67	75.33	8.00
GSHA-53	68	male	169.80	77.60	26.91	40.00	97.00	98.00	0.99	53.00	36.70	174.67	98.67	8.00
GSHA-54	67	male	176.40	83.00	26.67	43.50	99.00	102.00	0.97	55.50	36.70	193.50	89.00	20.00
GSHA-55	70	female	160.00	86.50	33.79	40.00	109.00	118.00	0.92	63.00	36.00	139.33	87.33	13.00
GSHA-56	68	female	167.30	67.30	24.04	34.00	85.00	102.00	0.83	56.00	36.90	137.00	85.00	11.00
GSHA-57	76	female	162.20	96.60	36.72	37.50	119.00	126.00	0.94	67.00	36.20	108.50	64.50	12.00
GSHA-58	74	female	165.40	72.00	26.32	35.50	104.00	104.00	1.00	58.50	36.90	186.67	106.00	14.00
GSHA-59	68	male	184.40	91.00	26.76	43.00	101.50	104.00	0.98	57.00	36.50	138.00	76.50	16.00
GSHA-60	77	female	168.30	98.80	34.88	39.00	110.00	123.00	0.89	68.00	36.90	160.00	89.00	11.00
GSHA-61	57	female	173.40	63.60	21.15	36.50	73.50	93.50	0.79	52.00	36.80	125.67	75.67	12.00
GSHA-62	66	female	163.60	64.60	24.14	33.00	87.00	100.00	0.87	57.00	37.00	143.00	84.67	11.00
GSHA-63	55	female	172.00	76.50	25.86	34.00	81.00	103.00	0.79	63.00	36.50	119.67	79.00	12.00
GSHA-64	56	male	181.00	82.00	25.03	38.00	90.00	101.00	0.89	51.00	36.60	132.00	86.67	13.00
GSHA-65	54	male	193.00	103.00	27.65	43.00	108.00	114.00	0.95	61.00	36.50	115.33	75.33	9.00
GSHA-66	66	male	181.00	77.70	23.72	39.00	88.00	100.00	0.88	56.50	37.10	139.33	82.00	19.00
GSHA-67	56	female	166.00	94.00	34.11	38.00	109.00	124.00	0.88	68.00	36.50	115.67	81.33	13.00
GSHA-68	68	female	160.00	92.90	36.29	38.00	107.00	121.50	0.88	69.50	36.90	144.50	95.50	14.00
GSHA-69	86	male	177.00	71.00	22.66	39.00	100.00	102.00	0.98	51.00	37.10	152.67	85.33	12.00
GSHA-70	57	female	168.00	62.00	21.97	33.00	87.00	95.00	0.92	54.00	36.40	122.00	78.00	12.00
GSHA-71	50	female	171.00	59.00	20.18	33.00	76.00	91.00	0.84	55.00	36.80	119.00	74.33	16.00
GSHA-72	66	female	154.70	86.50	36.14	39.00	119.00	112.50	1.06	58.00	36.50	150.00	97.00	15.00
GSHA-73	65	female	158.90	76.00	30.10	36.50	105.00	107.00	0.98	60.50	36.80	145.67	89.67	12.00
GSHA-74	51	female	162.50	83.00	31.43	35.00	97.00	114.00	0.85	63.00	36.50	133.00	83.00	13.00
GSHA-75	73	female	146.00	58.50	27.44	34.00	86.00	103.00	0.83	54.00	36.10	141.67	93.00	66.00
GSHA-76	79	male	170.20	74.80	25.82	43.00	90.00	100.00	0.90	50.00	36.70	185.00	99.67	14.00
GSHA-77	86	female	162.10	69.90	26.60	35.50	93.00	102.00	0.91	51.50	36.30	176.67	109.00	10.00
GSHA-78	76	female	157.90	94.00	37.70	38.50	121.00	124.00	0.98	62.00	36.60	124.00	78.50	12.00
GSHA-79	55	male	179.50	79.00	24.52	39.00	96.50	100.00	0.97	53.50	36.50	134.33	92.67	11.00
GSHA-80	76	female	162.00	78.50	29.91	36.00	93.00	110.00	0.85	60.00	36.60	181.00	102.00	13.00
GSHA-81	68	female	165.40	53.70	19.63	31.00	74.00	90.00	0.82	47.00	36.70	153.00	93.50	18.00
GSHA-82	75	male	156.50	68.00	27.76	42.00	108.00	99.00	1.09	51.00	36.70	171.67	97.67	16.00
GSHA-83	76	male	178.20	86.00	27.08	42.00	100.00	105.00	0.95	54.00	36.60	135.00	79.00	13.00
GSHA-84	74	male	184.60	86.00	25.24	40.00	95.50	103.00	0.93	59.00	36.30	144.00	82.00	27.00
GSHA-85	65	female	160.90	76.00	29.36	36.00	98.00	107.00	0.92	59.00	37.10	170.00	95.50	12.00
GSHA-86	62	male	173.00	76.00	25.39	39.50	88.00	99.00	0.89	52.50	36.40	138.00	86.00	12.00
GSHA-87	72	female	161.10	103.90	40.03	42.00	124.50	131.00	0.95	62.00	36.70	128.50	86.00	9.00
GSHA-88	72	male	186.50	113.00	32.49	45.00	121.00	119.00	1.02	59.00	36.30	132.50	80.00	20.00
GSHA-89	72	male	176.00	83.00	26.79	42.00	104.00	101.00	1.03	52.00	36.80	133.50	78.50	15.00
GSHA-90	61	female	155.20	72.90	30.27	35.00	102.00	108.00	0.94	60.50	36.30	129.33	81.00	19.00
GSHA-91	56	female	166.60	57.10	20.57	31.50	69.00	98.00	0.70	53.00	37.00	116.33	74.67	11.00
GSHA-92	76	male	181.60	66.70	20.23	36.50	81.50	98.00	0.83	51.00	36.70	168.67	111.67	17.00
GSHA-93	69	female	152.50	61.20	26.32	35.00	93.00	97.00	0.96	51.00	36.70	129.50	84.00	13.00
GSHA-94	67	female	161.30	88.00	33.82	36.50	107.70	121.50	0.89	63.00	36.70	146.67	100.67	12.00

Table 4 ***Anthropometric groups***

Sample	Age categories in years	Age-categories approximate	Height Categories	BMI approximate	BMI Categories	Body Size Phenotype	Neck circumference	Waist-Hip-Circumference (WHO)	Waist-Hip Circumference (DGSP)	Thigh circumference Categories	Handedness	Graduation	Time to graduation Categories
GSHA-1	50-65	under 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	normal	under 60 cm	Right	FH/University	16-20 Years
GSHA-2	66-80	over 65	170-174 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-3	50-65	under 65	165-169 cm	lean	normal	normal weight, metabolically healthy	obese	normal	overweight	under 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-4	66-80	over 65	170-174 cm	lean	normal	normal weight, metabolically abnormal	obese	normal	normal	under 60 cm	Right	Matura	10-15 Years
GSHA-5	66-80	over 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-6	66-80	over 65	175-179 cm	lean	normal	normal weight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-7	66-80	over 65	160-164 cm	lean	normal	normal weight, metabolically abnormal	normal	normal	overweight	under 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-8	66-80	over 65	175-179 cm	lean	normal	normal weight, metabolically abnormal	normal	normal	normal	under 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-9	66-80	over 65	170-174 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Both sides	Lehrabschluss	under 10 Years
GSHA-10	66-80	over 65	160-164 cm	obese	preobesity	overweight, metabolically healthy	obese	normal	normal	over 60 cm	Right	Volksschule/Hauptschule	under 10 Years
GSHA-11	66-80	over 65	NA	obese	obesity class I	obese, metabolically abnormal	obese	obese	overweight	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-12	66-80	over 65	165-169 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-13	over 80	over 65	155-159 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Both sides	Lehrabschluss	10-15 Years
GSHA-14	over 80	over 65	150-154 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	under 60 cm	Both sides	Volksschule/Hauptschule	under 10 Years
GSHA-15	over 80	over 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	FH/University	16-20 Years
GSHA-16	50-65	under 65	170-174 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-17	50-65	under 65	160-164 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-18	66-80	over 65	155-159 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Volksschule/Hauptschule	under 10 Years
GSHA-19	66-80	over 65	165-169 cm	lean	normal	normal weight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	FH/University	16-20 Years
GSHA-20	50-65	under 65	180-184 cm	lean	normal	normal weight, metabolically healthy	obese	obese	adipositas	under 60 cm	Right	FH/University	over 20 Years
GSHA-21	66-80	over 65	155-159 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie	16-20 Years
GSHA-22	50-65	under 65	170-174 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	FH/University	16-20 Years
GSHA-23	66-80	over 65	175-179 cm	lean	normal	normal weight, metabolically abnormal	obese	normal	normal	under 60 cm	Right	FH/University	16-20 Years
GSHA-24	50-65	under 65	160-164 cm	lean	normal	normal weight, metabolically healthy	obese	normal	normal	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-25	50-65	under 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	under 60 cm	Left	Volksschule/Hauptschule	10-15 Years
GSHA-26	50-65	under 65	160-164 cm	lean	normal	normal weight, metabolically healthy	obese	normal	overweight	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-27	NA	under 65	150-154 cm	lean	normal	normal weight, metabolically healthy	normal	normal	normal	under 60 cm	Right	Matura und Kolleg bzw. Akademie	16-20 Years
GSHA-28	66-80	over 65	165-169 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-29	66-80	over 65	155-159 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-30	66-80	over 65	NA	NA	NA	NA	NA	NA	NA	NA	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-31	50-65	under 65	170-174 cm	lean	normal	normal weight, metabolically abnormal	normal	obese	overweight	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-32	66-80	over 65	160-164 cm	obese	preobesity	overweight, metabolically healthy	obese	obese	adipositas	under 60 cm	Right	Volksschule/Hauptschule	10-15 Years
GSHA-33	50-65	under 65	155-159 cm	lean	normal	normal weight, metabolically healthy	normal	normal	normal	under 60 cm	Right	FH/University	16-20 Years
GSHA-34	50-65	under 65	170-174 cm	lean	normal	normal weight, metabolically healthy	obese	normal	normal	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-35	66-80	over 65	160-164 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	FH/University	over 20 Years
GSHA-36	50-65	under 65	160-164 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-37	66-80	over 65	170-174 cm	lean	normal	normal weight, metabolically healthy	obese	normal	overweight	under 60 cm	Right	FH/University	16-20 Years
GSHA-38	66-80	over 65	170-174 cm	lean	normal	normal weight, metabolically healthy	obese	obese	adipositas	under 60 cm	Both sides	Hohere Schule ohne Matura	10-15 Years
GSHA-39	66-80	over 65	165-169 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Matura	16-20 Years
GSHA-40	50-65	under 65	150-154 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-41	NA	under 65	170-174 cm	lean	normal	normal weight, metabolically abnormal	normal	normal	normal	under 60 cm	Right	FH/University	16-20 Years
GSHA-42	NA	under 65	160-164 cm	lean	normal	normal weight, metabolically healthy	obese	obese	adipositas	under 60 cm	Right	Matura	10-15 Years
GSHA-43	66-80	over 65	170-174 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-44	50-65	under 65	180-184 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	Matura	10-15 Years
GSHA-45	66-80	over 65	150-154 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-46	50-65	under 65	155-159 cm	lean	normal	normal weight, metabolically healthy	normal	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie	over 20 Years
GSHA-47	66-80	over 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	Matura	10-15 Years
GSHA-48	66-80	over 65	160-164 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-49	66-80	over 65	165-169 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-50	66-80	over 65	165-169 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-51	50-65	under 65	160-164 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-52	66-80	over 65	155-159 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	normal	over 60 cm	Right	Volksschule/Hauptschule	under 10 Years
GSHA-53	66-80	over 65	165-169 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	overweight	under 60 cm	Left	Volksschule/Hauptschule	under 10 Years
GSHA-54	66-80	over 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	FH/University	16-20 Years
GSHA-55	66-80	over 65	160-164 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Matura	10-15 Years
GSHA-56	66-80	over 65	165-169 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-57	66-80	over 65	160-164 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Both sides	Hohere Schule ohne Matura	10-15 Years
GSHA-58	66-80	over 65	165-169 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	FH/University	10-15 Years
GSHA-59	66-80	over 65	180-184 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	Hohere Schule ohne Matura	16-20 Years
GSHA-60	66-80	over 65	165-169 cm	obese	obesity class I	overweight, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-61	50-65	under 65	170-174 cm	lean	normal	normal weight, metabolically healthy	obese	normal	normal	under 60 cm	Right	Volksschule/Hauptschule	10-15 Years
GSHA-62	66-80	over 65	160-164 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-63	50-65	under 65	170-174 cm	obese	preobesity	overweight, metabolically healthy	obese	normal	normal	over 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-64	50-65	under 65	180-184 cm	obese	preobesity	overweight, metabolically healthy	obese	normal	normal	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-65	50-65	under 65	NA	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	over 60 cm	Right	Volksschule/Hauptschule	under 10 Years
GSHA-66	66-80	over 65	180-184 cm	lean	normal	normal weight, metabolically abnormal	obese	normal	normal	under 60 cm	Right	FH/University	16-20 Years
GSHA-67	50-65	under 65	165-169 cm	obese	obesity class I	obese, metabolically normal	obese	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-68	66-80	over 65	160-164 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-69	over 80	over 65	175-179 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	Matura	10-15 Years
GSHA-70	50-65	under 65	165-169 cm	lean	normal	normal weight, metabolically healthy	obese	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-71	50-65	under 65	170-174 cm	lean	normal	normal weight, metabolically healthy	obese	obese	overweight	under 60 cm	Right	FH/University	16-20 Years
GSHA-72	66-80	over 65	150-154 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	under 60 cm	Both sides	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-73	50-65	under 65	155-159 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-74	50-65	under 65	160-164 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-75	66-80	over 65	NA	obese	preobesity	overweight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	Hohere Schule ohne Matura	over 20 Years
GSHA-76	66-80	over 65	170-174 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	FH/University	10-15 Years
GSHA-77	over 80	over 65	160-164 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-78	66-80	over 65	155-159 cm	obese	obesity class II	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-79	50-65	under 65	175-179 cm	lean	normal	normal weight, metabolically abnormal	obese	obese	overweight	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-80	66-80	over 65	160-164 cm	obese	preobesity	overweight, metabolically healthy	obese	obese	adipositas	over 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-81	66-80	over 65	165-169 cm	lean	normal	normal weight, metabolically abnormal	normal	normal	overweight	under 60 cm	Right	FH/University	16-20 Years
GSHA-82	66-80	over 65	155-159 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Left	FH/University	16-20 Years
GSHA-83	66-80	over 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-84	66-80	over 65	180-184 cm	obese	preobesity	overweight, metabolically abnormal	obese	normal	overweight	under 60 cm	Right	FH/University	over 20 Years
GSHA-85	50-65	under 65	160-164 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-86	50-65	under 65	170-174 cm	obese	preobesity	overweight, metabolically healthy	obese	normal	normal	under 60 cm	Right	Lehrabschluss	10-15 Years
GSHA-87	66-80	over 65	160-164 cm	obese	NA	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Volksschule/Hauptschule	under 10 Years
GSHA-88	66-80	over 65	NA	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	FH/University	16-20 Years
GSHA-89	66-80	over 65	175-179 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Left	Matura und Kolleg bzw. Akademie	10-15 Years
GSHA-90	50-65	under 65	155-159 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	FH/University	16-20 Years
GSHA-91	50-65	under 65	165-169 cm	lean	normal	normal weight, metabolically healthy	normal	normal	normal	under 60 cm	Right	Hohere Schule ohne Matura	10-15 Years
GSHA-92	66-80	over 65	180-184 cm	lean	normal	normal weight, metabolically healthy	normal	normal	normal	under 60 cm	Right	FH/University	16-20 Years
GSHA-93	66-80	over 65	150-154 cm	obese	preobesity	overweight, metabolically abnormal	obese	obese	adipositas	under 60 cm	Right	Matura	10-15 Years
GSHA-94	66-80	over 65	160-164 cm	obese	obesity class I	obese, metabolically abnormal	obese	obese	adipositas	over 60 cm	Right	Matura	10-15 Years

Age

The age of the participants ranged from 46 to 86 years. The median age was 68 ± 7.8 years. For further testing, the samples were divided into two groups "Age under 65 years" (n=34) for samples with an age of up to 65 years and "Age above 65 years" (n=60) for samples with an age >65 years. These two groups were later divided into subgroups <50 (n=3), 50-65 (n=31), 66-80 (n=55) and >80 (n=5). However, the subgroup "<50" was excluded from analysis due to low group size.

Sex

The samples were divided into two groups labelled "male" (n=36) and "female" (n=58) depending on the stated sex in the anthropometric data.

Height

The height of all participants ranged from 146cm to 193cm. For further testing the samples were divided into nine groups in 5cm steps. The following groups were therefore generated: <150cm (n=1), 150-154cm (n=6), 155-159cm (n=11), 160-164cm (n=21), 165-169cm (n=15), 170-174cm (n=16), 175-179cm (n=13), 180-184cm (n=7), 185-189cm (n=2), ≥190cm (n=1). However, the groups "<150cm", "185-189cm" and "≥190cm" were excluded from analysis due to low groups size and another sample was excluded in this category because no height measurement was carried out for its participant (n=1).

BMI-body mass index

The body mass index was calculated from the measured weight and the size of the participants. Overall, the BMI ranged from 19.54 kg/m^2 to 40.03 kg/m^2 and the median was 26.32 ±3.73 kg/m^2. Based on the WHO grouping, the samples were sorted into the respective groups according to their BMI values (79). The following groups were present among the participants: normal BMI: 18.5-24.9 (n=35), pre-obesity BMI: 25.0-29.9 kg/m^2 (n=38), obesity class I BMI: 30.0-34.9 kg/m^2 (n=12), obesity class II BMI: 35.0-39.9 kg/m^2 (n=7) and obesity class III BMI >40.0 kg/m^2 (n=1). However, obesity class III was excluded from analysis due to low group size and another sample was excluded in this category because no weight and body size measurements were carried out on the participant (n=1).
In addition, BMI was also analysed in two groups, based on whether a subject's BMI level was below 25.0 kg/m^2 and lean (n=35) or above 25.0 kg/m^2 and therefore considered obese (n=58).

Body size phenotypes

For this category, subjects were sorted into 5 groups following strict criteria that have been proposed and implemented in previous studies (33,80).

BMI of obese (BMI >30 kg/m^2), overweight (BMI 25-29.9 kg/m^2) and normal (BMI <25 kg/m^2) subjects were subdivided by the cardiometabolic health status of individuals in "metabolically healthy" and "metabolically abnormal" using the following criteria. Individuals were considered metabolically abnormal when they had ≥2 of the following conditions: blood pressure ≥130/85 mmHg or consumption of antihypertensive medication; fasting triglycerides ≥150 mg/dL; HDL <40mg/dL (men), <50 mg/dL (women) or consumption of lipid-lowering medication; fasting glucose ≥100 mg/dL or consumption of antidiabetic medication; HOMA-IR >3, and CRP >3 mg/L. Thus, the following groups were created: normal weight, metabolically healthy (n=16), normal weight, metabolically abnormal (n=19), overweight, metabolically healthy (n=6), overweight, metabolically abnormal (n=33), obese, metabolically healthy (n=1) and obese, metabolically abnormal (n=18). Due to a low group size, the group "obese, metabolically healthy "was excluded from analysis. One subject was also excluded from this analysis since no anthropometric data was provided.

Neck circumference

The neck circumference of the participants ranged from 30-46cm, respectively for men from 36.5-46 cm and for women from 30-42cm. For further testing the samples were divided into two groups, based on sex-specific cut off values established in a previous study (81). Samples were divided in group 1 "obese" (n=82) or group 2 "normal" (n=10) depending on their respective neck circumference. The cut off values to be considered obese were ≥37.5cm for men, and ≥ 32.5cm for women. One sample was excluded in this category because no measurement of the neck circumference was carried out for its participant (n=1).

Waist-Hip-Ratio (WHR)

The Waist-hip-ratio was calculated from the measured waist circumference and the hip circumference. Overall, the ratio ranged from 0.70-1.11. The WHO data on the WHR served as reference values to divide the samples into two groups (82). Waist-hip-ratios above 0.96 for men and 0.83 for women were considered "obese" (n=61), whereas lower ratios were considered "normal" (n=32). One sample was excluded in this category because no measurement was carried out for its participant (n=1).

A more detailed breakdown based on the quotient was made according to the information provided by the DGSP (Deutsche Gesellschaft für Sportmedizin und Prävention): normal [men: <0.9; women: <0.8] (n=18), overweight [men: 0.9-0.99; women 0.8-0.84] (n=25), adipositas [men: >0.99; women: >0.84] (n=50)

Thigh-circumference

The thigh circumference varied between 47-86 cm. The median was 55.00 ±4.67 cm. For further testing, the samples were divided into two groups, based on sex-specific cut off values established in a previous study (83). Samples with thigh circumferences <60 cm were sorted in group 1 "<60 cm" (n=67), whereas samples with circumferences ≥ 60 cm were sorted in group 2 "≥ 60 cm" (n=26). One sample was excluded in this category because no measurement was carried out for its participant (n=1).

Body temperature

Body temperature from all subjects ranged from 36.0 to 37.2 °C and the median body temperature was 36.7 ±0.22 °C. Unfortunately, when the temperature was recorded, it was not noted which method was chosen for the measurement.

Handedness

In this category, the samples were divided into three groups, based on the information provided by the participants as to whether they were right-handed (n=84), left-handed (n=4) or both (n=6).

Highest education achieved

In this category, the samples were divided into 6 groups, based on the information provided by the participants on their highest degree achieved. The following degrees were present among the participants: Volksschule/Hauptschule (n=10), Matura und Kolleg bzw. Akademie (n=14), Matura (n=9), Lehrabschluss (n=21), Höhere Schule ohne Matura (n=19), Fachhochschule/Universität (n=21).

Duration until graduation

In this category, the time in years taken to reach the highest level of education was examined. The duration ranged from 5-66 years. The samples were also divided into four groups based on the duration: <10 years (n=8), 10-15 years (n=61), 16-20 years (n=20), >20 years (n=5).

2.5.2 Medical conditions and medication

2.5.2.1 Cardiovascular system

Table 5 ***Cardiovascular system***

Sample	Cardiovaskular riskfactors	Hypertension	Hypercholesterolaemia	Cardiovacular event	Hypertension ESC 2018	Effecively treated hypertension?	Smoker in last 12 months
GSHA-1	Yes	Yes	Yes	No	Hypertension I	poorly-treated	Non-smoker
GSHA-2	Yes	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-3	Yes	No	Yes	No	Normal	no known hypertension	Non-smoker
GSHA-4	Yes	Yes	Yes	Yes	Hypertension II	poorly-treated	Non-smoker
GSHA-5	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-6	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-7	Yes	Yes	No	No	High-Normal	well-treated	Non-smoker
GSHA-8	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-9	Yes	Yes	No	No	Normal	well-treated	Non-smoker
GSHA-10	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-11	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-12	No	No	No	No	Hypertension II	no known hypertension	Smoker
GSHA-13	Yes	No	Yes	No	Normal	no known hypertension	Non-smoker
GSHA-14	Yes	Yes	No	No	Hypertension III	poorly-treated	Non-smoker
GSHA-15	Yes	No	Yes	No	Normal	no known hypertension	Non-smoker
GSHA-16	Yes	Yes	No	No	Hypertension I	poorly-treated	Non-smoker
GSHA-17	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-18	Yes	Yes	Yes	No	High-Normal	well-treated	Non-smoker
GSHA-19	No	No	No	No	Hypertension II	no known hypertension	Non-smoker
GSHA-20	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-21	No	No	No	No	Hypertension II	no known hypertension	Non-smoker
GSHA-22	Yes	Yes	No	No	High-Normal	well-treated	Non-smoker
GSHA-23	No	No	No	No	Hypertension II	no known hypertension	Non-smoker
GSHA-24	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-25	No	No	No	No	Hypertension I	no known hypertension	Smoker
GSHA-26	Yes	No	Yes	Yes	Normal	no known hypertension	Non-smoker
GSHA-27	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-28	Yes	Yes	No	Yes	High-Normal	well-treated	Non-smoker
GSHA-29	No	No	No	No	Hypertension II	no known hypertension	Non-smoker
GSHA-30	Yes	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-31	Yes	Yes	No	No	Hypertension I	poorly-treated	Non-smoker
GSHA-32	Yes	Yes	Yes	No	Normal	well-treated	Non-smoker
GSHA-33	No	No	No	No	Normal	no known hypertension	Smoker
GSHA-34	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-35	No	No	No	Yes	Hypertension I	no known hypertension	Non-smoker
GSHA-36	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-37	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-38	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-39	No	No	No	No	Hypertension II	no known hypertension	Non-smoker
GSHA-40	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-41	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-42	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-43	Yes	Yes	Yes	Yes	Normal	well-treated	Non-smoker
GSHA-44	No	No	No	No	Hypertension II	no known hypertension	Smoker
GSHA-45	Yes	Yes	No	No	Hypertension II	poorly-treated	Non-smoker
GSHA-46	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-47	Yes	Yes	Yes	No	Hypertension II	poorly-treated	Non-smoker
GSHA-48	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-49	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-50	Yes	No	Yes	No	Hypertension I	no known hypertension	Non-smoker
GSHA-51	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-52	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-53	Yes	Yes	Yes	No	Hypertension II	poorly-treated	Non-smoker
GSHA-54	Yes	Yes	Yes	No	Hypertension III	poorly-treated	Non-smoker
GSHA-55	Yes	Yes	Yes	No	Hypertension I	poorly-treated	Non-smoker
GSHA-56	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-57	Yes	Yes	No	Yes	Normal	well-treated	Non-smoker
GSHA-58	Yes	Yes	No	No	Hypertension III	poorly-treated	Non-smoker
GSHA-59	Yes	Yes	Yes	No	High-Normal	well-treated	Non-smoker
GSHA-60	Yes	Yes	No	No	Hypertension II	poorly-treated	Non-smoker
GSHA-61	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-62	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-63	No	No	No	No	Normal	no known hypertension	Smoker
GSHA-64	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-65	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-66	Yes	Yes	No	No	Hypertension I	poorly-treated	Non-smoker
GSHA-67	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-68	Yes	Yes	No	No	Hypertension I	poorly-treated	Non-smoker
GSHA-69	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-70	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-71	No	No	No	No	Normal	no known hypertension	Smoker
GSHA-72	Yes	Yes	No	No	Hypertension I	poorly-treated	Non-smoker
GSHA-73	Yes	No	Yes	No	Hypertension I	no known hypertension	Non-smoker
GSHA-74	Yes	Yes	No	No	High-Normal	well-treated	Smoker
GSHA-75	Yes	Yes	No	No	Hypertension I	poorly-treated	Non-smoker
GSHA-76	No	No	No	No	Hypertension III	no known hypertension	Non-smoker
GSHA-77	Yes	Yes	Yes	No	Hypertension II	poorly-treated	Non-smoker
GSHA-78	Yes	Yes	No	No	Normal	well-treated	Non-smoker
GSHA-79	No	No	No	No	Hypertension I	no known hypertension	Smoker
GSHA-80	No	No	No	No	Hypertension III	no known hypertension	Non-smoker
GSHA-81	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-82	Yes	Yes	No	No	Hypertension II	poorly-treated	Non-smoker
GSHA-83	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-84	No	No	No	No	Hypertension I	no known hypertension	Non-smoker
GSHA-85	Yes	Yes	No	No	Hypertension II	poorly-treated	Non-smoker
GSHA-86	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-87	Yes	Yes	Yes	Yes	High-Normal	well-treated	Non-smoker
GSHA-88	Yes	Yes	Yes	No	High-Normal	well-treated	Non-smoker
GSHA-89	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-90	No	No	No	No	High-Normal	no known hypertension	Non-smoker
GSHA-91	No	No	No	No	Normal	no known hypertension	Non-smoker
GSHA-92	Yes	Yes	Yes	Yes	Hypertension III	poorly-treated	Non-smoker
GSHA-93	Yes	No	No	No	High-Normal	no known hypertension	Smoker
GSHA-94	Yes	Yes	No	No	Hypertension II	poorly-treated	Non-smoker

Cardiovascular risk factors

In this group we focused on existing cardiovascular risk factors as defined by the Framingham study. Which are obesity (represented by the body mass index), hypercholesterolemia, high blood pressure, smoking, male sex, diabetes mellitus, high LDL or low HDL cholesterol levels and age. We assessed whether a participant had any of the stated risk factors. The samples were then divided into two groups, if at least one risk factor was present, the sample was sorted into group 1 “risk factor present” (n=43), otherwise they were sorted into group 2 “no risk factor” (n=51).

Hypertension

For this category, the samples were divided into two groups, based on the data provided by the study participants on their medical conditions and the current medication. In the presence of known hypertonia or prescribed medication for the treatment of high blood pressure, the samples were sorted into group 1 " hypertension " (n=34). All other samples were sorted in group 2 "no hypertension " (n=60).

Hypercholesterolemia

For this category, the samples were divided into two groups based on the data provided by the study participants on their medical conditions and the current medication. In the presence of known hypercholesterolemia or prescribed medication for the treatment of high cholesterol levels, the samples were sorted into group 1 " hypercholesterolemia " (n=20). All other samples were sorted in group 2 "no hypercholesterolemia " (n=74).

Cardiovascular event

This category analysed any cardiovascular event, based on the data provided by the study participants on their medical conditions and previous illnesses. The following cardiovascular events were found among the participants: stroke (n=3), myocardial infarction (n=1), pulmonary arterial embolism (n=2), arterial embolism (n=1) and venous thrombosis (n=1). The samples were divided in five distinct groups based on the cardiovascular events described. All other samples were sorted in group 6 "no cardiovascular event" (n=86).

Hypertension according to the ESC 2018 - Guidelines

In this category, the samples were divided into groups based on the participants' measured blood pressure values, according to the current classification by the European society of cardiology from 2018 (84). The blood pressure was measured according to the recommendations for blood pressure measurements in humans by the American Heart Association (85). Participants had their blood pressure checked three times on each arm. The average time between the first and the second measurement was 23 minutes, between the second and third measurement it was 40 minutes. The mean value for the systolic and diastolic pressure was then determined from three measurements for each arm. Finally, the average measurement with the higher systolic blood pressure was used for the further analysis. According to their respective blood pressure levels the participants were then sorted into one of the following groups: **normal** <130/85mmHg (n=24), **high-normal** 130/85-139/89mmHg (n=22), **hypertension I°** 140/90-159/99 mmHg (n=26), **hypertension II°** 160/100-179/109 mmHg (n=16), **hypertension III°** ≥180/110 mmHg (n=6). The blood pressure was measured only twice in 6 participants.

Effectively treated hypertension

In this category, the samples were divided into three groups based on whether the participants knew about existing hypertension or were currently treated with blood pressure controlling medications and whether the current blood pressure was within the normal range. All participants with pre-existing hypertension that had measured blood pressure levels above **high-normal** (>139/89mmHg) were therefore categorized as “poorly adjusted hypertension” (n=21). The other groups were “well-adjusted hypertension” (n=13) and “no known hypertension” (n=60).

Smoking

In this category, the samples were divided into two groups based on the information on smoking behaviour over the past 12 months. If the participants had been smoking during this period, the samples were divided into group 1 “smoker” (n=9). All other samples were sorted in group 2 "non-smoker" (n=85).

2.5.2.2 Gastrointestinal system

Table 6 ***Gastrointestinal system***

Sample	Abdominal surgery	Appendectomie	Cholecystectomie	Hysterectomie	Gastrointestinal disorder	GERD
GSHA-1	No	No	No	No	No	No
GSHA-2	Yes	Yes	No	No	Yes	No
GSHA-3	No	No	No	No	No	No
GSHA-4	No	No	No	No	No	No
GSHA-5	No	No	No	No	No	No
GSHA-6	No	No	No	No	No	No
GSHA-7	No	No	No	No	No	No
GSHA-8	Yes	No	No	No	No	No
GSHA-9	No	No	No	No	Yes	No
GSHA-10	Yes	Yes	Yes	No	No	No
GSHA-11	Yes	No	Yes	No	No	No
GSHA-12	Yes	No	No	Yes	No	No
GSHA-13	Yes	No	No	Yes	No	No
GSHA-14	Yes	Yes	No	No	No	Yes
GSHA-15	No	No	No	No	No	No
GSHA-16	Yes	No	No	No	No	No
GSHA-17	Yes	Yes	No	No	No	No
GSHA-18	Yes	No	No	No	No	Yes
GSHA-19	Yes	Yes	No	No	No	No
GSHA-20	Yes	Yes	No	No	No	No
GSHA-21	No	No	No	No	No	No
GSHA-22	No	No	No	No	No	No
GSHA-23	Yes	Yes	No	No	No	No
GSHA-24	No	No	No	No	No	No
GSHA-25	No	No	No	No	No	No
GSHA-26	No	No	No	No	No	No
GSHA-27	No	No	No	No	No	No
GSHA-28	Yes	Yes	Yes	Yes	No	No
GSHA-29	Yes	Yes	No	No	No	No
GSHA-30	Yes	No	No	No	No	No
GSHA-31	Yes	Yes	No	No	Yes	No
GSHA-32	Yes	Yes	No	No	No	No
GSHA-33	No	No	No	No	No	No
GSHA-34	No	No	No	No	No	No
GSHA-35	No	No	No	No	No	No
GSHA-36	Yes	Yes	No	No	No	No
GSHA-37	No	No	No	No	No	No
GSHA-38	Yes	No	Yes	Yes	No	No
GSHA-39	No	No	No	No	No	Yes
GSHA-40	Yes	No	Yes	No	No	No
GSHA-41	No	No	No	No	Yes	No
GSHA-42	No	No	No	No	No	No
GSHA-43	No	No	No	No	No	No
GSHA-44	No	No	No	No	No	No
GSHA-45	Yes	Yes	No	Yes	No	No
GSHA-46	No	No	No	No	No	No
GSHA-47	No	No	No	No	No	No
GSHA-48	No	No	No	No	No	No
GSHA-49	No	No	No	No	No	No
GSHA-50	Yes	No	No	Yes	Yes	No
GSHA-51	Yes	No	No	Yes	No	No
GSHA-52	No	No	No	No	No	No
GSHA-53	Yes	Yes	Yes	No	No	No
GSHA-54	No	No	No	No	No	No
GSHA-55	Yes	Yes	Yes	No	No	No
GSHA-56	Yes	No	No	Yes	No	No
GSHA-57	Yes	Yes	No	No	No	No
GSHA-58	Yes	Yes	Yes	No	No	No
GSHA-59	No	No	No	No	No	No
GSHA-60	Yes	No	No	Yes	No	No
GSHA-61	Yes	Yes	No	No	No	No
GSHA-62	Yes	No	No	Yes	No	No
GSHA-63	No	No	No	No	No	No
GSHA-64	No	No	No	No	No	No
GSHA-65	Yes	Yes	No	No	Yes	No
GSHA-66	No	No	No	No	No	No
GSHA-67	No	No	No	No	No	No
GSHA-68	Yes	No	Yes	No	No	No
GSHA-69	Yes	No	Yes	No	No	No
GSHA-70	Yes	Yes	No	Yes	No	No
GSHA-71	Yes	Yes	No	No	No	No
GSHA-72	Yes	Yes	No	Yes	No	No
GSHA-73	No	No	No	No	Yes	No
GSHA-74	No	No	No	No	No	No
GSHA-75	Yes	No	No	No	No	No
GSHA-76	Yes	Yes	No	No	No	No
GSHA-77	Yes	Yes	No	No	Yes	No
GSHA-78	Yes	No	Yes	No	No	No
GSHA-79	Yes	Yes	No	No	No	No
GSHA-80	Yes	Yes	No	No	No	No
GSHA-81	Yes	No	No	No	Yes	No
GSHA-82	No	No	No	No	No	No
GSHA-83	No	No	No	No	No	No
GSHA-84	No	No	No	No	Yes	No
GSHA-85	Yes	No	No	Yes	No	No
GSHA-86	Yes	Yes	No	No	No	No
GSHA-87	Yes	No	No	Yes	No	No
GSHA-88	No	No	No	No	No	No
GSHA-89	Yes	No	No	No	No	No
GSHA-90	Yes	Yes	No	No	No	No
GSHA-91	No	No	No	No	No	No
GSHA-92	Yes	No	Yes	No	No	No
GSHA-93	Yes	Yes	Yes	No	No	No
GSHA-94	Yes	Yes	No	Yes	No	No

Abdominal surgery

For this category groups were formed on the criteria whether the participants had undergone any surgery, which involved an opening of the abdominal cavity. The performed surgeries were then tested in subgroups (**appendectomy**, **cholecystectomy**, **hysterectomy**).

Samples were divided in two groups based on whether they had undergone the respective surgery or not.

In the subcategory **appendectomy**, all subjects that have had an appendectomy were sorted in group 1 "appendectomy" (n=30), while all other subjects were sorted in group 2 "no appendectomy" (n=64).

In the subcategory **cholecystectomy**, all subjects that have had a cholecystectomy were sorted in group 1 "cholecystectomy" (n=13), while all other subjects were sorted in group 2 "no cholecystectomy" (n=81).

In the subcategory **hysterectomy**, all subjects that have had a hysterectomy were sorted in group 1 "hysterectomy" (n=15), while all other subjects were sorted in group 2 "no hysterectomy" (n=79).

Gastrointestinal disorders

This category analysed gastroenterological disorders, affecting the lower gastrointestinal tract, based on the data provided by the study participants on their medical conditions and the current medication. The following disorders were present among the participants: diverticulitis (n=3), intestinal polyps (n=3), haemorrhoids (n=4), obstipation (n=2) and no disorder (n=82). The samples were divided in two distinct groups based whether a gastrointestinal order was present (n=12).

GERD – gastroesophageal reflux disease

For this category, the samples were divided into two groups based on the data provided by the study participants on their medical conditions. In the presence of known GERD, the samples were sorted into group 1 " GERD " (n=3). All other samples were sorted in group 2 "no GERD " (n=91). Due to low group sizes this category was excluded from analysis.

2.5.2.3 Other medical conditions

Table 7 ***Oher medical conditions***

Sample	Diabetes mellitus	Hypothyroidism	Hypothyroidism/ Hashimoto	Tumour
GSHA-1	No	No	No	No
GSHA-2	No	No	No	No
GSHA-3	No	No	No	No
GSHA-4	No	No	No	No
GSHA-5	No	Yes	Yes	No
GSHA-6	No	No	No	No
GSHA-7	No	No	No	No
GSHA-8	No	No	No	Yes
GSHA-9	No	No	No	No
GSHA-10	No	No	No	No
GSHA-11	No	No	No	No
GSHA-12	No	No	No	No
GSHA-13	No	Yes	Yes	No
GSHA-14	No	No	No	No
GSHA-15	No	No	No	No
GSHA-16	No	No	No	No
GSHA-17	No	No	No	No
GSHA-18	No	Yes	Yes	No
GSHA-19	No	No	No	Yes
GSHA-20	No	No	No	Yes
GSHA-21	No	No	No	No
GSHA-22	No	No	No	No
GSHA-23	No	No	No	No
GSHA-24	No	No	No	No
GSHA-25	No	No	No	Yes
GSHA-26	No	Yes	Yes	No
GSHA-27	No	No	No	No
GSHA-28	No	No	No	Yes
GSHA-29	No	No	No	Yes
GSHA-30	No	No	No	No
GSHA-31	No	No	No	No
GSHA-32	No	No	No	No
GSHA-33	No	No	No	No
GSHA-34	No	No	No	No
GSHA-35	No	No	No	No
GSHA-36	No	No	No	No
GSHA-37	No	No	No	No
GSHA-38	No	Yes	Yes	No
GSHA-39	No	Yes	Yes	No
GSHA-40	No	No	No	No
GSHA-41	No	No	No	No
GSHA-42	No	No	No	No
GSHA-43	Type 2	No	No	No
GSHA-44	No	No	No	No
GSHA-45	No	Yes	Yes	Yes
GSHA-46	No	Yes	Yes	No
GSHA-47	No	No	No	No
GSHA-48	No	No	No	No
GSHA-49	No	No	No	No
GSHA-50	No	Yes	Hashimoto	No
GSHA-51	No	No	No	Yes
GSHA-52	No	No	No	No
GSHA-53	No	No	No	No
GSHA-54	No	Yes	Yes	No
GSHA-55	No	Yes	Yes	No
GSHA-56	No	No	No	No
GSHA-57	No	No	No	
GSHA-58	No	No	No	No
GSHA-59	Type 2	No	No	No
GSHA-60	No	No	No	No
GSHA-61	No	No	No	No
GSHA-62	No	No	No	Yes
GSHA-63	No	Yes	Yes	No
GSHA-64	No	No	No	No
GSHA-65	No	No	No	No
GSHA-66	No	Yes	Yes	No
GSHA-67	No	Yes	Yes	No
GSHA-68	No	Yes	Hashimoto	No
GSHA-69	No	No	No	No
GSHA-70	No	No	No	No
GSHA-71	No	No	No	No
GSHA-72	No	No	No	Yes
GSHA-73	No	Yes	Yes	No
GSHA-74	No	No	No	No
GSHA-75	No	No	No	No
GSHA-76	No	No	No	No
GSHA-77	No	No	No	No
GSHA-78	No	No	No	Yes
GSHA-79	No	No	No	No
GSHA-80	No	No	No	No
GSHA-81	No	Yes	Hashimoto	No
GSHA-82	No	No	No	No
GSHA-83	No	No	No	No
GSHA-84	No	No	No	Yes
GSHA-85	No	No	No	No
GSHA-86	No	No	No	No
GSHA-87	Type 2	No	No	No
GSHA-88	Type 2	No	No	No
GSHA-89	No	No	No	Yes
GSHA-90	No	No	No	No
GSHA-91	No	No	No	No
GSHA-92	No	No	No	No
GSHA-93	No	No	No	No
GSHA-94	No	Yes	Hashimoto	No

Diabetes

For this category, the samples were divided into two groups based on the data provided by the study participants on their medical conditions and the current medication. In the presence of known diabetes or the use of anti-diabetic drugs, the samples were sorted into group 1 "diabetes" (n=4). All other samples were sorted in group 2 "no diabetes" (n=90). Due to low groups sizes, this category was excluded from analysis.

Hypothyroidism

For this category, the samples were divided into two groups based on the data provided by the study participants on their medical conditions and the current medication. In the presence of known hypothyroidism or taking thyroid medication, the samples were sorted into group 1 "hypothyroidism" (n=18). Four of these participants reported having Hashimoto's thyroiditis (n=4). All other samples were sorted in group 2 "no hypothyroidism" (n=76).

Migraine

For this category, the samples were divided into two groups, based on the data provided by the study participants on their medical conditions and the current medication. In the presence of known migraine attacks, the samples were sorted into group 1 " migraine " (n=3). All other samples were sorted in group 2 "no migraine " (n=91). Due to the low prevalence of migraine in our subjects and the low group size, we excluded this category from analysis.

Tumour

For this category, the samples were divided into two groups based on the data provided by the study participants on their medical conditions. All samples from participants who had a form of benign or malignant neoplasia in the past were divided into group 1 “tumour” (n=13). All other samples were sorted in group 2 "no tumour " (n=81).

2.5.2.4 Medication

Table 8 ***Medication***

Sample	PPI	Anticoagulation	Statin usage	Vit-D Substitution	Nutritional supplements
GSHA-1	No	No	Yes	No	No
GSHA-2	No	OAC	No	No	Yes
GSHA-3	No	No	Yes	No	No
GSHA-4	No	Antiplatelet therapy	Yes	No	Yes
GSHA-5	No	No	No	No	No
GSHA-6	No	No	No	No	No
GSHA-7	No	Antiplatelet therapy	No	No	No
GSHA-8	No	No	No	No	No
GSHA-9	No	OAC	No	No	No
GSHA-10	Yes	Antiplatelet therapy	No	No	No
GSHA-11	No	No	No	No	Yes
GSHA-12	No	Antiplatelet therapy	No	Yes	Yes
GSHA-13	Yes	OAC	Yes	Yes	Yes
GSHA-14	Yes	Antiplatelet therapy	No	No	No
GSHA-15	No	No	Yes	No	No
GSHA-16	No	No	No	No	No
GSHA-17	No	No	No	No	No
GSHA-18	Yes	No	Yes	No	Yes
GSHA-19	No	No	No	No	No
GSHA-20	No	No	No	No	No
GSHA-21	No	No	No	No	No
GSHA-22	No	No	No	No	No
GSHA-23	No	No	No	No	No
GSHA-24	No	No	No	No	No
GSHA-25	No	No	No	No	No
GSHA-26	No	OAC	No	No	No
GSHA-27	No	No	No	Yes	No
GSHA-28	No	Antiplatelet therapy	No	Yes	Yes
GSHA-29	No	No	No	No	Yes
GSHA-30	No	No	No	Yes	No
GSHA-31	No	No	No	No	Yes
GSHA-32	No	Antiplatelet therapy	Yes	No	No
GSHA-33	No	No	No	Yes	No
GSHA-34	No	No	No	No	Yes
GSHA-35	No	No	No	No	Yes
GSHA-36	No	No	No	No	No
GSHA-37	No	No	No	No	No
GSHA-38	No	No	No	No	Yes
GSHA-39	No	No	No	No	No
GSHA-40	No	No	No	No	No
GSHA-41	No	No	No	No	Yes
GSHA-42	No	No	No	No	No
GSHA-43	No	OAC	Yes	No	No
GSHA-44	No	No	No	No	No
GSHA-45	No	Antiplatelet therapy	No	No	No
GSHA-46	No	No	No	Yes	No
GSHA-47	No	Antiplatelet therapy	Yes	No	Yes
GSHA-48	No	No	No	No	Yes
GSHA-49	No	No	No	No	No
GSHA-50	No	OAC	Yes	Yes	No
GSHA-51	No	No	No	No	No
GSHA-52	No	No	No	No	Yes
GSHA-53	No	No	No	No	Yes
GSHA-54	No	Antiplatelet therapy	Yes	No	No
GSHA-55	No	OAC	No	Yes	No
GSHA-56	No	No	No	No	No
GSHA-57	No	No	No	Yes	No
GSHA-58	No	No	No	No	No
GSHA-59	No	Antiplatelet therapy	Yes	Yes	No
GSHA-60	No	No	No	Yes	No
GSHA-61	No	No	No	No	Yes
GSHA-62	No	No	No	Yes	No
GSHA-63	No	No	No	No	No
GSHA-64	No	No	No	No	No
GSHA-65	No	No	No	No	No
GSHA-66	No	No	No	No	Yes
GSHA-67	No	No	No	No	No
GSHA-68	No	Antiplatelet therapy	No	Yes	Yes
GSHA-69	No	Antiplatelet therapy	No	No	No
GSHA-70	No	No	No	Yes	No
GSHA-71	No	No	No	No	No
GSHA-72	No	No	No	No	Yes
GSHA-73	No	Antiplatelet therapy	Yes	Yes	No
GSHA-74	No	No	No	No	No
GSHA-75	Yes	No	No	Yes	No
GSHA-76	No	No	No	No	Yes
GSHA-77	No	No	No	No	No
GSHA-78	No	No	No	No	Yes
GSHA-79	No	No	No	No	No
GSHA-80	No	No	No	No	No
GSHA-81	No	No	No	No	Yes
GSHA-82	Yes	No	No	No	No
GSHA-83	No	Antiplatelet therapy	No	No	No
GSHA-84	No	No	No	No	No
GSHA-85	No	No	No	No	Yes
GSHA-86	No	No	No	No	No
GSHA-87	Yes	OAC	Yes	Yes	No
GSHA-88	No	Antiplatelet therapy	Yes	Yes	No
GSHA-89	No	No	No	No	No
GSHA-90	No	No	No	No	Yes
GSHA-91	No	No	No	Yes	No
GSHA-92	No	Antiplatelet therapy	Yes	No	No
GSHA-93	No	No	No	No	No
GSHA-94	No	No	No	Yes	Yes

PPI

For this category, the samples were divided into two groups based on the data provided by the study participants on their current medication. All samples from participants, who were currently treated with proton pump inhibitors, were divided into group 1 “PPI treatment” (n=7). All other samples were sorted in group 2 "no PPI treatment " (n=87).

Anticoagulation

For this category, the samples were divided into different groups based on the data provided by the study participants on their current usage of anticoagulant medication. The following anticoagulant medications were used by the participants: clopidogrel (n=3), acetylsalicylic acid (n=17), marcumar (n=3) and NOAK (n=2). No participant had more than one prescribed anticoagulant. The samples were sorted into three groups, depending on whether the subjects took oral anticoagulation (n=5), antiplatelet therapy (n=20) or no anticoagulants (n=69).

Treatment with statins

In this category, the samples were divided into two groups, based on the data provided by the study participants on their current medication. All samples from participants who are currently treated with statins were divided into group 1 “Statin treatment” (n=16). All other samples were sorted into group 2 "no statin treatment " (n=78). None of the participants was treated with Lovastatin, the majority used Simvastatin or Atorvastatin.

Vitamin-D substitution

For this category, the samples were divided into two groups based on the data provided by the study participants on their current medication. All samples from participants, who were currently treated with vitamin d supplements were divided into group 1 “vitamin D substitution” (n=21). All other samples were sorted into group 2 "no vitamin D substitution" (n=73).

Nutritional supplement

In this category, the samples were divided into two groups based on the data provided by the study participants on their current medication. All samples from participants who were currently taking any form of dietary supplement other than vitamin D were assigned to group 1 “nutritional supplement” (n=25). Herbal medicine and homeopathics were not considered nutritional supplements and therefore not included in this category. All other samples were sorted into group 2 "no nutritional supplement " (n=69).

2.5.3 Blood tests

Blood tests have been performed on 93 participants. The laboratory reference values for these blood tests were adopted from the LKH Graz. The different blood parameters were examined in the categories low, normal, or high and on the basis of their individual values. One participant did not provide a blood sample and was therefore excluded from analysis in these categories. The hormone blood tests were carried out by the endocrinology laboratory, which also provided the reference levels for the different hormones. Four participants did not provide blood for the endocrinological laboratory and were therefore excluded from analysis in these categories.

2.5.3.1 Electrolytes

Table 9 ***Electrolyte groups sizes and mean levels***

	Group size n	Mean
SODIUM	93	140.70 ±1.84
low	0	-
normal	93	140.70 ±1.84
high	0	-
POTASSIUM	93	4.13 ±0.32
low	1	3.4 ±0.00
normal	90	4.12 ±0.28
high	2	5.12 ±0.05
CALCIUM	9	2.38 ±0.097
low	3	2.16 ±0.02
normal	89	2.38 ±0.09
high	1	2.69 ±0.00
CHLORIDE	93	102 ±2.29
low	0	-
normal	93	102 ±2.29
high	0	-

Sodium

Sodium levels from all participants ranged from 135 mmol/L to 145 mmol/L. The mean sodium level was 140.7 ±1.84 mmol/L. As the laboratory reference values range from 135 – 145 mmol/L, all samples were inside this range and this category therefore consists of only one group. Thus, this category was excluded from analysis.

Potassium

Potassium levels from all participants ranged from 3.4 mmol/L to 5.2 mmol/L. The mean potassium level was 4.13 ±0.32 mmol/L. As the laboratory reference values range from 3.5 - 5.0 mmol/L, only three values were outside this range, and were excluded from the analysis due to their small number. Therefore, this category consists of only one group and thus, was excluded from analysis.

Calcium

Calcium levels from all participants ranged from 2.14 mmol/L to 2.69 mmol/L. The mean calcium level was 2.38 ±0.10 mmol/L. As the laboratory reference values range from 2.2 - 2.65 mmol/L, only three values were outside this range, and were excluded from the analysis due to their small number. Therefore, this category consists of only one group and thus, was excluded from analysis.

Chloride

Chloride levels from all participants ranged from 96 mmol/L to 107 mmol/L. The mean chloride level was 102.00 ±2.29 mmol/L. As the laboratory reference values range from 95 – 110 mmol/L, all samples were inside this range and this category therefore consists of only one group. Thus, this category was excluded from analysis.

2.5.3.2 Hepatico-biliary markers

Table 10 ***Hepatico-biliary group sizes and mean levels***

	Group size n	Mean	Mean (female)	Mean (male)
ALT	93	21.54 ±7.64	19.68 ±5.82	24.47 ±9.12
low	0	-	-	-
normal	89	20.60 ±6.26	-	-
high	4	42.5 ±5.22	-	-
AST	93	24.88 ±7.34	23.68 ±6.14	26.78 ±8.58
low	0	-	-	-
normal	79	22.57 ±4.29	-	-
high	14	37.92 ±7.34	-	-
AP	93	63.80 ±16.97	66.021 ±16.27	60.28 ±17.45
low	5	35.00 ±3.41	-	-
normal	87	64.78 ±14.82	-	-
high	1	122 ±0.00	-	-
BILIRUBIN	93	0.65 ±0.34	-	-
low	0	-	-	-
normal	87	0.59 ±0.25	-	-
high	6	1.53 ±0.35	-	-
CHOLINESTERASE	93	8215.67 ±1580.17	8217.35 ±1595.03	8213 ±1556.34
low	0	-	-	-
normal	89	7966.023 ±1366.82	-	-
high	4	11247.5 ±141.34	-	-
GGT	93	22.84 ±14.06	18.96 ±10.52	28.97 ±16.54
low	0	-	-	-
normal	86	20.07 ±8.76	-	-
high	7	56.86 ±20.77	-	-

ALT

ALT levels from all participants ranged from 7 U/L to 49 U/L. The mean ALT level for women was 19.68 ±5.82 U/L and for men 24.47 ±9.12 U/L. Overall, the mean ALT level was 21.54 ±7.64 U/L. The laboratory reference values were sex-specific and ranged from 0-35 U/L for women and from 0 – 45 U/L for men. Only four values were outside this range, and therefore excluded from the analysis due to their small number. Therefore, this category consists of only one group and thus, was excluded from analysis.

AST

AST levels from all participants ranged from 14 U/L to 58 U/L. The mean AST level for women was 23.68 ±6.14 U/L and for men 26.78 ±8.58 U/L. Overall, the mean AST level was 24.88 ±7.34 U/L. The laboratory reference values were sex-specific and ranged from 0-30 U/L for women and from 0 – 35 U/L for men. 14 values exceeded this range and were therefore labelled “high” (n=14), all other samples were sorted in the group “normal” (n=79).

Alkaline phosphatase

Alkaline phosphatase (AP) levels from all participants ranged from 30 U/L to 122 U/L. The mean AP level for women was 66.02 ±16.27 U/L and for men 60.28 ±17.45 U/L. Overall, the mean AP level was 63.80 ±16.97 U/L. The laboratory reference values were sex-specific and ranged from 35-105 U/L for women and from 40 - 130 U/L for men. Therefore, samples were sorted into three different groups: low (n=5), normal (n=87) and high (n=1). Due to its low sample size, group "high" was excluded from this analysis.

Bilirubin

Bilirubin levels from all participants ranged from 0.24 mg/dL to 2.12 mg/dL. The mean bilirubin level was 0.65 ±0.34 mg/dL. The laboratory reference values ranged from 0.1 - 1.2 mg/dL. Therefore, samples were sorted into two different groups: normal (n=87) and high (n=6).

Cholinesterase

Cholinesterase levels from all participants ranged from 5571 U/L to 11723 U/L. The mean cholinesterase level for women was 8217.35 ±1595.03 U/L and for men 8213.00 ±1556.34 U/L. Overall, the mean cholinesterase level was 8215.67 ±1580.17 U/L. The laboratory reference values were sex-specific and ranged from 3900-11000 U/L for women and from 4600 - 13000 U/L for men. Therefore, samples were sorted into two different groups: normal (n=89) and high (n=4). Due to its low sample size, group "high" was excluded from this analysis. Therefore, this category consists of only one group and thus, was excluded from analysis.

Gamma-GT (GGT)

GGT levels from all participants ranged from 7 U/L to 103 U/L. The mean GGT level for women was 18.96 ±10.52 U/L and for men 28.97 ±16.54 U/L. Overall, the mean GGT level was 22.85 ±14.06 U/L. The laboratory reference values were sex-specific and ranged from 0 - 38 U/L for women and from 0 - 55 U/L for men. Therefore, samples were sorted into two different groups: normal (n=86) and high (n=7).

2.5.3.3 Metabolic markers

Table 11 ***Metabolic marker group sizes and mean levels***

	Group size n	Mean	Mean (female)	Mean (male)
TOTAL PROTEIN	93	7.25 ±0.35	-	-
low	3	6.23 ±0.25	-	-
normal	89	7.28 ±0.27	-	-
high	1	8.4 ±0.00	-	-
CRP	93	1.78 ±2.26	-	-
low	0	-	-	-
normal	86	1.27 ±1.10	-	-
high	7	8.03 ±3.31	-	-
GLUCOSE	93	97.15 ±22.04	-	-
low	0	-	-	-
normal	72	89.44 ±5.87	-	-
high	21	123.57 ±33.63	-	-
URIC ACID	93	5.40 ±1.23	5.08 ±1.14	5.9 ±1.20
low	0	-	-	-
normal	72	4.97 ±0.96	-	-
high	21	6.88 ±0.83	-	-
UREA	93	35.04 ±10.73	-	-
low	0	-	-	-
normal	78	31.62 ±7.48	-	-
high	15	52.87 ±6.63	-	-
CREATININE	93	0.89 ±0.17	0.81 ±0.13	1.01 ±0.16
low	0	-	-	-
normal	83	0.85 ±0.13	-	-
high	10	1.19 ±0.13	-	-
LDH	93	196.68 ±36.18	-	-
low	1	109 ±0.00	-	-
normal	82	188.69 ±23.58	-	-
high	10	271 ±28.28	-	-
CREATINEKINASE	93	132.96 ±98.73	115.12 ±63.75	161.19 ±132.08
low	0	-	-	-
normal	69	90.20 ±28.08	-	-
high	24	255.88 ±123.05	-	-

Total protein

Total protein levels from all participants ranged from 5.9 g/dL to 8.4 g/dL. The mean total protein level was 7.25 ±0.35 g/dL. As the laboratory reference values range from 6.6 - 8.3 g/dL, only four values were outside this range, and were excluded from the analysis due to their small number. Therefore, this category consists of only one group and thus, was excluded from analysis.

C-reactive protein (CRP)

CRP levels from all participants ranged from 0 mg/dL to 14.2 mg/dL. The mean CRP level was 1.78 ±2.26 mg/dL. The laboratory reference values ranged from 0.6 - 5.0 mg/dL. Levels below the measurable range (<0.6 mg/dL) were classified as 0.0 mg/dL for analysis. Therefore, samples were sorted into two different groups: normal (n=86) and high (n=7).

Glucose

Glucose levels from all participants ranged from 75 mg/dL to 238 mg/dL. The mean glucose level was 97.25 ±22.04 mg/dL. The laboratory reference values ranged from 70 - 100 mg/dL. Therefore, samples were sorted into two different groups: normal (n=72) and high (n=21).

Uric acid

Uric acid levels from all participants ranged from 2.4 mg/dL to 9.6 mg/dL. The mean level for women was 5.08 ±1.14 mg/dL and for men 5.90 ±1.20 mg/dL. Overall, the mean uric acid level was 5.4 ±1.23 mg/dL. The laboratory reference values were sex-specific and ranged from 2.4 - 5.7 mg/dL for women and from 3.4 - 7.0 mg/dL for men. Therefore, samples were sorted into two different groups: normal (n=72) and high (n=21).

Urea

Urea levels from all participants ranged from 16 mg/dL to 68 mg/dL. The mean urea level was 35.04 ±1ß.73 mg/dL. The laboratory reference values ranged from 10 - 45 mg/dL. Therefore, samples were sorted into two different groups: normal (n=78) and high (n=15).

Creatinine

Creatinine levels from all participants ranged from 0.54 mg/dL to 1.39 mg/dL. The mean level for women was 0.81 ±0.13 mg/dL and for men 1.01 ±0.16 mg/dL. Overall, the mean creatinine level was 0.89 ±0.17 mg/dL. The laboratory reference values were sex-specific and ranged from 0 - 1.00 mg/dL for women and from 0 - 1.21 mg/dL for men. Therefore, samples were sorted into two different groups: normal (n=83) and high (n=10).

Lactate dehydrogenase (LDH)

LDH levels from all participants ranged from 109 U/L to 329 U/L. The mean LDH level was 196.68 ±36.18 U/L. The laboratory reference values ranged from 120 - 240 U/L. Therefore, samples were sorted into three different groups: low (n=1), normal (n=82) and high (n=10). Due to its low sample size, group "low" was excluded from this analysis.

Creatinine kinase

Creatinine kinase levels from all participants ranged from 38 U/L to 667 U/L. The mean level for women was 115.12 ±63.75 U/L and for men 161.19 ±132.08 U/L. Overall, the mean creatinine kinase level was 132.96 ±98.73 U/L. The laboratory reference values were sex-specific and ranged from 0-145 U/L for women and from 0-170 U/L for men. Therefore, samples were sorted into two different groups: normal (n=69) and high (n=24).

2.5.3.4 Lipid markers

Table 12 ***Lipid marker group sizes and mean levels***

	Group size n	Mean
CHOLESTEROL	93	211.97 ±45.15
low	0	-
normal	37	169.47 ±21.47
high	56	240.04 ±33.16
LDL	93	131.38 ±38.88
low	0	-
normal	17	104.37 ±18.70
high	76	165.63 ±29.78
HDL	93	56.88 ±19.71
low	18	33.67 ±9.79
normal	75	62.45 ±17.28
high	0	-
TRIGLYCERIDE	93	97.92 ±47.71
low	0	-
normal	82	85.37 ±28.77
high	11	184.55 ±39.07

Cholesterol

Cholesterol levels from all participants ranged from 105 mg/dL to 327 mg/dL. The mean cholesterol level was 211 ±45.15 mg/dL. The laboratory reference values ranged from 0 - 200 mg/dL. Therefore, samples were sorted into two different groups: normal (n=37) and high (n=56).

Low density lipoprotein (LDL)

LDL levels from all participants ranged from 41 mg/dL to 253 mg/dL. The mean LDL level was 131.38 ±38.88 mg/dL. The laboratory reference values varied depending on the individual ESC-score of each participant (86). Cut off levels were: 0-116 mg/dL for low risk, 0-100 mg/dL for moderate risk, 0-70 mg/dL for high-risk and 0-55 mg/dL for very high-risk patients. Samples were then sorted into two different groups following their respective cut off level: normal (n=17) and high (n=76).

High density lipoprotein (HDL)

HDL levels from all participants ranged from 0 mg/dL to 124 mg/dL. The mean HDL level was 56.88 ±19.71 mg/dL. The laboratory cut off level for a normal HDL level was >40 mg/dL. Therefore, samples were sorted into two different groups: normal (n=75) and low (n=18).

Triglycerides

Triglyceride levels from all participants ranged from 36 mg/dL to 332 mg/dL. The mean triglyceride level was 97.92 ±47.71 mg/dL. The laboratory reference values ranged from 0 - 150 mg/dL. Therefore, samples were sorted into two different groups: normal (n=82) and high (n=11).

2.5.3.5 Hormones

Table 13 ***Hormone group sizes and mean levels***

	Group size n	Mean	Mean (female)	Mean (male)
1.25 - Dihydroxy vitamin D3	89	93.045 ±55.72	-	-
low	33	38.94 ±15.26	-	-
normal	53	118.04 ±35.82	-	-
high	3	346.67 ±5.78	-	-
25 – Hydroxy vitamin D3	90	25.67 ±12.26	-	-
low	67	20.64 ±6.11	-	-
normal	19	34.51 ±6.03	-	-
high	4	67.85 ±3.13	-	-
Aldosterone	89	8.46 ±13.24	-	-
low	27	0.00 ±0.00	-	-
normal	60	10.02 ±7.09	-	-
high	2	74.35 ±30.14	-	-
Renine	89	29.31 ±49.45	-	-
low	15	3.15 ±1.70	-	-
normal	70	23.61 ±18.44	-	-
high	4	227.25 ±75.30	-	-
C-Peptide	90	1.71 ±0.89	-	-
low	6	0.68 ±0.05	-	-
normal	58	1.32 ±0.28	-	-
high	26	2.80 ±0.90	-	-
Insulin	90	11.84 ±9.07	-	-
low	1	2.30 ±0.00	-	-
normal	84	10.35 ±4.74	-	-
high	5	38.88 ±17.80	-	-
Leptin	89	23.87 ±20.03	31.35 ±21.19	11.18 ±8.00
low	1	3.50 ±0.00	-	-
normal	16	5.13 ±2.27	-	-
high	72	28.32 ±19.77	-	-
PTH	90	54.90 ±18.72	54.40 ±17.04	55.725 ±21.17
low	0	-	-	-
normal	70	47.37 ±11.36	-	-
high	20	81.24 ±15.27	-	-
SHBG	90	69.43 ±32.31	75.65 ±35.04	59.19 ±23.92
low	1	14.20 ±0.00	-	-
normal	76	61.43 ±24.02	-	-
high	13	120.42 ±25.38	-	-
Progesterone	88	0.49 ±0.36		
low	-	-	-	-
normal	-	-	-	-
high	-	-	-	-
Oestradiol	88	35.39 ±21.08	-	-
low	-	-	-	-
normal	-	-	-	-
high	-	-	-	-
Free testosterone	87	5.31 ±5.34	1.51 ±0.76	11.54 ±3.42
low	7	7.16 ±1.1	-	-
normal	78	5.17 ±5.6	-	-
high	2	4.44 ±0.23	-	-
Testosterone	90	1.74 ±2.14	0.25 ±0.14	4.20 ±1.53
low	13	0.87 ±1.00	-	-
normal	76	1.9 ±2.26	-	-
high	1	0.97 ±0.00	-	-
TSH	90	1.82 ±1.15	-	-
low	2	0.01 ±0.01	-	-
normal	81	1.62 ±0.82	-	-
high	7	4.61 ±0.47	-	-
fT3	90	4.57 ±0.57	-	-
low	0	-	-	-
normal	89	4.55 ±0.48	-	-
high	1	7.5 ±0	-	-
fT4 (Thyroxine)	90	14.8 ±2.35	-	-
low	0	-	-	-
normal	90	14.8 ±2.35	-	-
high	0	-	-	-

1.25 - Dihydroxy vitamin D_3

1.25 - Dihydroxy vitamin D_3 levels from all participants ranged from 0 pmol/L to 253 pmol/L. Levels below the measurable range (<18 pmol/L) were classified as 0.0 pmol/L for analysis. The mean level was 93.04 ±55.72 pmol/L. The laboratory reference values ranged from 63 - 228 pmol/L. Therefore, samples were sorted into three different groups: normal (n=53), low (n=33), and high (n=3). Due to its low samples size, group "high" was excluded from analysis. In addition, one sample could not be measured, because there was not enough material for analysis.

25 – Hydroxy vitamin D_3

25 - Hydroxy vitamin D_3 levels from all participants ranged from 0 ng/mL to 73.1 ng/mL. Levels below the measurable range (<7 ng/mL) were classified as 0.0 ng/mL for analysis. The mean level was 25.67 ±12.6 ng/mL. The laboratory reference values ranged from 30 - 60 ng/mL. Therefore, samples were sorted into three different groups: normal (n=19), low (n=67), and high (n=4). Due to its low samples size, group "high" was excluded from analysis.

Aldosterone

Aldosterone levels from all participants ranged from 0 ng/dL to 104 ng/dL. Levels below the measurable range (<3.7 ng/dL) were classified as 0.0 ng/dL for analysis. The mean level was 8.43 ±13.24 ng/dL. The laboratory reference values ranged from 3.7 – 43.2 ng/dL, because the blood samples were taken, while the participants were sitting down. Therefore, samples were sorted into three different groups: normal (n=60), low (n=27), and high (n=2). Due to its low samples size, group "high" was excluded from analysis. In addition, one sample could not be measured, because there was not enough material for analysis.

Renin

Renin levels from all participants ranged from 0 µU/mL to 357.3 µU/mL. Levels below the measurable range (<1.8 µU/mL) were classified as 0.0 µU/mL for analysis. The mean level was 29.31 ±49.45 µU/mL. The laboratory reference values ranged from 5.3 – 99.1 µU/mL, because the blood samples were taken, while the participants were sitting down. Therefore, samples were sorted into three different groups: normal (n=70), low (n=15), and high (n=4). Due to its low samples size, group "high" was excluded from analysis. In addition, one sample could not be measured, because there was not enough material for analysis.

C-peptide

C-peptide levels from all participants ranged from 0.63 ng/mL to 5.19 ng/mL. The mean level was 1.71 ±0.89 ng/mL. The laboratory reference values ranged from 0.78 – 1.89 ng/mL. Therefore, samples were sorted into three different groups: normal (n=58), low (n=6), and high (n=26).

Insulin

Insulin levels from all participants ranged from 2.3 mU/L to 73.5 mU/L. The mean level was 11.84 ±9.07 mU/L. The laboratory reference values ranged from 3 – 25 mU/L. Therefore, samples were sorted into three different groups: normal (n=84), low (n=1), and high (n=5). Due to its low samples size, group "low" was excluded from analysis.

Leptin

Leptin levels from all participants ranged from 2.1 ng/mL to 126.5 ng/mL. The mean leptin level for women was 31.35 ±21.19 ng/mL and for men 11.18 ±8.00 ng/mL. Overall, the mean level was 23.87 ±20.03 ng/mL. The laboratory reference values were sex-specific and ranged from 3.7 - 11.1 ng/mL for women and from 2 - 5.6 ng/mL for men. Therefore, samples were sorted into three different groups: normal (n=16), low (n=1), and high (n=72). Due to its low samples size, group "low" was excluded from analysis. In addition, one sample could not be measured, because there was not enough material for analysis.

Parathormone (PTH)

PTH levels from all participants ranged from 24 pg/mL to 120.2 pg/mL. The mean level was 54.90 ±18.72 pg/mL. The laboratory reference values ranged from 15 – 65 pg/mL. Therefore, samples were sorted into two different groups: normal (n=70) and high (n=20).

Sex hormone-binding globulin (SHBG)

SHBG levels from all participants ranged from 14.2 nmol/L to 165.9 nmol/L. The mean SHBG level for women was 75.65 ±35.04 nmol/L and for men 59.19 ±23.92 nmol/L. Overall, the mean level was 69.43 ±32.31 nmol/L. The laboratory reference values were sex-specific and ranged from 19 - 117 nmol/L for women and from 16 - 76 nmol/L for men. Therefore, samples were sorted into three different groups: normal (n=76), low (n=1), and high (n=13). Due to its low samples size, group "low" was excluded from analysis.

Progesterone

Progesterone levels from all participants ranged from 0.0 ng/mL to 1.91 ng/mL. The median progesterone level for women was 0.27 ±0.17 ng/mL and for men 0.79 ±0.28 ng/mL. Overall, the median level was 0.41 ±0.3 ng/mL. Levels below the measurable range (<0.09 ng/mL) were classified as 0.0 ng/mL for analysis. The laboratory reference levels were varied for sex and the phase of the menstrual cycle. However, no data was provided on the menstrual cycle of our subjects and thus we excluded this category from analysis.

Oestradiol

Oestradiol levels from all participants ranged from 0.0 ng/mL to 315 pg/mL. The median oestradiol level for women was 28.9 ±22.66 pg/mL and for men 38.3 ±7.96 pg/mL. Overall, the median level was 32.4 ±18.05 pg/mL. Levels below the measurable range (<20 pg/mL) were classified as 0.0 pg/mL for analysis. The laboratory reference levels were varied for sex and the phase of the menstrual cycle. However, no data was provided on the menstrual cycle of our subjects and thus we excluded this category from analysis.

Free testosterone

Free testosterone levels from all participants ranged from 0.49 pg/mL to 19.88 pg/mL. The mean free testosterone level for women was 1.51 ±0.76 pg/mL and for men 11.54 ±3.42 pg/mL. Overall, the mean level was 5.31 ±5.34 pg/mL. The laboratory reference values were sex-specific and ranged from 0.29 – 3.18 pg/mL for women and from 8.69 – 54.69 pg/mL for men. Therefore, samples were sorted into three different groups: normal (n=78), low (n=7), and high (n=2). Due to its low samples size, group "high" was excluded from analysis. In addition, three samples could not be measured, because there was not enough material for analysis.

Testosterone

Testosterone levels from all participants ranged from 0 ng/mL to 7.86 ng/mL. Levels below the measurable range (<0.07 ng/mL) were classified as 0.0 ng/mL for analysis. The mean testosterone level for women was 1.51 ±0.76 ng/mL and for men 11.54 ±3.42 ng/mL. Overall, the mean level was 5.31 ±5.34 ng/mL. The laboratory reference values were sex-specific and ranged from 0.14 – 0.77 ng/mL for women and from 2.41 – 8.3 ng/mL for men. Therefore, samples were sorted into three different groups: normal (n=76), low (n=13), and high (n=1). Due to its low samples size, group "high" was excluded from analysis.

Thyroid-stimulating hormone (TSH)

TSH levels from all participants ranged from 0 µU/mL to 5.24 µU/mL. The mean level was 1.82 ±1.15 µU/mL. The laboratory reference values ranged from 0.10 – 4 µU/mL. Therefore, samples were sorted into three different groups: normal (n=81), low (n=2), and high (n=7). Due to its low samples size, group "low" was excluded from analysis.

fT_3 (Triiodothyronine)

Triiodothyronine levels from all participants ranged from 3.5 pmol/L to 7.5 pmol/L. The mean level was 4.57 ±0.57 pmol/L. The laboratory reference values ranged from 3 – 6.3 pmol/L. Therefore, samples were sorted into two different groups: normal (n=89) and high (n=1). Due to its low samples size, group "high" was excluded from analysis. Therefore, this category consists of only one group and thus, was excluded from analysis.

fT_4 (Thyroxine)

Thyroxine levels from all participants ranged from 9.7 pmol/L to 22.7 pmol/L. The mean level was 14.8 ±2.35 pmol/L. The laboratory reference values ranged from 9.5 – 24 pmol/L. Since all samples were in normal range, this category consists of only one group and was excluded from analysis.

2.6 Adjusting for multiple testing

Due to the large number of tests we had to adjust our significance levels using the Benjamin-Hochberg-method. Adjusted significance levels (marked as adjusted p-level in the following sections) were generated for each supergroup as illustrated in table 14.

Table 14 ***Adjusted significance levels***

Supergroup and number of groups included	Adjusted significance levels (adjusted p-levels)
Anthropometric metadata (n=15)	p=0.0033
Anthropometric groups (n=13)	p=0.0038
Cardiovascular system (n=7)	p=0.021
Gastrointestinal system (n=5)	p=0.01
Other medical conditions (n=2)	p=0.025
Medication (n=5)	p=0.04
Electrolytes (n=4)	p=0.0125
Hepatico-biliary markers (n=6)	p=0.0166
Metabolic markers (n=8)	p=0.00625
Lipid markers (n=4)	p=0.025
Hormones (n=16)	p=0.0313

3 Results

This section presents our observations in detail and highlights known associations or correlations.

Archaeal signatures were present in 69.15% in archaeal PCR and 71.28% in archaeal qPCR. In comparison, universal PCR revealed archaeal signatures only in 28.72% of all subjects. Overall archaeal abundance was lower than bacterial abundance.

Significant associations were found in PCR and qPCR, however, due to the large number of tests (111 different categories, of which 64 were of categorial nature, that were tested on five different taxonomic levels and again for qPCR), we had to adjust the p-level for multiple testing, using the Benjamin-Hochberg-method. The significance levels were adjusted for each super-group which included up to 15 distinct groups. The exact composition of these super-groups and their individual p-levels can be viewed in detail in section 2.5 .

After the adjustment, we still had significant associations and correlations of microbiota or archaea with insulin-, SHBG-, triglyceride-, bilirubin-levels as well as with hypercholesterolemia, hypertension, cardiovascular events, PPI usage, statin usage and appendectomy.

However, the most striking observation with the highest significance was the association of methanogens and insulin.

3.1 Insulin

Insulin levels were measured by the hormone laboratory of the Endocrinology Department. The levels from all participants ranged from 2.3 mU/L to 73.5 mU/L and the mean level was 11.84 ±9.07 mU/L. Samples were divided in three groups based on their respective insulin levels and according to the laboratory reference values of the hormone laboratory (3 – 25 mU/L): low (n=1), normal (n=84) and high (n=5). Due to its low samples size, group “low” was excluded from analysis.

3.1.1 Archaea targeted PCR

The archaea targeted PCR showed a highly significant association of relative archaeal abundance and normal insulin levels on multiple taxonomic levels, i.e. superkingdom (Fig. 4), phylum (Fig. 5), class (Fig. 6) and genus level (Fig. 7). We also adjusted our p-level for multiple testing (adjusted p-level was p=0.0313)

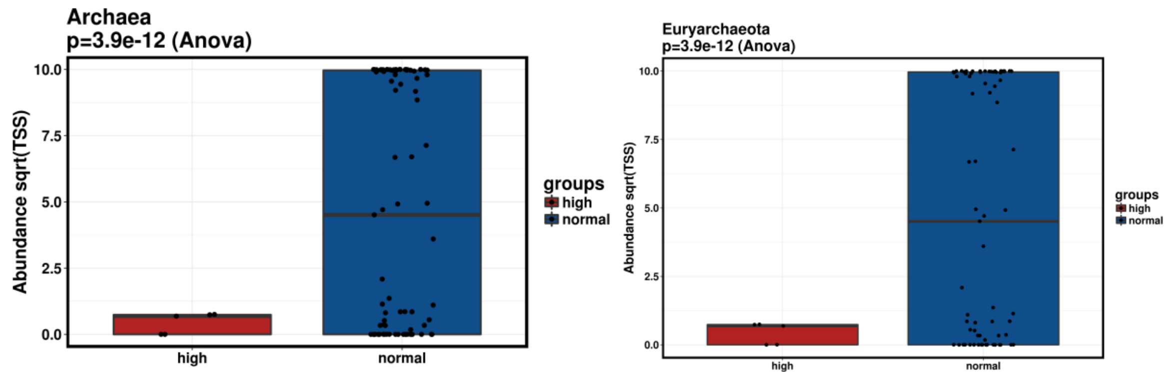

Figure 4 ***Reduced archaeal abundance (superkingdom) – Archaeal dataset***

Figure 5 ***Reduced archaeal abundance (phylum level) – Archaeal dataset***

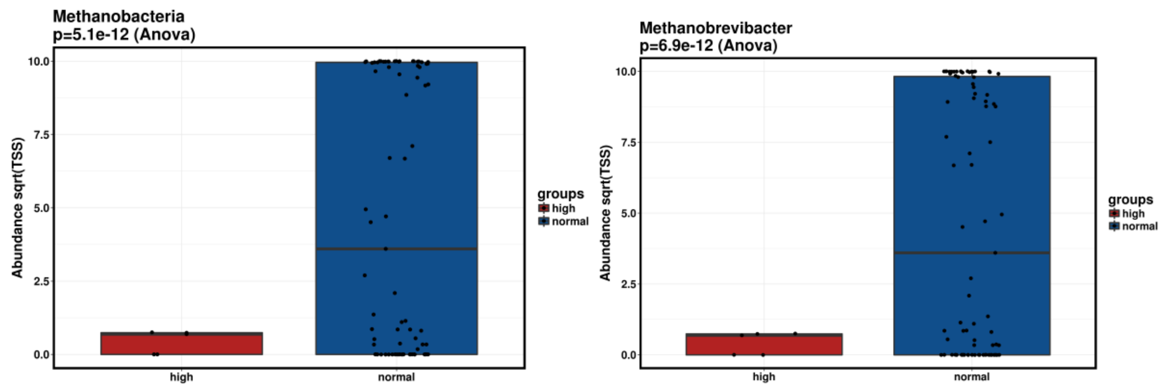

Figure 6 ***Reduced archaeal abundance (class level) – Archaeal dataset***

Figure 7 ***Reduced archaeal abundance (genus level) – Archaeal dataset***

The group with high insulin levels (>25 mU/L), had a median insulin level of 30.3 ±11.66 mU/L, whereas the group with normal insulin levels (3-25 mU/L) had a median insulin level of 9.9 ±3.71 mU/L. Interestingly, the relative abundance of *Methanobrevibacter* was reduced in all five subjects who were in the group with the elevated insulin levels. Up to now, no association between *Methanobrevibacter* and insulin levels has been made or reported. In addition, *Methanosphaera* was also associated with normal insulin levels (Fig. 8).

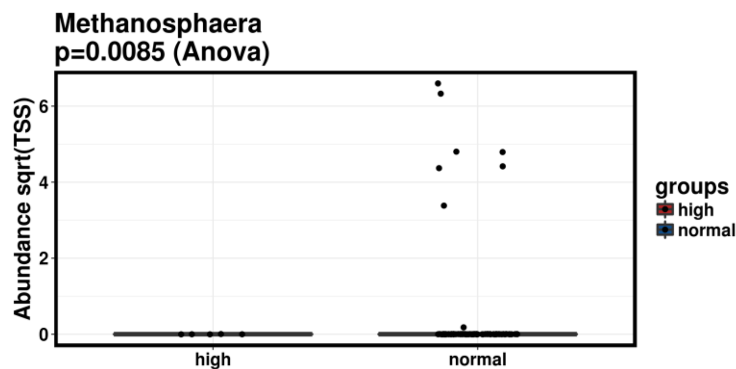

Figure 8 ***Methanosphaera is associated with normal insulin levels – Archaeal dataset***

While the association of *Methanobrevibacter* is highly significant (p=0.0000000000069), the association of *Methanosphaera* is less significant (p=0.0085).

Although there is no direct evidence that methanogens interact directly with insulin or glucose, they are heavily involved in bacterial metabolism, for example by the promotion of bacterial fermentation and thereby the production of short-chain-fatty acids (SCFA). SCFA can stimulate the GLP-1 and peptide-YY secretion via g-protein coupled receptors (GPR41 and GPR43), which in turn increase glucose-dependent insulin secretion and promote islet-cell function and proliferation (2). Other bacterial metabolites, which modulate insulin-sensitivity via FGF-19, FXR and TGR-5 are bile acids (2,18).

Since methanogenic archaea promote bacterial fermentation and modulate the microbial metabolism, which in turn modulates insulin secretion and glucose tolerance, they are involved in the energy-metabolism of the host and thereby might also indirectly influence glucose metabolism and insulin secretion. This could be reflected in the reduced abundance of methanogens in subjects with high insulin levels, which could be indicating dysbiosis and thus a dysfunctional insulin-glucose metabolism.

3.2 Hypertension according to the ESC 2018 Guidelines

In this category, the samples were divided into groups based on the participants' measured blood pressure values, according to the current classification by the European society of cardiology from 2018 (84). The blood pressure was measured according to the recommendations for blood pressure measurements in humans by the American Heart Association (85). For more details on the process of gathering data and procession see chapter 2.4.3.1. According to their respective blood pressure levels the participants were sorted into one of the following groups: **normal** <130/85mmHg (n=24), **high-normal** 130/85-139/89mmHg (n=22), **hypertension I**° 140/90-159/99 mmHg (n=26), **hypertension II**° 160/100-179/109 mmHg (n=16), **hypertension III°** ≥180/110 mmHg (n=6). The blood pressure was measured only twice in 6 participants.

3.2.1 Archaea targeted PCR

The archaeal PCR revealed an association of the relative abundance of archaea and blood pressure on the phylum (Fig. 9) and genus level (Fig. 10). However, after adjusting the p-level for multiple testing, the results were no longer significant (our adjusted p-level was p=0.021). Relative archaeal abundance was increased in the group with high-normal blood pressure and the group with hypertension II°. In contrast, a reduced relative archaeal abundance was observed in the groups with normal blood pressure and the group with hypertension I°. While an association of Euryarchaeota and *Methanobrevibacter* with different grades of blood pressure was observed, this was not shown for *Methanosphaera*. However, *Methanosphaera* showed a significant (p=0.0081) inverse association with medically documented hypertension (Fig. 11) and was only observable in subjects without documented hypertension in our archaeal PCR. For this category, the samples were divided into two groups, based on the data provided by the study participants on their medical conditions and the current medication. In the presence of known hypertonia or prescribed medication for the treatment of high blood pressure, the samples were sorted into group 1 " hypertension " (n=34). All other samples were sorted in group 2 "no hypertension " (n=60). Although no association of archaea and blood pressure is yet known, they are theoretically able to influence the blood pressure via the promotion of SCFA-production and TMAO-degradation, which are both known to interact with the blood pressure (2). In addition, it is presumed that dysbiosis could trigger obstructive sleep apnoea (OSAS), which in turn is known to induce hypertension (2).

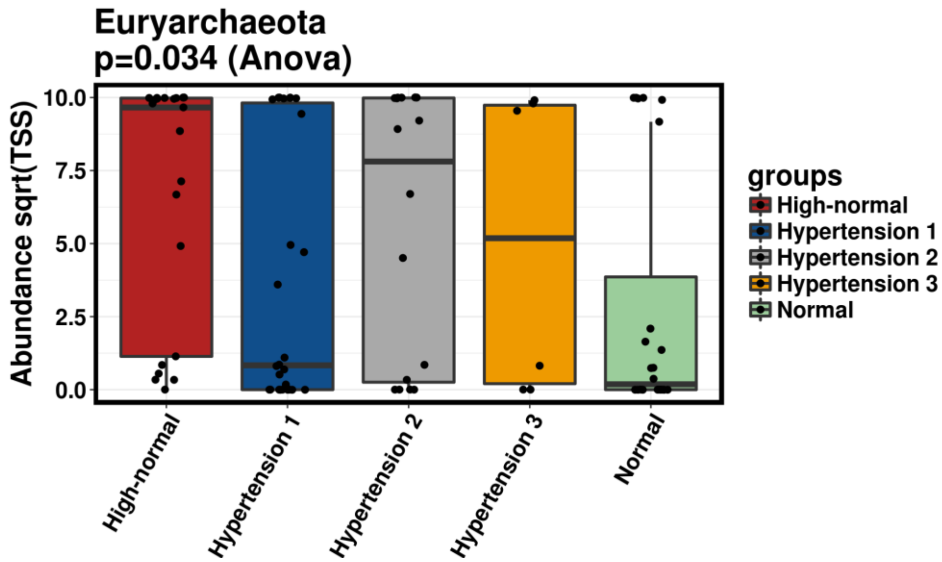

Figure 9 ***Different grades of arterial hypertension are associated with Euryarchaeota on the phylum level – Archaeal dataset***

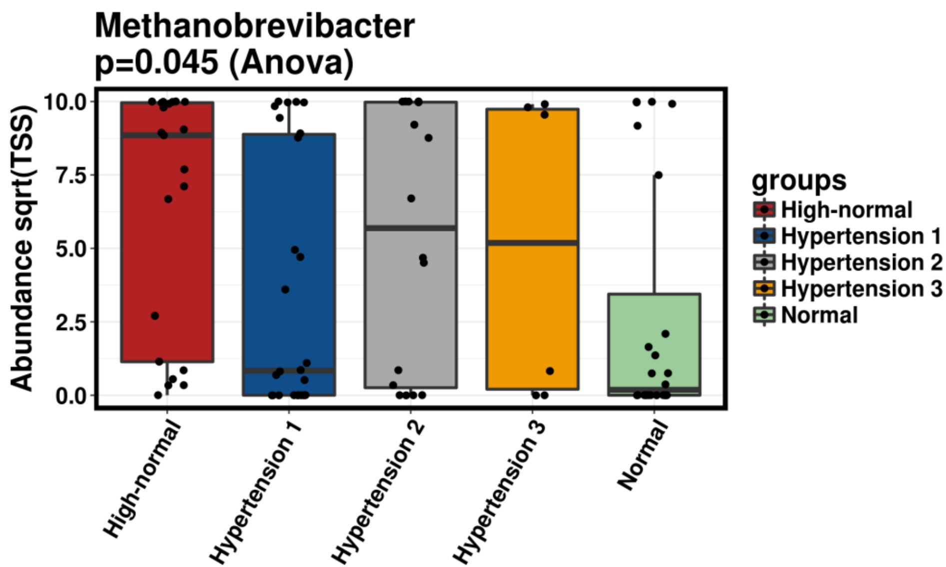

Figure 10 ***Different grades of arterial hypertension are associated with the abundance of Methanobrevibacter – Archaeal dataset***

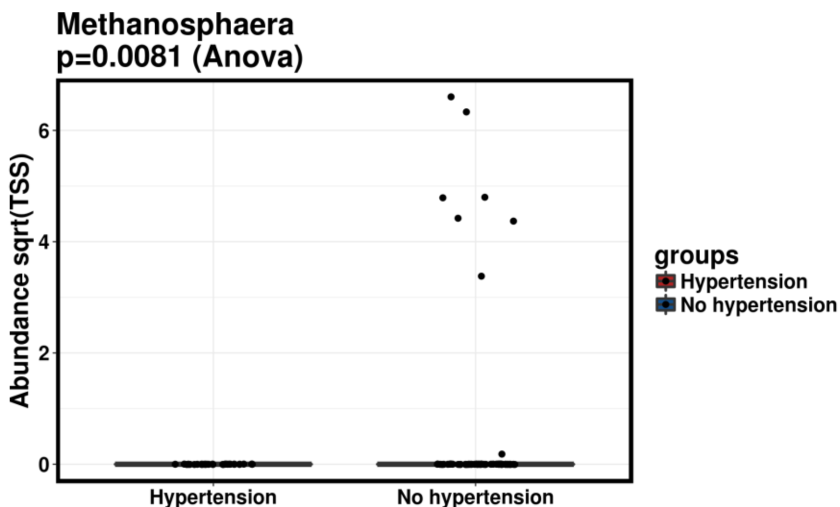

Figure 11 ***Methanosphaera is inversely associated with arterial hypertension – Archaeal dataset***

3.3. Urea

Urea levels from all participants ranged from 16 mg/dL to 68 mg/dL. The mean urea level was 35.04 ±1ß.73 mg/dL. The laboratory reference values ranged from 10 - 45 mg/dL. Therefore, samples were sorted into two different groups: normal (n=78) and high (n=15).

3.3.1 Archaea targeted PCR

Archaea targeted PCR revealed a positive correlation of relative archaeal abundance and blood urea levels on the phylum level (Fig. 12) and genus level (Fig. 13). However, after adjusting the p-level for multiple testing, the results were no longer significant (our adjusted p-level was p=0.00625). On the genus level, *Methanobrevibacter* showed a positive correlation with increasing urea levels. Although some archaea, like Thaumarchaeota are known to metabolize urea, this has not been reported for methanogenic archaea. So far, no correlation of methanogenic archaea and blood urea levels has been reported in humans. However, studies in ruminants, i.e. bulls, reported an increased abundance of *Methanobrevibacter* after a diet supplemented with high urea levels (87).

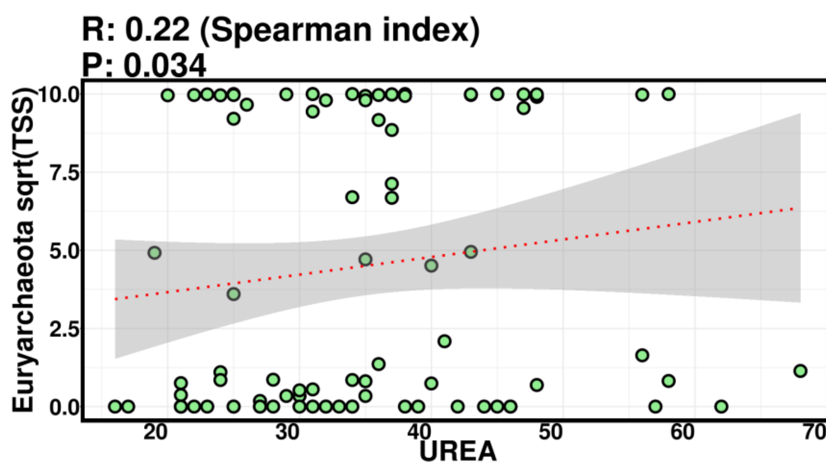

Figure 12 ***Increasing abundance of Euryarchaeota with increasing urea levels – Archaeal dataset***

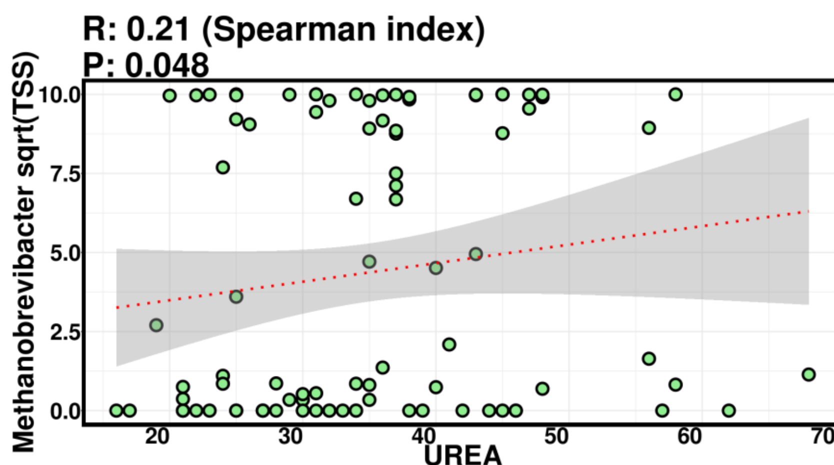

Figure 13 ***Increasing abundance of Methanobrevibacter with increasing urea levels – Archaeal dataset***

3.4 Triglycerides

Triglyceride levels from all participants ranged from 36 mg/dL to 332 mg/dL. The mean triglyceride level was 97.92 ±47.71 mg/dL. The laboratory reference values ranged from 0 - 150 mg/dL. Therefore, samples were sorted into two different groups: normal (n=82) and high (n=11). However, group analysis revealed no significant association.

3.4.1 Archaea targeted PCR

Archaeal PCR revealed a significant negative correlation of relative archaeal abundance and increasing serum triglyceride levels on the phylum (p=0.013, Fig. 14) and genus level (p=0.021, Fig. 15). These observations were also significant after adjusting for multiple testing (adjusted p-level was p=0.025). On the genus level, we observed this correlation for *Methanobrevibacter.* So far, there are no reports concerning an association of archaea and serum triglyceride levels. However, there is evidence, that the microbiome can modulate and stimulate de-novo lipogenesis for example via bile acids and bile acid receptors like FRX or TGR-5 (88). In addition, some studies reported increased fasting serum triglycerides in individuals with low microbial diversity (2). In addition, archaea are associated with highly diverse microbiomes (34). This could lead to the conclusion that individuals with low microbial diversity and thus elevated triglyceride levels also display a reduced archaeal abundance.

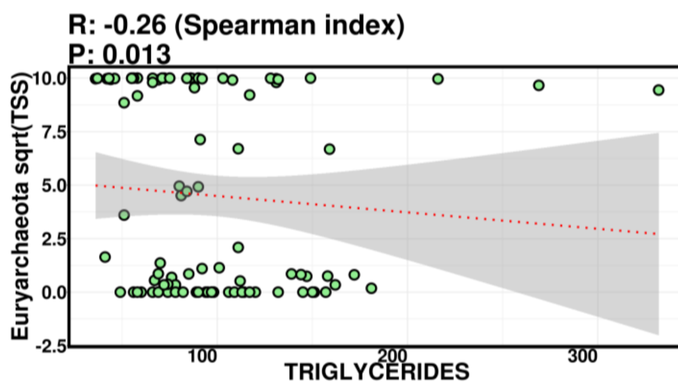

Figure 14 ***Decreasing abundance of Euryarchaeota with increasing triglyceride levels – Archaeal dataset***

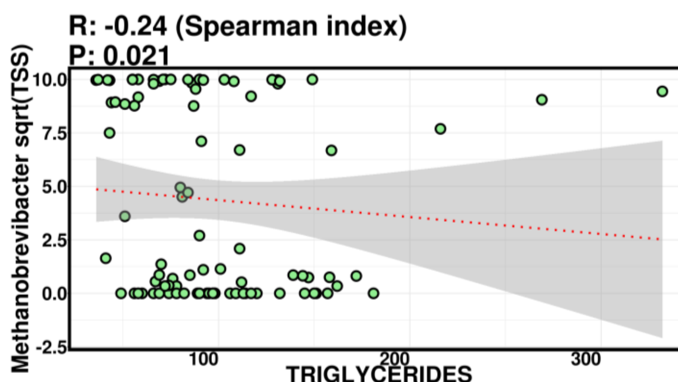

Figure 15 ***Decreasing abundance of Methanobrevibacter with increasing triglyceride level – Archaeal dataset***

3.5 Calcium

Calcium levels from all participants ranged from 2.14 mmol/L to 2.69 mmol/L. The mean calcium level was 2.38 ±0.10 mmol/L. Since the laboratory reference values range from 2.2 - 2.65 mmol/L, all subjects exhibited physiological serum calcium levels.

3.5.1 Archaea targeted qPCR

Archaeal qPCR revealed a significant (p=0.036) negative correlation of absolute archaeal abundance and serum calcium levels (Fig. 16) using the Pearson correlation index. However, after adjusting the p-level for multiple testing, the result was no longer significant (our adjusted p-level was p=0.0125). So far, no direct association of archaea and serum calcium levels has been reported. However, their involvement in calcium absorption in the gut is plausible because they promote bacterial fermentation and the production of SCFA. The production of SCFA lowers the pH, which is presumed to reduce mineral complexation and facilitate calcium absorption (89). In addition, SCFA are highly involved in signalling pathways, which could modulate calcium absorption (89). However, despite these observations, SCFA levels do not correlate with calcium absorption, leaving their importance in this mechanism for debate. Overall, the archaeal involvement in calcium metabolism is poorly understood and more research is needed to illuminate their role.

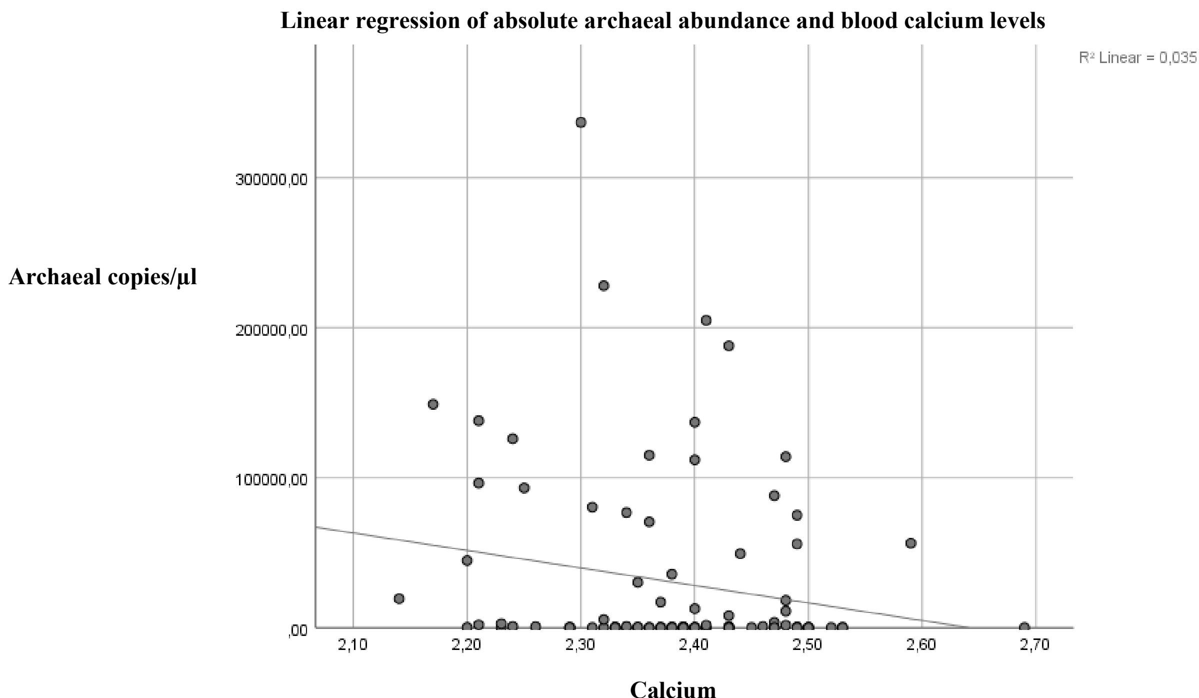

Figure 16 ***Archaeal abundance decreases with increasing calcium levels – Archaeal dataset***

3.6 Body temperature

Body temperature from all subjects ranged from 36.0 to 37.2 °C. The median body temperature was 36.7 ±0.22 °C. Unfortunately, when the temperature was recorded, it was not noted which method was chosen for the measurement.

3.6.1 Archaea targeted PCR

Archaeal PCR revealed a significant ($p=0.018$) positive correlation for the relative abundance of *Methanosphaera* and increasing body temperature (Fig. 17).

3.6.2 Universally targeted PCR

In addition, the universal PCR revealed a decreasing microbial diversity with increasing body temperature on the RSV-($p=0.0058$)(Fig. 18) and genus level ($p=0.024$)(Fig. 19).

However, after adjusting the p-level for multiple testing, these results were no longer significant (our adjusted p-level was $p=0.0033$).

While there are no reports on an association of *Methanosphaera* and body temperature, there are animal studies on changes in the microbiome depending on an increasing environmental temperature and thus increased body temperature (90). One of the reported changes was a reduction in the abundance of Firmicutes (90). However, it is highly debatable whether this observation can be transferred to humans and whether a mere reduction of Firmicutes leads to reduced diversity.

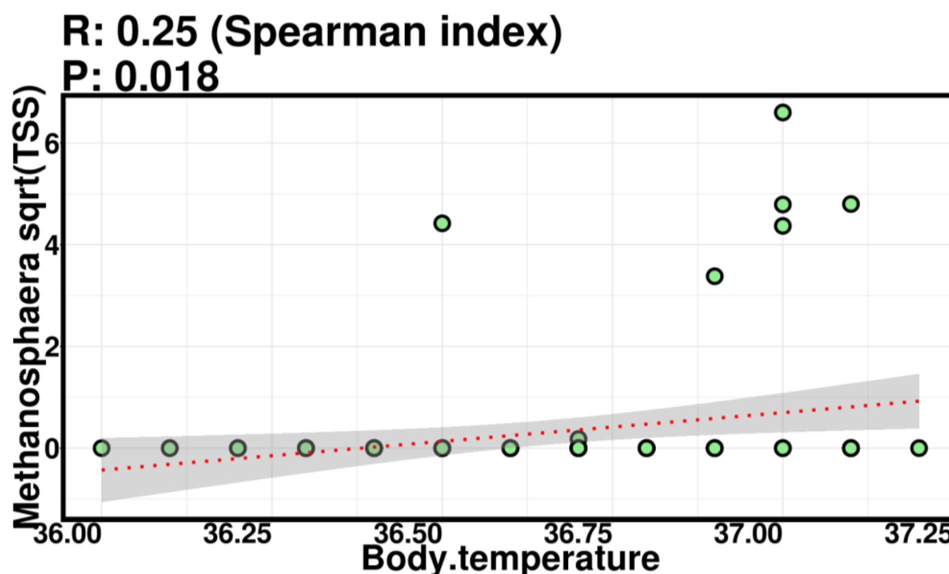

Figure 17 ***Relative abundance of Methanosphaera is increasing with increasing body temperature – Archaeal dataset***

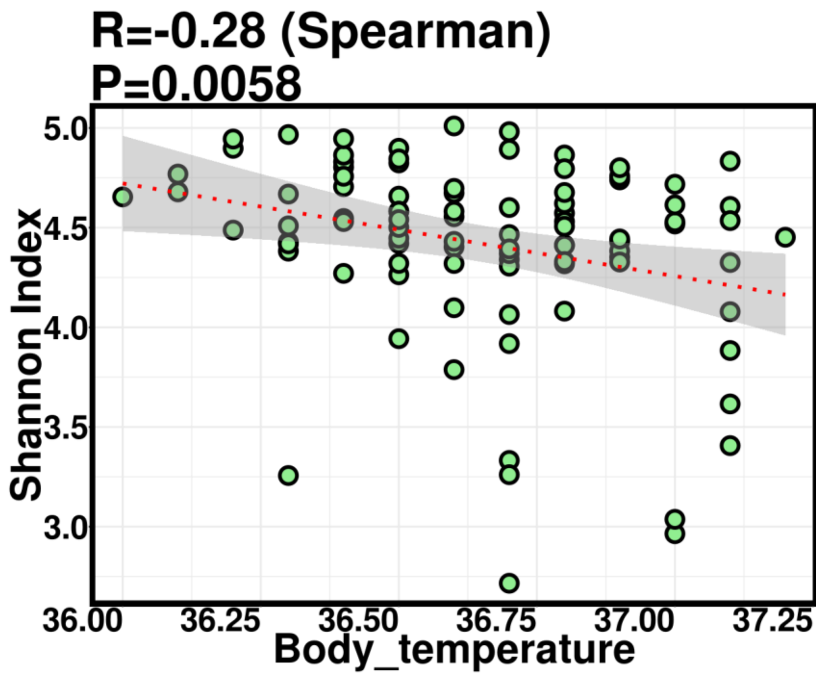

Figure 18 ***Decreasing microbial diversity with increasing temperature (RSV level) – Universal dataset***

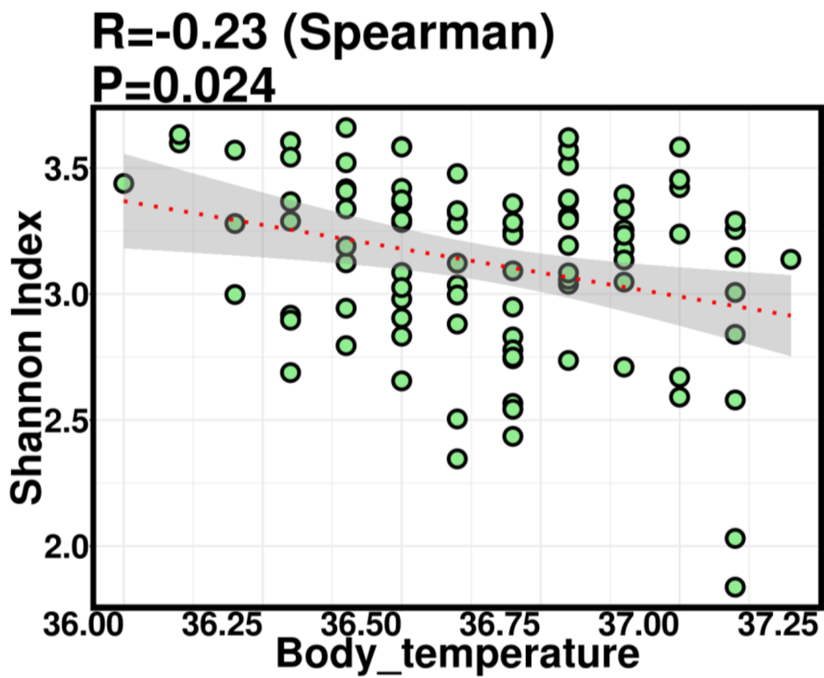

Figure 19 ***Decreasing microbial diversity with increasing body temperature (genus level) – Universal dataset***

3.7 Proton-pump-inhibitor usage

For this category, the samples were divided into two groups based on the data provided by the study participants on their current medication. All samples from participants, who were currently treated with proton pump inhibitors, were divided into group 1 “PPI usage” (n=7). All other samples were sorted in group 2 "no PPI usage " (n=87).

3.7.1 Archaea targeted PCR

In the archaea targeted PCR *Methanosphaera* was only observable in subjects without PPI usage, and this observation was significant (p=0.0085) (Fig. 20).

3.7.2 Universally targeted PCR

Universal PCR revealed an increased microbial diversity in subjects with PPI usage on the class (Fig. 21) and phylum level (Fig. 22). In addition, the group with PPI usage showed an association with higher relative abundances of *Streptococcus*, *Bacilli*, *Desulfovibrio*, *Shigella* and *Dialister* in a subsequent LefSe-Analysis on the genus level (Fig 23).

These observations were also significant after adjusting for multiple testing (adjusted p-level was p=0.04). The detection and over-representation of oral bacteria like *Streptococcus*, as well as an increase in potentially pathogenic members of *Escherichia coli* (*Shigella*) in the faecal microbiome has already been reported in other studies and could lead to dysbiosis (91–93). However, the data situation is currently inconsistent regarding the changes in diversity. While some studies report an increase in diversity when taking PPI (91), others report a decrease (92) or no change at all (94,95). To date there are no reports on the inverse association of *Methanosphaera* and PPI usage. While some archaea are usually associated with high microbial diversity, this observation could be partially due to changes in the microbial composition following PPI usage, which could in turn lead to dysbiosis (34,93). However, the association of archaea and high microbial diversity has only been reported for *Methanobrevibacter* but not for *Methanosphaera* (34).

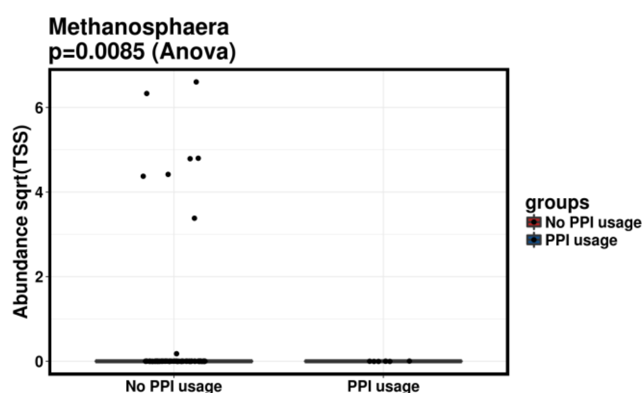

Figure 20 ***Methanosphaera is only detectable in subjects with no PPI-usage – Archaeal dataset***

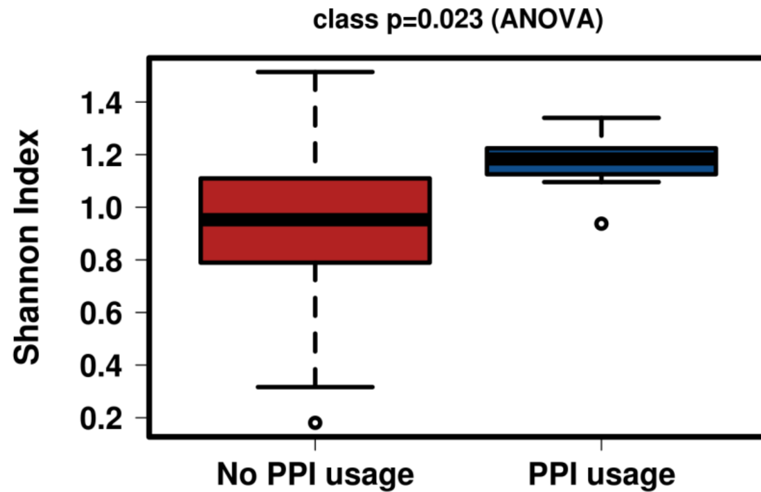

Figure 21 ***Increased microbial diversity associated with PPI-usage (class level) – Universal dataset***

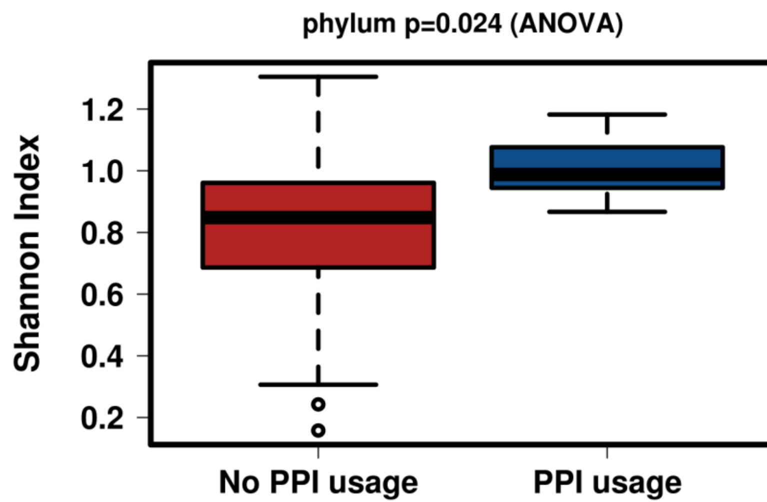

Figure 22 ***Increased microbial diversity is associated with PPI-usage (phylum level) – Universal dataset***

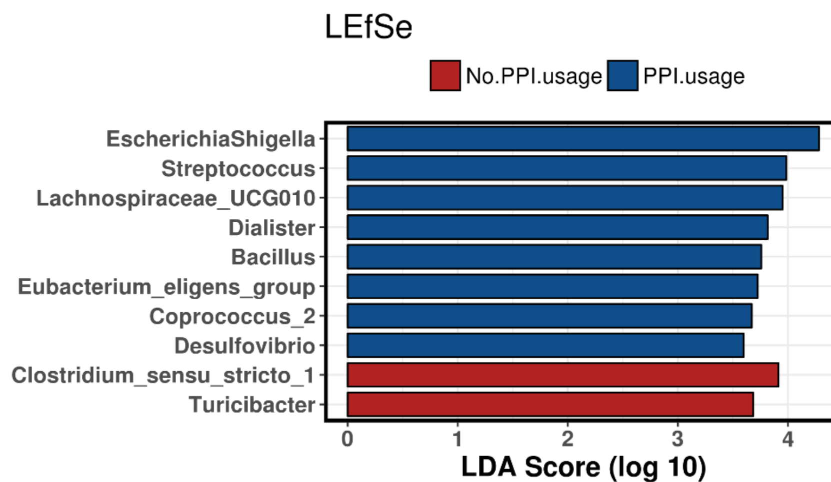

Figure 23 ***Association of different bacteria and current PPI-usage – Universal dataset***

3.8 Bilirubin

Bilirubin levels from all participants ranged from 0.24 mg/dL to 2.12 mg/dL. The mean bilirubin level was 0.65 ±0.34 mg/dL. The laboratory reference values ranged from 0.1 - 1.2 mg/dL. Therefore, samples were sorted into two different groups: normal (n=87) and high (n=6).

3.8.1 Archaea targeted PCR

The archaeal PCR revealed a significant (p=0.0085) association of *Methanosphaera* with normal serum bilirubin levels (Fig. 24).

3.8.2 Universally targeted PCR

Similar results were observed for the microbial diversity on the genus level in the universal PCR (p=0.0018) (Fig. 25).

These observations were also significant after adjusting for multiple testing (adjusted p-level was p=0.0166). While there are no reports on an association of *Methanosphaera* and serum bilirubin levels, there are reports, that bilirubin can exert a selective pressure on microbes, quite similar to bile acids (96). Another study observed a disrupting effect of bilirubin on the cell membrane of gram-positive bacteria, while it protected gram-negative bacteria from ROS (97). Thereby bilirubin modulates bacterial function and microbial diversity. The observed results might demonstrate this modulation.

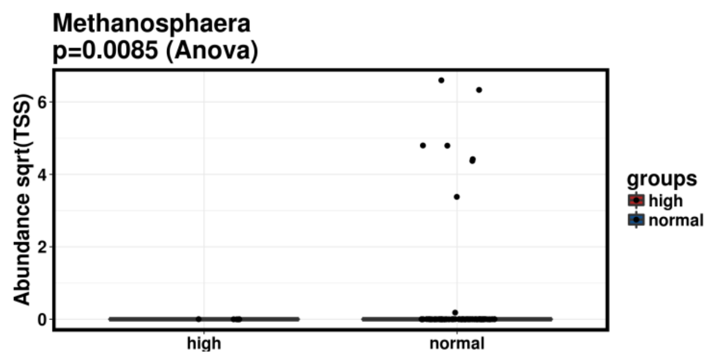

Figure 24 ***Methanosphaera is associated with normal bilirubin levels – Archaeal dataset***

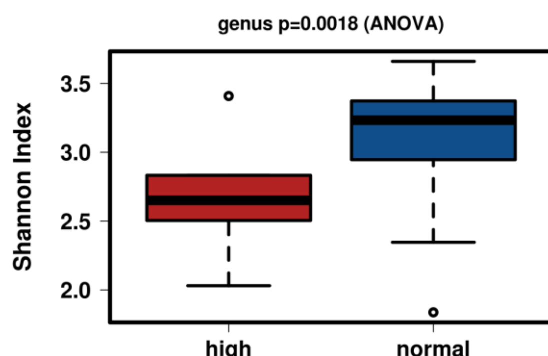

Figure 25 ***High bilirubin levels are associated with a decrease in microbial diversity (genus level) – Universal dataset***

3.9 Appendectomy

For this category, groups were formed on the criteria whether the participants had undergone an appendectomy. Therefore, two groups were formed: group 1 “appendectomy” (n=30) and group 2 “no appendectomy” (n=64).

3.9.1 Archaea targeted PCR

Archaea targeted PCR revealed a significant (p=0.0082) association of *Methanosphaera* and subjects with no recorded appendectomy (Fig. 26). This observation was also significant after adjusting for multiple testing (adjusted p-level was p=0.01). To date, there have been no reported direct associations of *Methanosphaera* and the absence of an appendix vermiformis, respectively an appendectomy.

However, a recent study reported a reduced methane production in subjects, who had an appendectomy (98). This study also hypothesized, that the appendix might contribute to the housing of methanogens and thus increase methane production (98). The same mechanism could be responsible for the high methane levels in diverticulosis, possibly even aggravating this condition (61,98).

Figure 26 ***Methanosphaera is only detectable in subjects without an appendectomy – Archaeal dataset***

3.10 Hypercholesterolaemia

For this category, the samples were divided into two groups based on the data provided by the study participants on their medical conditions and their current medication. In the presence of known hypercholesterolemia or prescribed medication for the treatment of high cholesterol levels, the samples were sorted into group 1 " hypercholesterolemia " (n=20). All other samples were sorted in group 2 "no hypercholesterolemia " (n=74). This category was not based on current cholesterol levels.

3.10.1 Archaea targeted PCR

Archaea targeted PCR revealed a significant (p=0.0084) association of *Methanosphaera* and no recorded hypercholesterolemia (Fig. 27). This observation was also significant after adjusting for multiple testing (adjusted p-level was p=0.021). This observation could be explained due to the fact that all subjects with recorded hypercholesterolemia were treated with statins. Statins have been reported to disrupt archaeal cell membrane synthesis and thus inhibit their growth (44,46). This effect was also present in our study, as can be observed in chapter 3.11. However, this effect was not observed for *Methanobrevibacter* in any of these categories.

In addition, some archaea like *Methanomassiliicoccus luminyensis* can influence lipid metabolism by the degradation of the bacterial metabolite TMAO (2,18). TMAO has been reported to contribute to a disrupted lipid metabolism and the development of atherosclerosis through the inhibition of bile acid synthesis and an altered expression of scavenger receptors in the endothelium and on macrophages (2). Thereby, a reduction of TMAO could prevent hyperlipidaemia, hypercholesterolaemia and atherosclerosis. However, this metabolic function has not yet been reported for *Methanosphaera*.

Figure 27 ***Methanosphaera is inversely associated with hypercholesterolaemia – Archaeal dataset***

3.11 Statin usage

In this category, the samples were divided into two groups, based on the data provided by the study participants concerning their current medication. All samples from participants who were currently treated with statins were divided into group 1 “Statin usage” (n=16). All other samples were sorted into group 2 "no statin usage " (n=78). None of the participants was treated with Lovastatin, the majority used Simvastatin or Atorvastatin.

3.11.1 Archaea targeted PCR

In the archaea targeted PCR we observed a significant (p=0.0084) association of *Methanosphaera* and the group with no statin usage (Fig. 28). This observation was also significant after adjusting for multiple testing (adjusted p-level was p=0.04). This observation is in line with reports of the effect of statins on methanogenic archaea *in vitro* (44,46). However, the inhibition of the archaeal HMG-CoA-reductase, and thus their inhibition of growth has only been reported for the statins Lovastatin and Mevastatin, two substances that were not in use by any of our study’s subjects (47,48). Interestingly, the subjects in our study primarily used Atorvastatin and Simvastatin, for which archaeal growth inhibition was not reported (44).

In addition, this effect was not observed for *Methanobrevibacter* or on a higher taxonomic level.

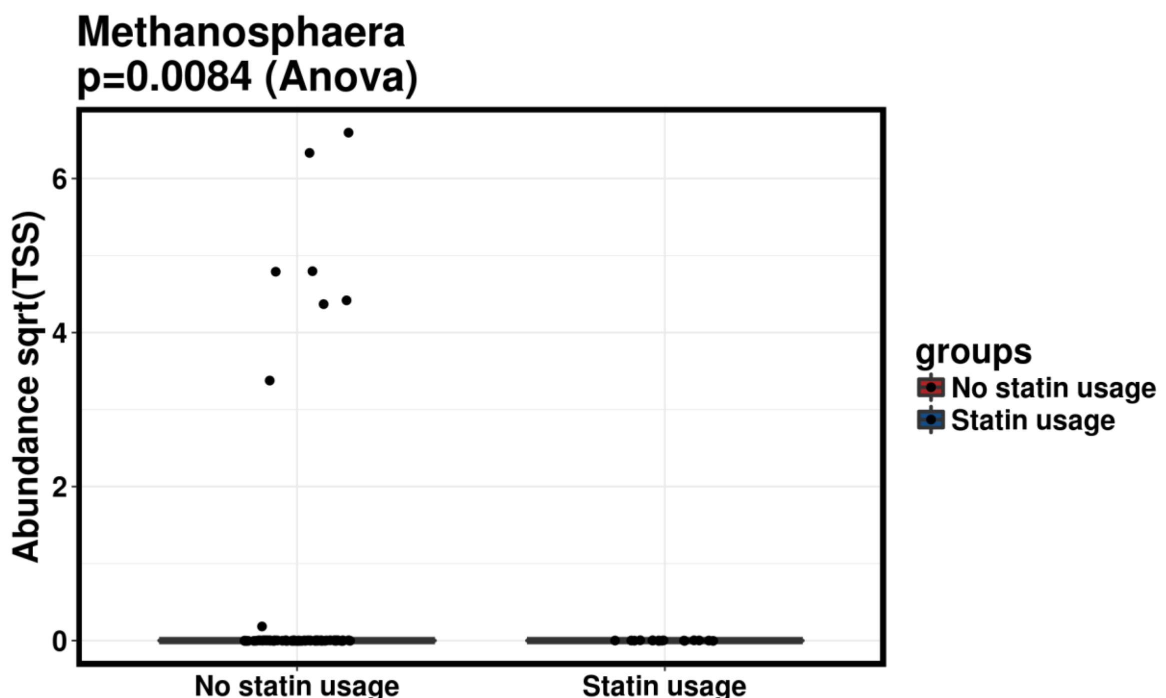

Figure 28 ***Methanosphaera is only detectable in subjects with no statin treatment – Archaeal dataset***

3.12 Cardiovascular events

This category analysed any cardiovascular event, based on the data provided by the study participants concerning their medical conditions and previous illnesses. The following cardiovascular events were found among the participants: stroke (n=3), myocardial infarction (n=1), pulmonary arterial embolism (n=2), arterial embolism (n=1) and venous thrombosis (n=1). The samples were divided into two groups based on whether any cardiovascular events have been recorded. Thereby two groups were created: group 1 “cardiovascular event” (n=8) and group 2 "no cardiovascular event" (n=86).

3.12.1 Archaea targeted PCR

The archaea targeted PCR revealed a significant (p=0.0085) association of *Methanosphaera* and the group with no recorded cardiovascular events (Fig. 29). This observation was also significant after adjusting for multiple testing (adjusted p-level was p=0.021). To date there are no reported associations of *Methanosphaera* and cardiovascular events. However, as hypercholesterolaemia is a risk factor for cardiovascular disease, this observation could be related to our previously described results on statin use and the presence of hypercholesterolaemia. Regarding this fact, five subjects in the group with cardiovascular events had recorded hypercholesterolaemia and out of these five subjects, four were treated with statins. Again, this observation was not made for *Methanobrevibacter* or on any higher taxonomic level.

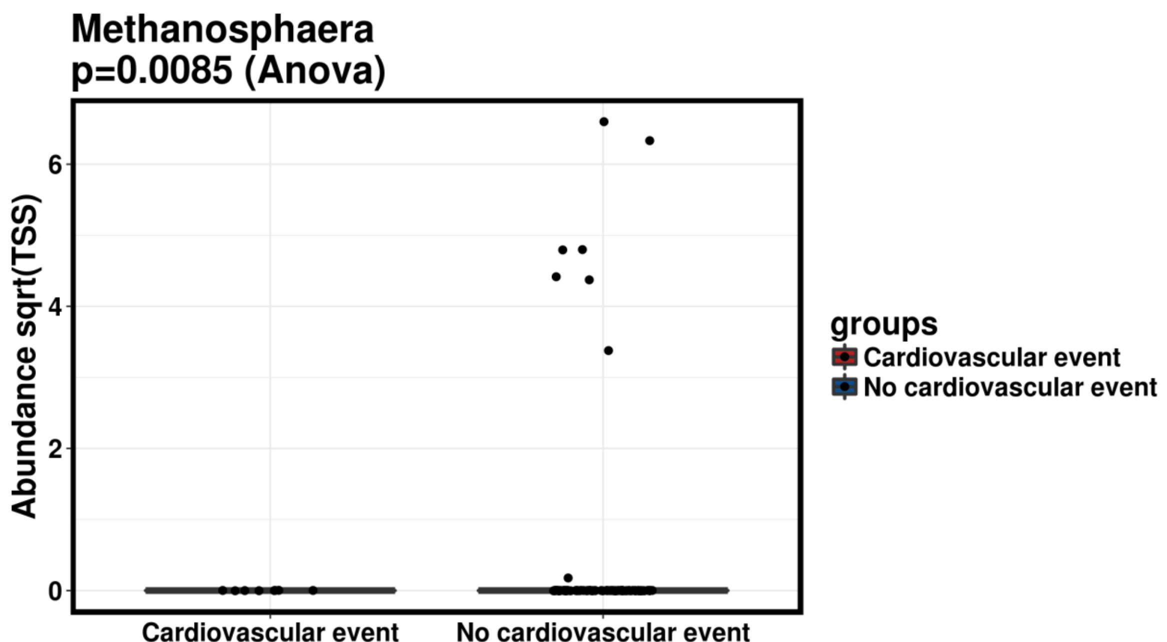

Figure 29 ***Methanosphaera is only detectable in subjects without cardiovascular events – Archaeal dataset***

3.13 C-reactive protein

CRP levels from all participants ranged from <0.6 mg/dL to 14.2 mg/dL. The mean CRP level was 1.78 ±2.26 mg/dL. The laboratory reference values ranged from 0.6 - 5.0 mg/dL. Levels below the measurable range (<0.6 mg/dL) were classified as 0.0 mg/dL for analysis. Therefore, samples were sorted into two different groups: normal (n=86) and high (n=7).

3.13.1 Archaea targeted PCR

Archaea targeted PCR revealed a significant (p=0.0085) association of *Methanosphaera* and the group with normal CRP levels (Fig. 30). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.00625). This observation could support the concept of *Methanosphaera* being a commensal gut inhabitant. There is evidence, that methanogenic archaea, such as *Methanosphaera* are well adapted and specialized to the human gut, for example by the expression of adhesin-like proteins (ALP) for intercellular and mucosal adhesion, the possession of bile salt hydrolase to evade potentially toxic bile acid and the ability to modify polysaccharides for a better adherence to biotic and abiotic surfaces (43). In addition, the intestinal epithelial cells show no inflammatory reaction to *Me. stadtmanae*, a phenomenon that is typical for commensals (8). However, it has been reported, that *Me. stadtmanae* can trigger a strong inflammatory reaction via monocyte-derived dendritic cells and leukocytes (43). In addition, in subjects with crohn's disease the abundance of *Me. stadtmanae* and specific antibodies directed at *Me. stadtmanae* are increased (8). However, it is debatable whether these observations are due to the influence of *Me. stadtmanae* or the loss of integrity of the intestinal epithelial barrier.

Based on our observations, it can be stated that *Methanosphaera* is probably not associated with a pronounced systemically measurable inflammation.

Methanosphaera
p=0.0085 (Anova)

Figure 30 **Methanosphaera is associated with normal CRP levels – Archaeal dataset**

3.14 Absolute microbial abundance

Absolute microbial abundance ranged from 1,350,000 to 1,010,000,000 copies/g stool with a median abundance of 167,000,000.00 ±136,542,021.28. In contrast, archaeal abundance was much lower and ranged from 0.00 to 336,903.945 copies/g stool. Median archaeal abundance was 100.45 ±29,871.85 copies/g stool. Overall, we found archaeal reads in 65 subjects in qPCR. In these 65 subjects, archaea made up on average 0.04 ±0.087% of the total microbes.

3.14.1 Archaea targeted and universally targeted qPCR

Archaeal and universal qPCR revealed a significant (p=0.02) correlation of archaeal abundance and overall microbial abundance using the Pearson correlation index (Fig. 31). However, after adjusting for multiple testing this observation was no more significant (adjusted p-level was p=0.0033). This observation is in line with reports on the association of methanogenic archaea and microbiomes with high gene counts, i.e. highly diverse microbiomes (34). In addition, this correlation could also be an expression of the strong dependence of archaea on bacterial substrates, whose presence they need to thrive (43). Furthermore, it could be argued that this correlation is caused by the fact that archaea are also detected in the universal qPCR and thus correlate with themselves. However, the fact that the universal primers have a significantly lower sensitivity to archaeal rRNA than the archaea-specific primers we use indicates otherwise.

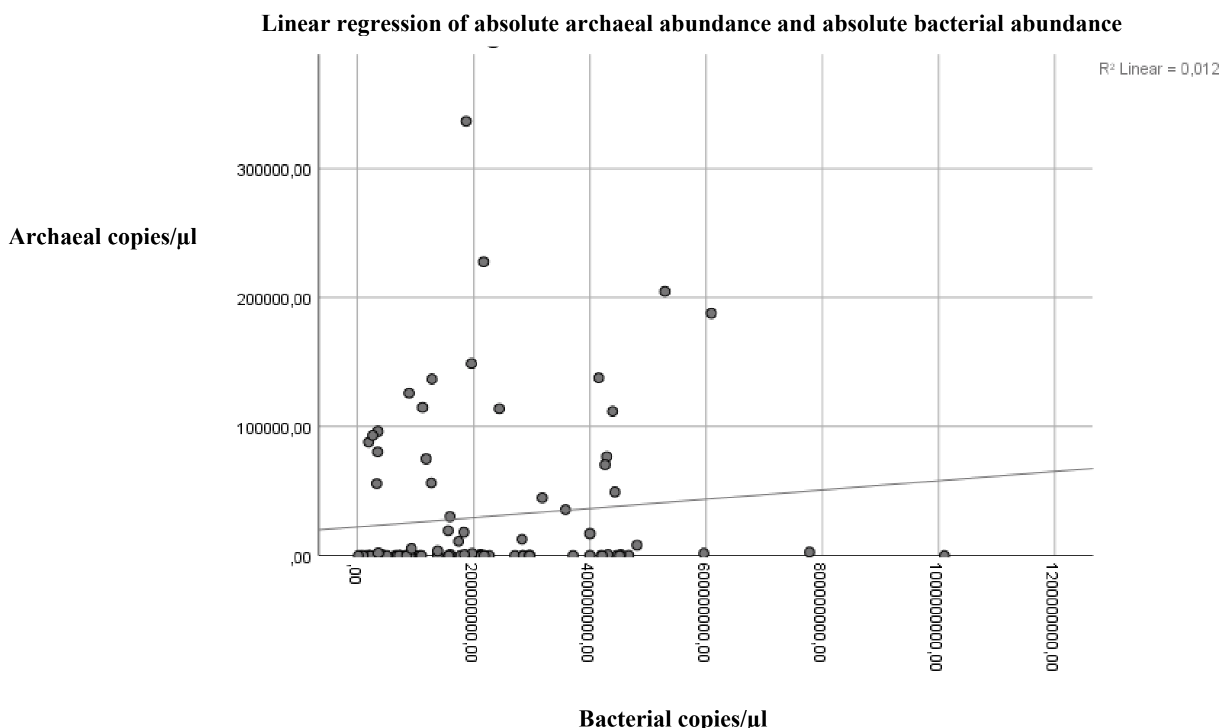

Figure 31 ***Absolute abundance of archaea increases with increasing absolute bacterial abundance – both datasets***

3.15 1.25-Dihydroxyvitamin D_3

1.25 - Dihydroxy vitamin D_3 levels from all participants ranged from <7 ng/mL to 73.1 ng/mL. Levels below the measurable range (<7 ng/mL) were classified as 0.0 ng/mL for analysis. The mean level was 25.67 ±12.6 ng/mL. The laboratory reference values ranged from 30 - 60 ng/mL.

3.15.1 Universally targeted PCR

Universal PCR revealed a significant (p=0.047) negative correlation of microbial diversity and increasing 1.25-Dihydroxyvitamin D_3 levels on the RSV level (Fig 32). However, after adjusting the p-level for multiple testing, the result was no longer significant (our adjusted p-level was p=0.0313). 1.25-Dihydroxyvitamin D_3 is the active form of the prohormone vitamin D and is involved in the calcium-metabolism and immune regulation, where it is presumed to have an anti-inflammatory effect (99). Changes in the microbiome following vitamin D supplementation have been reported in mice and humans (99,100). A reduction of the members of the phylum Firmicutes and an increase of the phylum Bacteroidetes were reported in these studies (99,100). It could be argued that the decrease of members of the Firmicutes phylum might result in the observed decrease of the overall microbial diversity. In addition, it has been reported, that some bacteria can modulate 1.25-Dihydroxyvitamin D_3 levels by processing and activating vitamin D in a manner similar to humans (99). However, the exact relationship of vitamin D_3 and the microbiome is still unclear, because we have only small knowledge of the effects of vitamin D_3 on bacteria (99).

Interestingly, we observed no change in microbial diversity in association with the status of vitamin D_3 supplementation in the medical history.

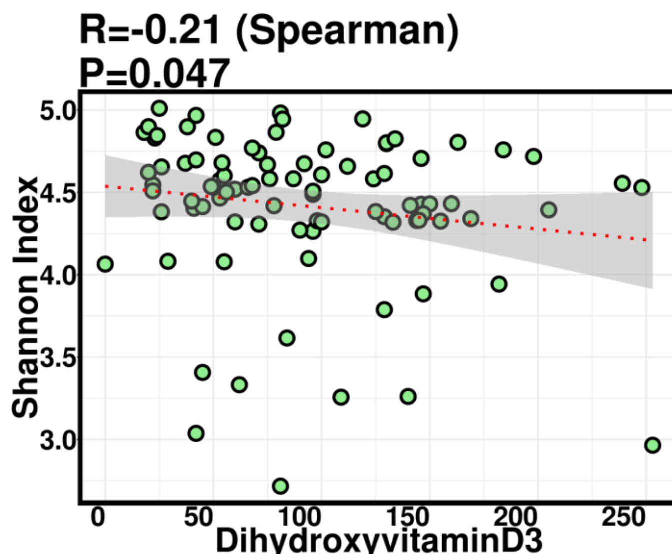

Figure 32 ***Decreasing microbial diversity with increasing 1.25-Vitamin D_3 (RSV level) – Universal dataset***

3.16 Aldosterone

Aldosterone levels from all participants ranged from <3.7 ng/dL to 104 ng/dL. Levels below the measurable range (<3.7 ng/dL) were classified as 0.0 ng/dL for analysis. The mean level was 8.43 ±13.24 ng/dL. The laboratory reference values ranged from 3.7 – 43.2 ng/dL, because the blood samples were taken, while the participants were seated. Therefore, samples were sorted into three different groups: normal (n=60), low (n=27), and high (n=2). Due to its low samples size, group "high" was excluded from analysis. In addition, one sample could not be measured, because there was not enough material for analysis.

3.16.1 Universal targeted PCR

Universal PCR revealed a significant (p=0.046) association of microbial diversity and the group with normal aldosterone levels on the class level (Fig. 33). However, after adjusting the p-level for multiple testing, the result was no longer significant (our adjusted p-level was p=0.0313). It is presumed, that the microbiome is involved in the modulation of the renin-angiotensin-aldosterone system (RAAS) via the production of activating and inhibiting metabolites and thus might influence blood pressure (101). While some bacteria can mediate aldosterone synthesis, others synthesize angiotensin-converting-enzyme (ACE)- or renin-inhibiting substances which modulate the RAAS and thereby the blood pressure (101). It has therefore been hypothesized, that dysbiosis or specific enterotypes might lead to hypertension (101–103). In the group with low aldosterone levels (n=27), most subjects had hypertension (n=14) or borderline high blood pressure (n=5) and a total of eight of these subjects were treated with antihypertensive medication, which in turn suppresses aldosterone levels. The association of a decreased microbial diversity and the group with low aldosterone levels could thereby be due to dysbiosis in those subjects with hypertension and a treatment with antihypertensive drugs. However, we did not observe a significant association of microbial diversity and hypertension in our study, which contradicts this hypothesis.

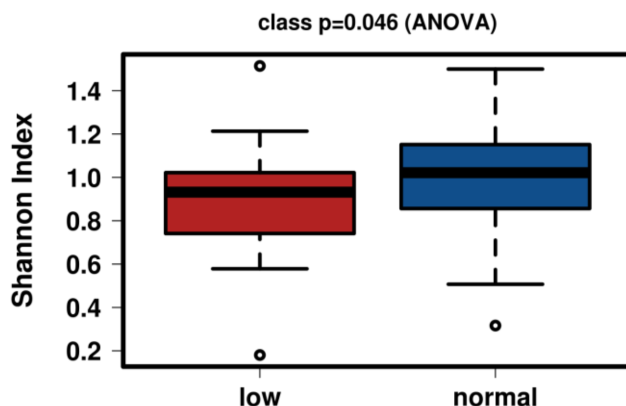

Figure 33 ***Normal aldosterone levels are associated with increased microbial diversity (class level) – Universal dataset***

3.17 Cholinesterase

Cholinesterase levels from all participants ranged from 5,571 U/L to 11,723 U/L. The mean cholinesterase level for women was 8,217.35 ±1,595.03 U/L and for men 8,213.00 ±1,556.34 U/L. Overall, the mean cholinesterase level was 8,215.67 ±1,580.17 U/L. The laboratory reference values were sex-specific and ranged from 3,900-11,000 U/L for women and from 4,600 – 13,000 U/L for men. Therefore, samples were sorted into two different groups: normal (n=89) and high (n=4).

3.17.1 Universally targeted PCR

Universal PCR revealed a significant (p=0.047) positive correlation of microbial diversity and increasing cholinesterase levels on genus level (Fig. 34). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.0166). So far, this specific correlation has not been reported in any other study. Since cholinesterase is a marker for liver function, the observation could indicate that a proper (metabolic) function of the liver is associated with increased microbial diversity. An association of liver cirrhosis and dysbiosis has been reported in this context in other studies (104,105). Alcoholic liver disease for example is associated with a decrease in the phylum Firmicutes and chronic alcoholism in general is associated with a reduction of beneficial commensals of the gut (104,105). However, it can only be speculated whether this reduction of firmicutes and beneficial microbiotas is actually measurable as a reduction in microbial diversity.

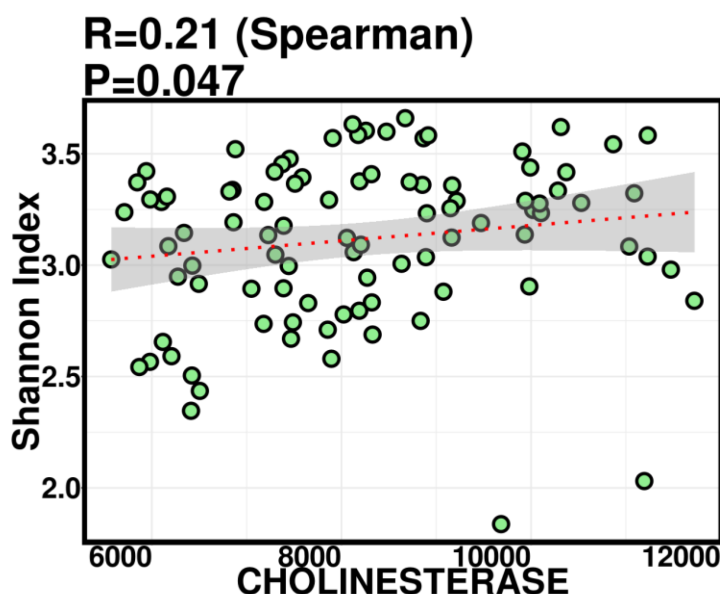

Figure 34 ***Microbial diversity increases with increasing cholinesterase (genus level) – Universal dataset***

3.18 Lactate dehydrogenase

LDH levels from all participants ranged from 109 U/L to 329 U/L. The mean LDH level was 196.68 ±36.18 U/L. The laboratory reference values ranged from 120 - 240 U/L. Therefore, samples were sorted into three different groups: low (n=1), normal (n=82) and high (n=10).

3.18.1 Universally targeted PCR

Universal PCR revealed a significant (p=0.036) positive correlation of microbial diversity and increasing LDH levels on the genus level (Fig. 35). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.00625). Serum LDH is a marker for cell lysis, for example in haemolysis or ischaemia. So far, the association of changes in the microbiome and LDH have only been described in conjunction with antibiotic induced dysbiosis or severe systemic inflammation, but not with changes in microbial diversity (106,107).

Unfortunately, it is almost impossible to provide a definitive statement on this observation, given that LDH is a very unspecific marker which is difficult to interpret without context to the individual subject's condition.

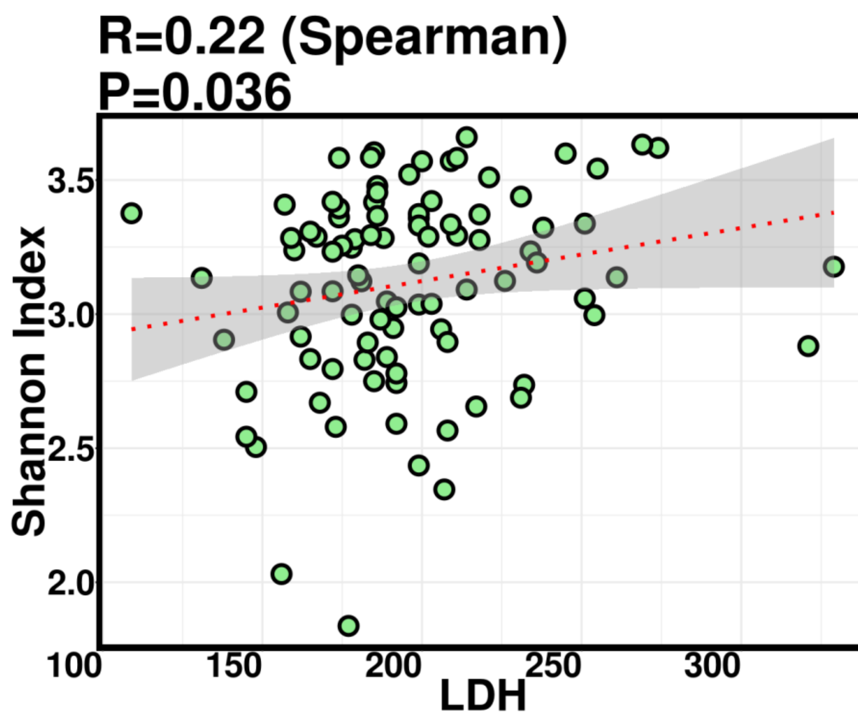

Figure 35 ***Microbial diversity increases with increasing LDH (genus level) – Universal dataset***

3.19 Sex hormone binding globulin

SHBG levels from all participants ranged from 14.2 nmol/L to 165.9 nmol/L. The mean SHBG level for women was 75.65 ±35.04 nmol/L and for men 59.19 ±23.92 nmol/L. Overall, the mean level was 69.43 ±32.31 nmol/L. The laboratory reference values were sex-specific and ranged from 19 - 117 nmol/L for women and from 16 - 76 nmol/L for men. Therefore, samples were sorted into three different groups: normal (n=76), low (n=1), and high (n=13). Due to its low samples size, group "low" was excluded from analysis.

3.19.1 Universally targeted qPCR

Universal qPCR revealed a significant (p=0.001) association of an increased microbial abundance and the group with normal SHBG levels (Fig. 36). This observation was also significant after adjusting for multiple testing (adjusted p-level was p=0.0313). In addition, a negative correlation of microbial abundance and increasing SHBG levels was also observed for our whole study population (p=0.02) (Fig, 37), as well as for male (p=0.031) (Fig. 38) and female subjects (p=0.013) (Fig. 39) using the Spearman correlation index. These results were also significant after adjusting for multiple testing (our adjusted p-level was p=0.0313). While there are so far no reports on the association of microbial abundance and SHBG levels, it is presumed that the microbiome is involved in the modulation of sex hormones and can be influenced by the hosts circulating sex hormones (108). One study even reported changes in the composition of the microbiome in respect to different levels of testosterone and oestradiol (108). However, while we were not able to confirm these observations for total testosterone or free testosterone in our study, we observed a correlation of archaeal abundance and oestradiol levels.

In conclusion, it can only be noted that this association should be investigated in detail.

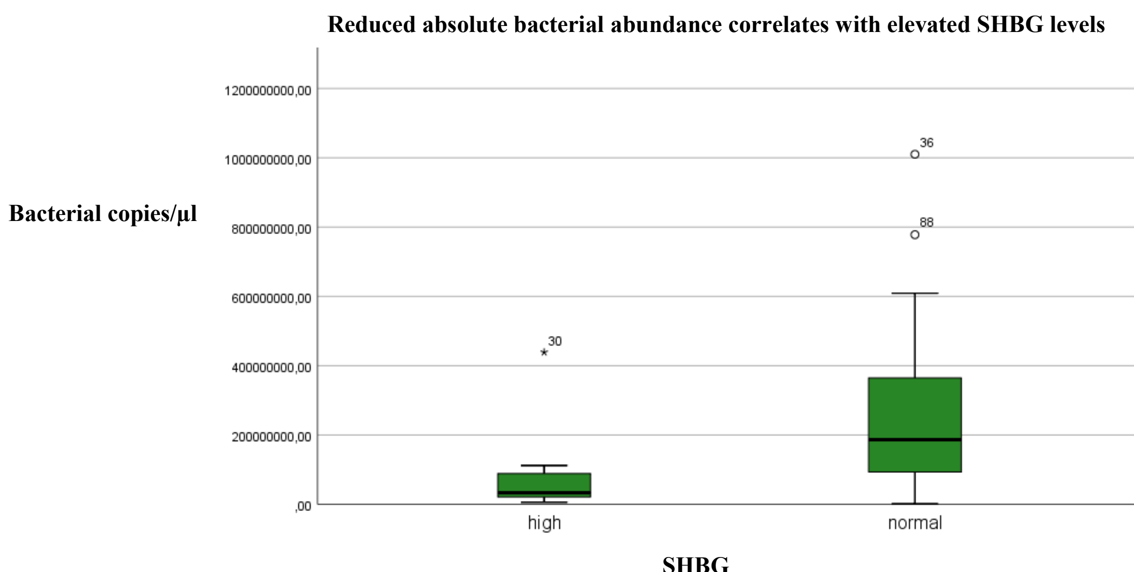

Figure 36 ***Reduced microbial abundance in subjects with high SHBG levels (both sexes) – Universal dataset***

Linear regression of absolute bacterial abundance and SHBG levels

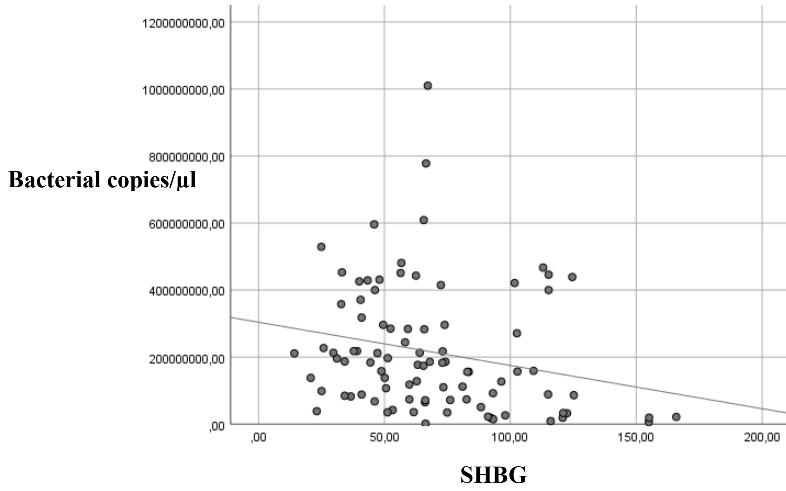

Figure 37 ***The abundance of microbiota decreases with increasing SHBG levels (both sexes) – Universal dataset***

Linear regression of absolute bacterial abundance and SHBG levels

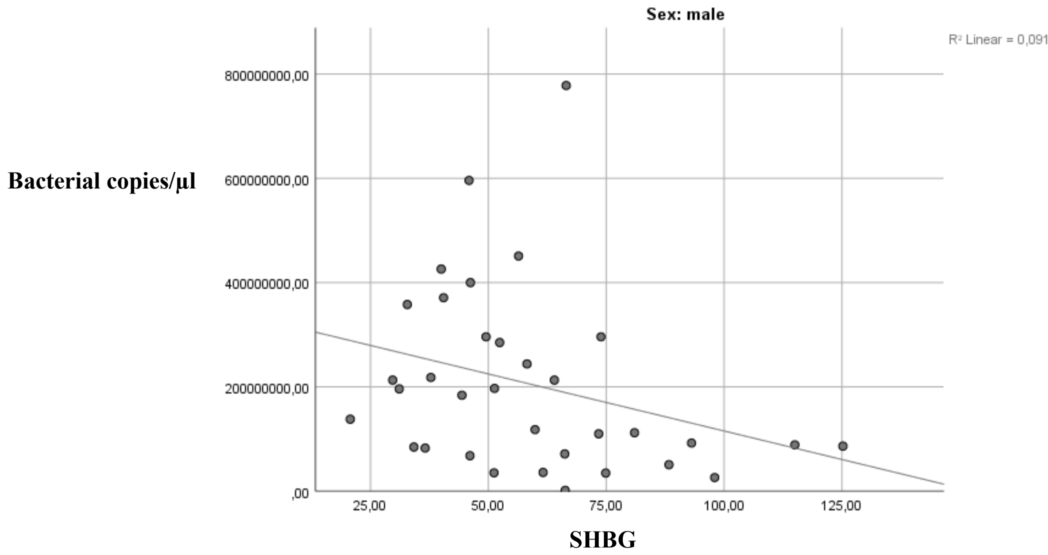

Figure 38 ***The abundance of microbiota decreases with increasing SHBG levels (male) – Universal dataset***

Linear regression of absolute bacterial abundance and SHBG levels

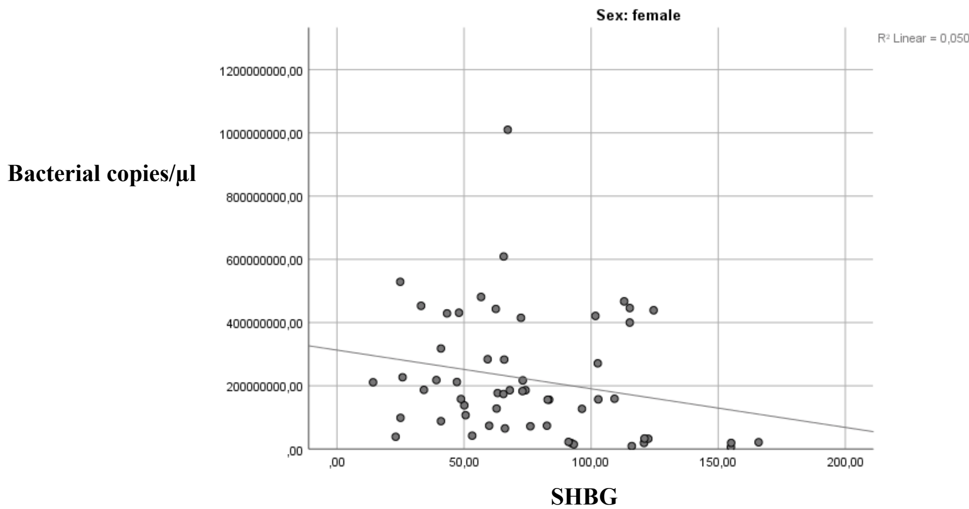

Figure 39 ***The abundance of microbiota decreases with increasing SHBG levels (female) – Universal dataset***

3.20 Thyroxine

Thyroxine levels from all subjects ranged from 9.7 – 22.7 pmol/L. Median thyroxine level was 14.35 ±1.76 pmol/L and the laboratory reference values provided by the endocrinological department from the LKH Graz ranged from 9,5 – 24 pmol/L.

3.20.1 Universally targeted qPCR

Universal qPCR revealed a significant (p=0.02) negative correlation of absolute microbial abundance and increasing thyroxine levels (Fig. 40) using the Spearman correlation index. This result was also significant after adjusting the p-level for multiple testing (our adjusted p-level was p=0.0313). While we did not observe an association of hypothyroidism, hyperthyroidism or fT_3 with the microbiome in our study, there are reports on such associations in other studies (109–111). For instance, studies have described an increased incidence of dysbiosis associated with autoimmune thyroid diseases (111). In addition, it is presumed that the microbiome plays a role in the co-occurrence of intestinal and thyroid diseases, such as coeliac disease and Hashimito's thyroiditis (111). It has also been shown that some bacteria can bind thyroid hormones and deiodize by intestinal deoidases, thus modulating the amount and ration of circulating thyroid hormones (109,111). Furthermore, an increased microbial diversity in subjects with hypothyroidism was also described, which was primarily attributed to the prolonged intestinal transit time in these subjects (109). However, as previously mentioned, we could not observe this association in subjects with hypothyroidism.

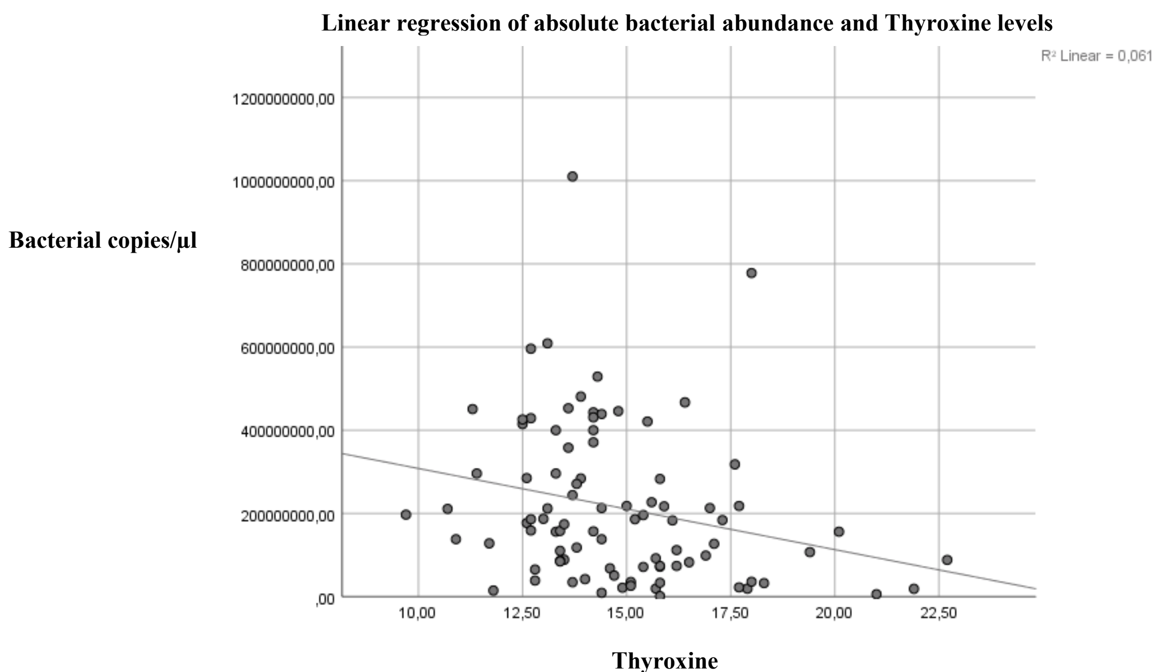

Figure 40 ***The abundance of microbiota decreases with increasing thyroxine levels – Universal dataset***

3.21 Low density lipoprotein

LDL levels from all participants ranged from 41 mg/dL to 253 mg/dL. The mean LDL level was 131.38 ±38.88 mg/dL. The laboratory reference values varied depending on the individual ESC-score of each participant (86). Cut off levels were: 0-116 mg/dL for low risk, 0-100 mg/dL for moderate risk, 0-70 mg/dL for high-risk and 0-55 mg/dL for very high-risk patients. Samples were then sorted into two different groups following their respective cut off level: normal (n=17) and high (n=76).

3.21.1 Universally targeted qPCR

Universal qPCR revealed a significant (p=0.042) positive correlation of absolute microbial abundance and increasing LDL levels using the Pearson correlation index (Fig. 41). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.025). This observation is in line with previous reports on the involvement of the microbiome in lipid metabolism (112–115). In detail, the microbiome seems to be involved in cholesterol metabolism, transport, and the promotion of certain lipoprotein classes (113). For example, it is known that certain bacterial families (*Erysipelotrichaceae* and *Lachnospiraceae*) correlate positively with increasing LDL levels (115). In contrast, probiotics with specific bacterial strains are able to reduce LDL and total cholesterol levels (114). Furthermore, there are reports suggesting that the microbiome is in fact accountable for the variation of LDL and other lipids (112).

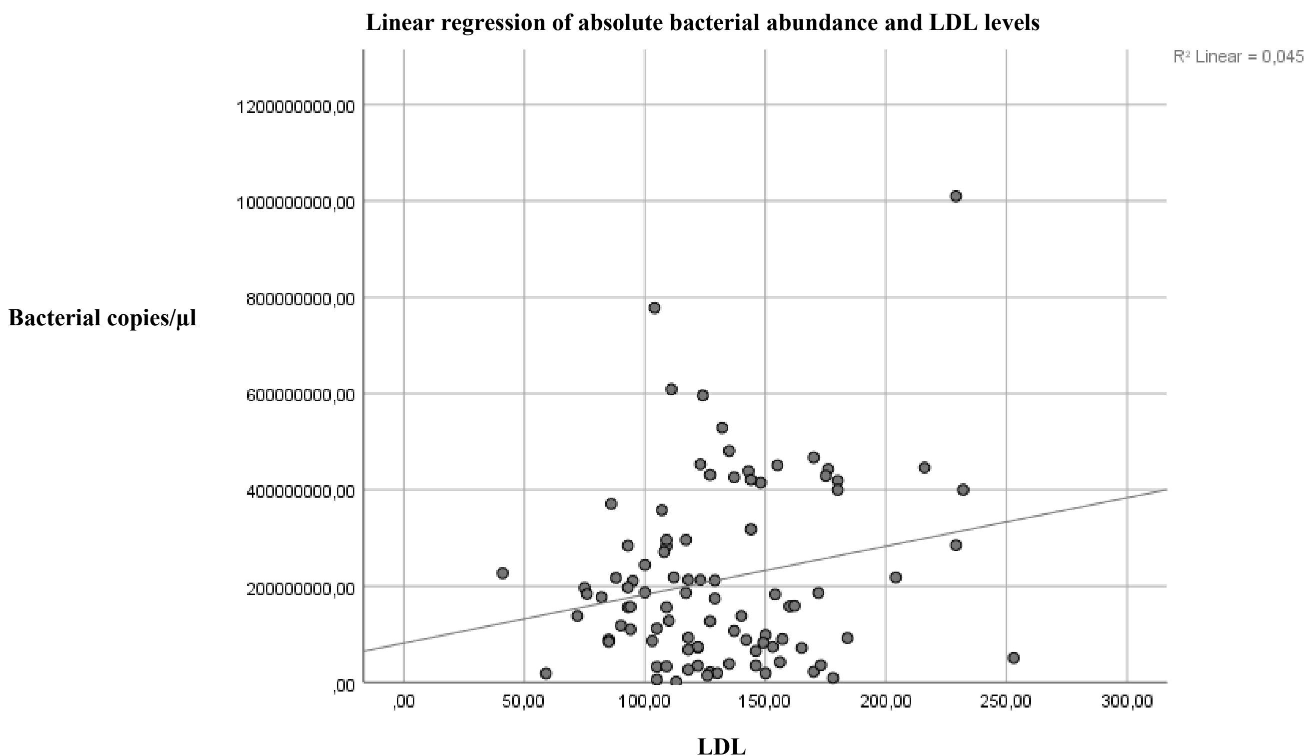

Figure 41 ***The abundance of microbiota increases with increasing LDL levels – Universal dataset***

3.22 Glucose

Glucose levels from all participants ranged from 75 mg/dL to 238 mg/dL. The mean glucose level was 97.25 ±22.04 mg/dL. The laboratory reference values ranged from 70 - 100 mg/dL. Therefore, samples were sorted into two different groups: normal (n=72) and high (n=21).

3.22.1 Universally targeted qPCR

Universal qPCR revealed a significant (p=0.027) positive correlation between absolute microbial abundance and increasing glucose levels using the Spearman correlation index (Fig. 42). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.00625). It is known that the microbiome is involved in glucose metabolism and glucose-homeostasis via the secretion of incretins (GLP-1), the production of SCFA, the bile acid metabolism and the regulation of adipose tissue (116,117). Furthermore, it has been suggested that the antidiabetic effect of metformin is partly due to a modulation of the microbiome (116). In other studies, in subjects with diabetes, improved glucose homeostasis was achieved after FMT with stool from patients who had been treated with metformin (116). In addition, diabetes and prediabetes are associated with dysbiosis and altering changes the microbial composition (116). Since microbiome and glucose homeostasis influence each other, the question of causality arises: does the disturbed glucose metabolism cause the dysbiosis or vice versa? However, to date there are no reports on a direct correlation of microbial abundance and increasing glucose levels.

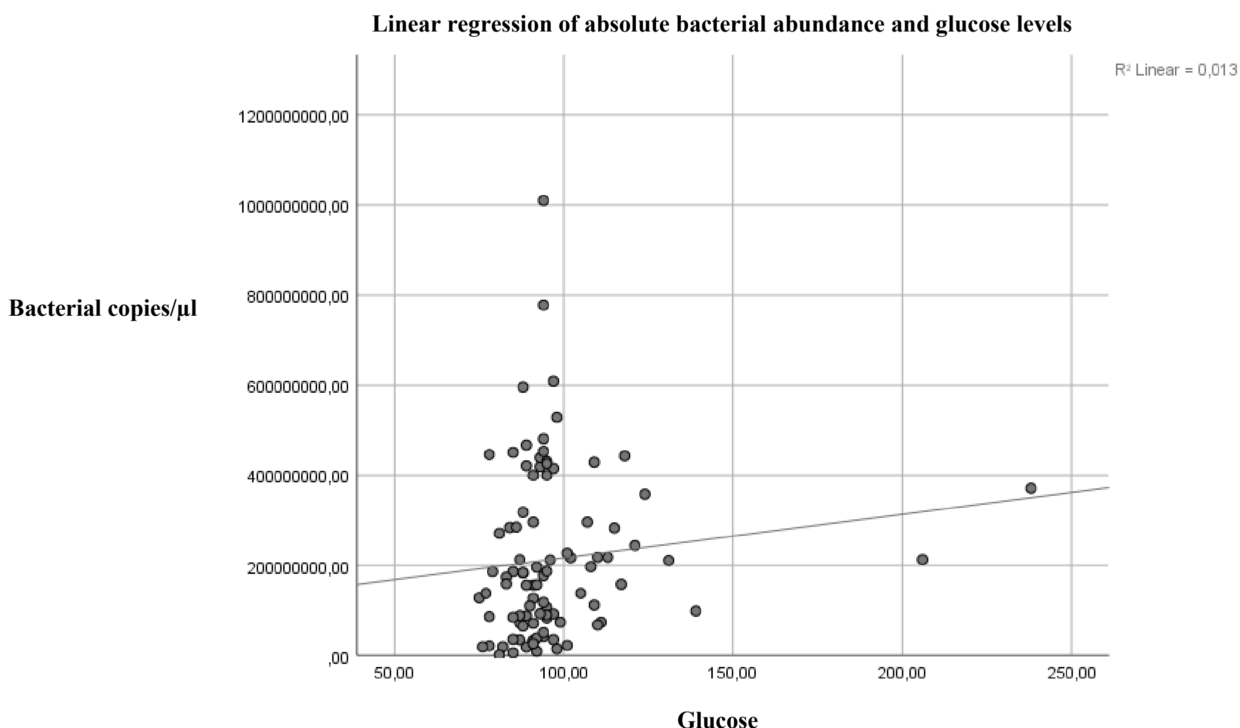

Figure 42 ***The abundance of microbiota increases with increasing glucose levels – Universal dataset***

3.23 Body mass index

The body mass index was calculated from the measured weight and the size of the participants. Overall, the BMI ranged from 19.54 kg/m² to 40.03 kg/m² and the median was 26.32 ±3.73 kg/m². Based on the WHO grouping, the samples were sorted into the respective groups according to their BMI values (79). The following groups were present among the participants: normal BMI: 18.5-24.9 (n=35), pre-obesity BMI: 25.0-29.9 kg/m² (n=38), obesity class I BMI: 30.0-34.9 kg/m² (n=12), obesity class II BMI: 35.0-39.9 kg/m² (n=7) and obesity class III BMI >40.0 kg/m² (n=1). However, the group with obesity class III was excluded from analysis due to low group size and another sample was excluded in this category because no weight and body size measurements were carried out on the participant (n=1).

3.23.1 Universally targeted qPCR

Universal PCR revealed a significant (p=0.042) association of microbial diversity and different BMI classes on the class level (Fig. 43). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.0038). In our study we observed a decreased microbial diversity in the groups with a BMI level from 30.0 – 39.9 kg/m² (obesity class I and II) when compared to the groups with a BMI level with 29.9 kg/m² or lower (normal and pre-obesity). This observation is in line with reports on microbial diversity in obese subjects (5–7,16,18,33,34). However, the entire evidence concerning this subject currently is still very contradictory, and the existence of this association therefore cannot be conclusively proven (33,34).

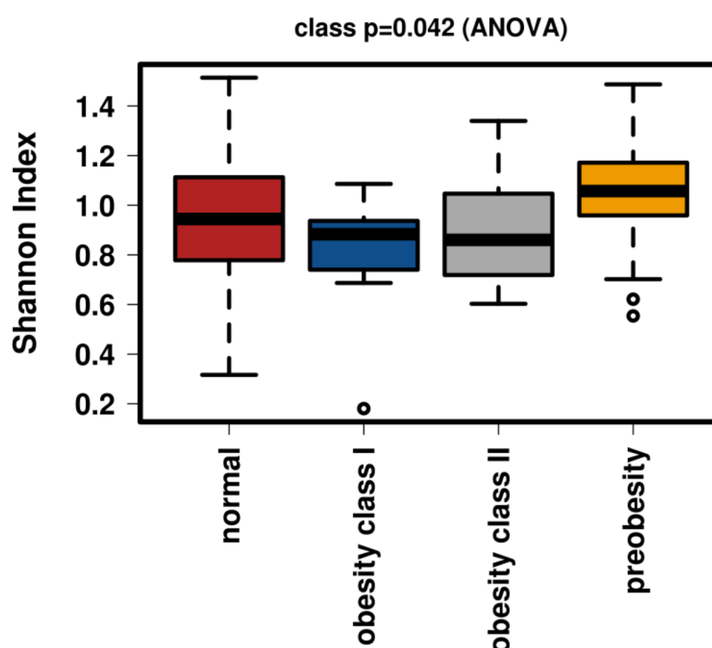

Figure 43 ***Different BMI-levels associated with changes in microbial diversity (class level) – Universal dataset***

3.24 Thigh circumference

Thigh circumference varied among all subjects between 47-86 cm. The median was 55.00 ±4.67 cm. For further testing the samples were divided into two groups, based on sex-specific cut off values established in a previous study(83). Samples with thigh circumferences <60cm were sorted in group 1 "<60cm" (n=67), whereas samples with circumferences ≥ 60cm were sorted in group 2 "≥ 60cm" (n=26). One sample was excluded in this category because no measurement was carried out for its participant (n=1).

3.24.1 Universally targeted PCR

Universal PCR revealed a significant (p=0.03) positive correlation of microbial diversity and increasing thigh circumference (Fig. 44). However, after adjusting for multiple testing, this observation was no longer significant (adjusted p-level was p=0.0038). Thigh circumference is an anthropometric marker, which is reported to correlate with body fat percentage (118). As mentioned in the chapter on possible associations of the BMI and microbial diversity (chapter 3.24), it is presumed that microbial diversity decreases with increasing BMI. This association was also reported for anthropometric marker, such as thigh circumference (118). However, as noted above, the current evidence is contradictory, which is also reflected in our study (33,34). Contrary to expectations, we observed an increase in diversity with increasing thigh circumference.

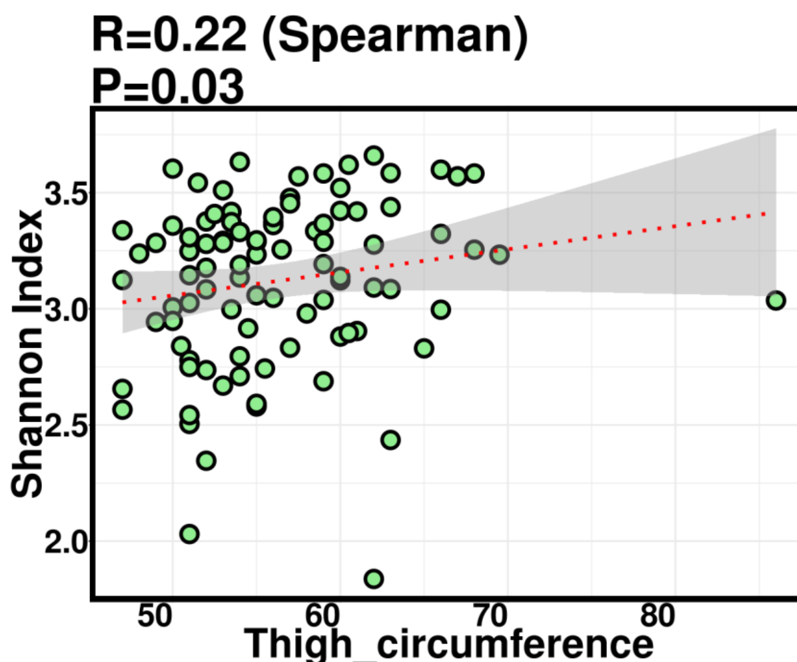

Figure 44 ***Microbial diversity associated with thigh circumference (genus level) – Universal dataset***

3.25 Age

The age of all participants ranged from 46 to 86 years. The median age was 68 ±7,8 years.

3.25.1 Universally targeted PCR

On the phylum level in the universal dataset, Firmicutes were inversely correlating with age (Fig. 45), while Bacteroidetes showed no association with age. The decrease in Firmicutes in combination with a stable abundance of Bacteroidetes leads to a decreasing Firmicutes-Bacteroidetes-ratio. This observation is in line with previous reports on the decreasing Firmicutes-Bacteroidetes-ratio with increasing age (31).

In addition, the phylum of Proteobacteria, was also significantly associated with increasing age (Fig. 46). However, after adjusting for multiple testing, these observations were no longer significant (adjusted p-level was p=0.0033). As mentioned earlier, the phylum Proteobacteria includes many opportunistic bacteria which might lead to an inflammatory microbial phenotype, thus promoting an instability of the microbiome's composition (1,7,10).

Furthermore, the observed changes in abundance could also be attributed to a change in diet, which often occurs, as described in section 1.1.5.3, with increasing age due to multiple factors (10,16).

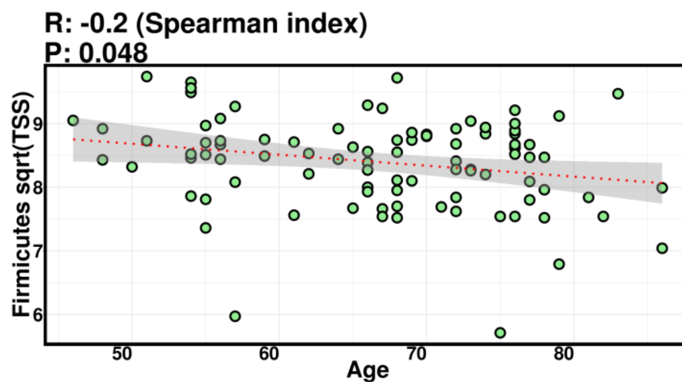

Figure 45 ***Abundance of Firmicutes decreases with increasing age – Universal dataset***

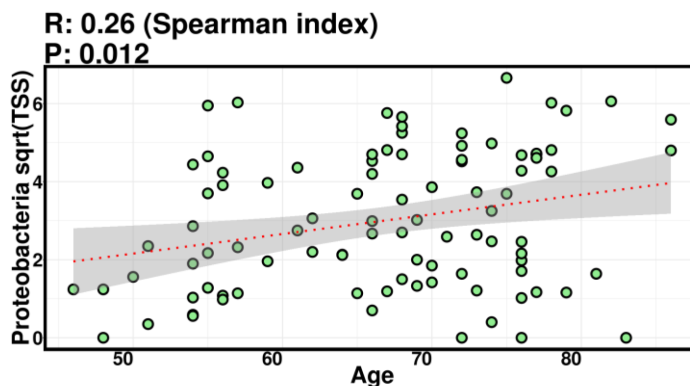

Figure 46 ***Abundance of Proteobacteria increases with increasing age – Universal dataset***

4 Discussion

4.1 Archaeal involvement in human metabolism

While the involvement of archaea in the human metabolism is long known, the exact mechanisms and pathways for interaction are currently unclear. Although our observations cannot shed light on the mechanism of these interactions either, they show nevertheless that archaea, and in this case especially the methanogens, play an important role in the human organism.

4.1.1 Archaeal involvement in insulin-glucose-homeostasis

The association of *Methanobrevibacter* and *Methanosphaera* with normal insulin levels observed in the archaea targeted PCR corroborates the role of archaea in glucose-homeostasis and archaea may therefore be pivotal in the genesis of diabetes mellitus. It therefore seems reasonable to investigate the involvement of methanogenic archaea with insulin homeostasis in detail in subjects with diabetes mellitus. Unfortunately, this was not possible in this study due to a low group size, since only four subjects had reported a diagnosed diabetes mellitus. Since the mean age of our study group is 66.85 ±9.51 years, prevalence of diabetes should be higher. This finding is discussed in detail in section 4.4.1 and section 4.4.3 on limitations.

In addition, our observations were not confirmed in the archaea targeted qPCR and we found no correlation between increasing insulin levels and relative archaeal abundance in the archaea targeted PCR. Thus, our observation might result from the unequal group size.

4.1.2 Archaeal involvement in lipid-metabolism

The observed association of *Methanobrevibacter* and triglyceride levels indicate archaeal involvement in human lipid metabolism. The decrease in relative archaeal abundance with increasing triglyceride levels could indicate an inhibition of archaeal growth by high triglyceride levels. On the other hand, it could also indicate that archaea-heavy microbiomes can metabolise the existing triglycerides better and more effectively to achieve lower lipid levels.

However, this observation was only made in the archaea targeted PCR and not confirmed in the archaea targeted qPCR. Furthermore, we could not detect any associations with other markers of lipid metabolism such as cholesterol, LDL, HDL or the reference levels for triglycerides.

4.2 Association of *Methanosphaera* and different characteristics

Our study revealed multiple significant associations regarding signatures of the archaeon *Methanosphaera*. However, *Methanosphaera* was found in only seven subjects, and thus the reliability of these observations is limited. This raises the question why we found so few samples with *Methanosphaera*. One possibility could be the use of faecal samples for microbial analysis, since archaea are highly associated with the intestinal mucosa and thus faecal samples might only display a fraction of the archaeal community. Nevertheless, most of these results are plausible and should be examined in future studies.
This sub-section highlights the associations found in relation to *Methanosphaera* and attempts to assess them on the basis of the current literature.

4.2.1 Statins

The archaeal cell membrane is composed of isoprenoid-glycerol-ethers, which are synthesized by the HMG-CoA-reductase and thus it has been reported, that statins can inhibit archaeal growth by disturbing membrane synthesis. However, this was only observed for the statins Lovastatin and Mevastatin and not for the more commonly used Atorvastatin and Simvastatin. In addition, the inhibiting effects of Lovastatin and Mevastatin on the growth of *Methanobrevibacter* and suppression of its methane production have only been observed *in vitro* (44). However, it might be possible, that Lovastatin and Mevastatin have direct inhibiting effects on the gene expression of *Methanobrevibacter* which are unrelated to the inhibition of the HMG-Co-A-reductase (44). Nevertheless, we observed a significant association of *Methanosphaera* and the group without statin usage in the archaea targeted PCR. In addition, this observation was not made for *Methanobrevibacter* which prove to be unaffected by the statins used in our subjects. Since *Methanosphaera* and *Methanobrevibacter* have a similar basic cell wall structure and are thus in principle susceptible to statins, (as has been shown at least for *Methanobrevibacter* in *in vitro* studies(44,47)), the question arises as to why only *Methanosphaera* is affected by a statin induced inhibition in our study. A possible explanation could be, that *Methanobrevibacter* is not susceptible to the dominating statins in our study group (Atorvastatin and Simvastatin), since *in vitro* test revealed no inhibition of growth via these statins (44). This could indicate, that *Methanosphaera* is more susceptible to statins and might even be inhibited by Simvastatin and Atorvastatin, which show no effect on *Methanobrevibacter*. Furthermore, the dosage of the used statins and possible effective intestinal statin-levels are unknown. In addition, we could not observe an association of archaea and the use of statins in the archaea

targeted qPCR. However, since this effect only affected *Methanosphaera* in the archaea targeted PCR and was also only observed in a small number of subjects, it is quite possible that this finding is masked by other archaea in the archaea targeted qPCR and is therefore not detectable.
Another finding was the significant association of *Methanosphaera* and hypercholesterolemia in our archaea targeted PCR. However, because this group is based on the subjects' medical history and current medication, it includes all subjects who were treated with statins and is only extended by four additional subjects who were not receiving statins. Nevertheless, these observations require more confirmation and therefore the effect of statins on archaea, especially *Methanosphaera*, should be examined closer in future studies. In addition, our observations might be the result of the generally low prevalence of *Methanosphaera* in our study and small group sizes.

4.2.2 Appendectomy

The observed association of *Methanosphaera* and the group without an appendectomy was quite surprising. While it has been reported by Pausan et al., that *Methanosphaera* are associated with the appendix in high relative abundances in appendix samples, there are to date no reports of an association of *Methanosphaera* and appendectomy (119). However, recent reports suggest that the appendix acts as a stable housing for archaea, as it is not so heavily affected by intestinal transit (98). This could also apply to patients with diverticulosis, whose diverticula provide a safe habitat for archaea. Patients with diverticulosis are associated with elevated breath methane, which in turn suggests an increased intestinal colonisation with methanogens (61).
However, it is debatable whether diverticula promote methanogenic colonisation *per se* or whether the production of methane by methanogens leads to an increase in intraluminal pressure and constipation, which in turn, might promote the development of diverticulosis.

Another study showed that patients who underwent appendectomy were less likely to be methane-producers when breath methane was analysed (98). There are several possible interpretations of this observation, but no definitive explanation. One hypothesis is that the loss of the appendix leads to a decrease in methane-producing archaea and thus to a reduced methane excretion (98). However, since the appendix only makes up a fraction of the surface area of the intestine and the majority of microbes are found in the mucosa of the colon, this explanation seems very unlikely (98). More likely, however, is the assumption that methane

producers harbour a particular composition of the microbiome which might prevent the development of appendicitis (98). In addition, the proposed function of the appendix as a safe house for the intestinal microbiome might enable re-inoculation of the large intestine and terminal ileum following intestinal perturbations (120). The exact function of the appendix may be unknown, but it definitely plays a role in maintaining intestinal homeostasis. Whether this role is beneficial or detrimental, however, cannot be conclusively determined. For example, an ambivalent effect of appendectomy has been described in inflammatory bowel disease (98). Although appendectomy seems to reduce the risk of developing ulcerative colitis (121), it also appears to increase the risk of developing crohn's disease (122).

However, it is important to note, that our observation was not made for *Methanobrevibacter* and might be the result of the low prevalence of *Methanosphaera* in our study group.

4.2.3 PPI

Archaea have previously been associated with highly diverse microbiomes and might even be indicative for the microbiome's health status (34). Thus, the observed association of *Methanosphaera* and the group without PPI usage might be the result of a dysbiosis induced by PPIs. However, this observation was not made in our study when examining *Methanobrevibacter*, which showed no association with PPI usage. Thus, this observation might be due to the low prevalence of *Methanosphaera* in our study population. Nevertheless, we observed an increased microbial diversity in the group with PPI usage, which might disturb or inhibit archaeal growth due to a changed microbial composition.

4.2.4 Bilirubin

Bilirubin has been reported to enact a selective pressure on microbes and can have a disrupting effect on the cell membrane of gram-positive bacteria, while protecting gram-negative bacteria from ROS (96,97). Bilirubin might thereby modulate microbial diversity and thus archaeal abundance. Therefore, this could be an explanation for the observed association of *Methanosphaera* and normal bilirubin levels. However, as was the case in our other observations regarding *Methanosphaera*, this observation was not made for *Methanobrevibacter* and might be the result of the low prevalence of *Methanosphaera* in our study group.

4.2.5 Hypertension

Hypertension can be caused by a multitude of different factors, of which some can be attributed or aggravated by the microbiome. One major pathway might be the production of TMAO, which has pro-atherosclerotic effects and thus might lead to hypertension and cardiovascular diseases (2). In addition, SCFA are another important product of bacterial fermentation, which is involved in many pathways, of which some are also presumed to be linked to hypertension (2). Since some archaea are capable of degrading TMAO as well as enhancing bacterial fermentation and thus increasing the SCFA production, they might influence blood pressure and hypertension.

The observed association of *Methanosphaera* and the group without hypertension might thereby represent this blood pressure modulation. In addition, it is also corroborated by the observed association of *Methanobrevibacter* and high-normal blood pressure, although this observation was not significant after adjusting for multiple testing.

Nevertheless, these findings might be due to the low prevalence of *Methanosphaera* in our study population and need confirmation in future studies.

4.3 Archaeal vs. universal primer

Since archaea and bacteria are fundamentally different in some biological aspects, their detection by using the same primers has been a challenge. This is demonstrated by our study, when the same category is examined and compared in the archaeal and universal dataset (Fig. 47 and Fig. 48). Hereby, archaeal PCR enables a higher detection of archaeal signatures and thus allows a more detailed and differentiated analysis.

A total of almost 70% of our subjects were found to have archaea, in which they accounted for about 0.04% of the faecal microbiome.

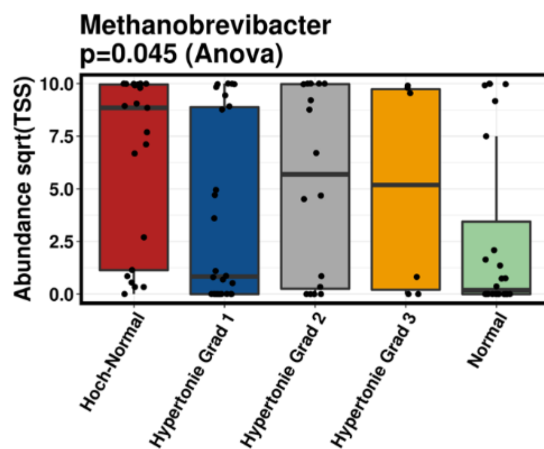

Figure 47 **Archaeal PCR**

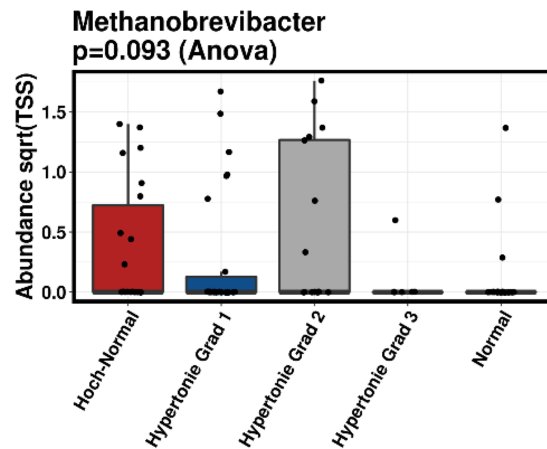

Figure 48 **Universal PCR**

4.4 Limitations

Our study is a pilot study for the actual GSHA, which is planned to include 3000 subjects. It served as a test run to detect possible problems and sources of error as well as to determine how the collected data can be used.
Despite our best efforts, our study revealed some flaws, which might result in bias. These limitations will be discussed in this section.

4.4.1 Study design

The GSHA is a cross-sectional study involving data collected by a wide variety of departments. It is therefore a comprehensive collection of a variety of data from 100 subjects, which should allow a widespread use of these data in future studies. However, since many different departments are involved in the data collection, important information can be lost. This was the case, for example, for the collected body temperature values, which did not include the body region where the temperature was measured.
In addition, due to the requirements of the GSHA, most subjects were of an age of 50 years and older, meaning our study population represents mostly older adults and elders.
Since Graz residents could not be randomly selected and written to, the study participants had to apply for the study themselves. The required number of participants was then randomly selected from the applicants.
Therefore, there is a high probability that our study population is biased by the restriction to voluntary participants, who may be paying close attention to their health or who already have health problems and therefore are not really representative for the population of Graz.

4.4.2 Metadata

Most of the medical data was collected via the patients' self-reports, which also led to incorrect or incomplete data. For example, there were discrepancies between reported medical conditions and current medication of some subjects. Therefore, when creating the metadata, we sometimes had to guess which information was correct; in case of doubt, the decision was always made in favour of an existing disease or medical condition. Furthermore, the drug names were repeatedly misspelled, which made it difficult to create our metadata.

4.4.3 Underrepresentation of endemic diseases

When creating the metadata, it was striking that certain diseases, which are endemic in Austria and Europe, were underrepresented in our study population. For example, we registered only three subjects with diabetes mellitus (n=3), although prevalence in this age group (45 – >75 years) ranges from 5.1 – 21.2 % (123), indicating that diabetes in most subjects was undiagnosed, not reported or not present due to a selection bias. This also applies to the prevalence of diverticulitis (n=3) in our study population, which should have been much higher. However, we cannot rule out the possibility, that these diseases were undiagnosed and thus underrepresented in our study.

4.4.4 Group size

Overall, the group size was low, which limits the interpretation of our results and their significance. We excluded groups with less than five percent of the total number of participants from the analysis. Furthermore, most significant observations were made with unequal groups. For example, our most significant observation, regarding the insulin level and an association with archaeal abundance was made with two very disproportionate groups (n=5 vs. n=84).

4.4.5 Archaeal detection

While we observed archaeal signatures in our archaeal dataset in about 70% of all subjects (n=65 in qPCR and n=67 in PCR), we only found 4 subjects who harboured *Methanomassiliicoccus* and 7 who harboured *Methanosphaera*. Due to its low representation, we did not perform any analyses for *Methanomassiliicoccus*. However, due to the low number of subjects with *Methanosphaera* it is debatable whether significant observations made on *Methanosphaera* in this study are in fact valid associations or just bias. Interestingly, when significant associations were observed, all subjects with *Methanosphaera* were in the same group, however, this is probably due to the low number of subjects harbouring *Methanosphaera*.

Another interesting fact regarding the presence of archaea in archaeal PCR was that there was a large number of subjects with high archaeal abundance as well as a large number of subjects with low to no archaeal abundance. However, there are only few subjects with an archaeal abundance that could be sorted in between those two extremes.

4.4.6 Faecal samples

Due to the high number of participants in this study and the intention to increase this number to 3000 subjects, it was necessary to select a method for the collection of microbial samples that can be carried out quickly and easily on the subjects. Therefore, we used faecal samples for the microbial analysis, as the collection of intestinal biopsies during an endoscopy is an invasive and time-consuming procedure which is difficult to perform in the context of this study and cannot be ethically justified without a reason for endoscopy. However, archaea are highly associated with the intestinal mucosa, which is evident by their extensive repertoire of adhesin-like-proteins, that are used for adhesion and interaction. Thus, faecal samples might not represent the intestinal microbiome and archaeome correctly.

4.4.7 Multiple testing

Due to the vast number of independent tests for each variable (111 different categories), we performed p-level correction for multiple testing using the Benjamin-Hochberg-correction for each group of tests. Each of the 111 categories was assigned to a "super" – category: Firstly, anthropometrics containing anthropometric numeric groups (n=15), anthropometric groups (n=13). Secondly, medical data encompassing cardiovascular system (n=7), gastrointestinal system (n=5), other medical conditions (n=2) and medication (n=6). Third blood tests encompassing electrolytes numeric (n=4), electrolyte groups (n=0), liver specific blood tests numeric (n=6), liver specific blood tests groups (n=4), metabolic markers numeric (n=8), metabolic markers groups (n=8), lipid markers numeric (n=4), lipid markers groups (n=3), hormone laboratory numeric (n=15) and lastly hormone laboratory groups (n=13). Individual adjusted levels for significance were eventually calculated using the Benjamin-Hochberg-method as listed in table_14.

5 Conclusion

Although the existence of archaea has been known for quite a considerate time and their presence in and on humans has been described several times, still little is known about these unique microorganisms. Therefore, it was a difficult task to put our observations in relation to the existing knowledge on archaea.

Nevertheless, we observed new associations, which have not yet been reported for archaea and different biological traits, such as insulin levels, triglyceride levels, bilirubin levels, SHBG levels, hypertension, statin or PPI usage and the absence of an appendectomy. In addition, we were able to show that about 70% of all subjects harboured archaea. Another interesting discovery was the absence of *Methanosphaera* in subjects with statin usage, since the inhibition of archaeal growth via statins has only been reported in vitro for *Methanobrevibacter* and the statins Lovastatin and Mevastatin (44). However, our group sizes were unequal and low for most analyses and thus these observations have to be interpretated with care. Overall, it is striking that we have identified many associations related to metabolic markers, indicating the strong involvement of archaea in the human metabolism. In addition, we could not find any correlation between the abundance of archaea and obesity, neither in the case of BMI nor in the case of body size phenotype. However, as noted before, the existing reports on the association of archaea and obesity are contradictory.

Although it was one of our goals to detect non-methanogenic archaea, we were not successful in this study. However, this could be attributed to the use of faecal samples instead of biopsies.

In conclusion, I would like to point out that this thesis was carried out in the course of a pilot study and was intended to explore possible links between archaea and humans, which could be further examined in future studies. In this respect, our observations, especially the ones concerning the relationship between insulin and the abundance of archaea should be examined in larger groups. Overall, more research is needed to explore the many facets of archaeal modulation and involvement in the microbiome and human metabolism.

For instance, the investigation of archaeal involvement in insulin- and glucose-homeostasis could be investigated and compared in healthy subjects and type 2 – diabetics. Additionally, the observed inhibitory effect of statins (especially Simvastatin and Atorvastatin) on *Methanosphaera* should be explored in more detail.

Another interesting aspect which requires further investigation is the role of the appendix and its influence on the composition of the archaeome, as well as methane production.

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7 Supplements

The listed tables (table 13-16) represent the complete extracted metadata, before groups were excluded due to low group size.

Table 15 ***Complete metadata (part 1)***

Sample	Age	Sex	Height	Weight	BMI-level	Neck circumference	Waist circumference	Hip circumference	WHR	Thigh circumference	Temperature	Mean systolic RR	Mean diastolic RR	Time to graduation	Time to graduation Categories	Age categories	Age-categories approximate	Height Categories	BMI Categories	Body Size Phenotype	Neck circumference (Saudi)	Neck circumference Categories	Waist-Hip Circumference (WHO)	Waist-Hip Circumference (DGSP)	Thigh circumference Categories	Handedness	Graduation
GSHA-1	56	male	175.5	79	25.65	40	90	101	0.89	53	36.8	150.00	90.00	16	16-20 Years	50-65	under 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	normal	under 60 cm	Right	FH/University
GSHA-2	77	female	172	76	25.69	34	96	107	0.90	61	36.5	134.00	82.50	11	10-15 Years	66-80	over 65	170-174 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Lehrabschluss
GSHA-3	54	female	165	63	23.14	33	77	96	0.80	55	37.1	114.50	73.00	13	10-15 Years	50-65	under 65	165-169 cm	normal	normal weight, metabolically healthy	obese	normal	normal	overweight	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-4	78	male	173	63	21.05	39	81	93	0.87	49	36.4	170.67	85.33	12	10-15 Years	66-80	over 65	170-174 cm	normal	normal weight, metabolically abnormal	obese	overweight	normal	normal	under 60 cm	Right	Matura
GSHA-5	72	male	178	85	26.83	41	93	100	0.93	56	36.9	132.00	85.00	12	10-15 Years	66-80	over 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	Lehrabschluss
GSHA-6	68	male	178	76	23.99	40	91	99	0.92	55	36.8	139.50	82.50	11	10-15 Years	66-80	over 65	175-179 cm	normal	normal weight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	Lehrabschluss
GSHA-7	74	female	161	56	21.60	31	82	99	0.83	52	36.8	135.33	83.00	12	10-15 Years	66-80	over 65	160-164 cm	normal	normal weight, metabolically abnormal	normal	normal	normal	overweight	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-8	78	male	176.6	69	22.12	37	85	101	0.84	51	36.5	134.00	82.33	13	10-15 Years	66-80	over 65	175-179 cm	normal	normal weight, metabolically abnormal	normal	overweight	normal	normal	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-9	76	male	172	88	29.75	43	112	109	1.03	55	37	124.00	72.67	5	under 10 years	66-80	over 65	170-174 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Both sides	Lehrabschluss
GSHA-10	77	female	164	69	25.65	36	85	110	0.77	60	36.6	114.67	78.33	8	under 10 years	66-80	over 65	160-164 cm	preobesity	overweight, metabolically healthy	obese	normal	normal	normal	over 60 cm	Right	Volksschule/Hauptschule
GSHA-11	72	male	187	121.9	34.86	46	115	118	0.97	86	36.6	156.00	87.00	12	10-15 Years	66-80	over 65	NA	obesity class I	obese, metabolically abnormal	obese	overweight	obese	overweight	over 60 cm	Right	Lehrabschluss
GSHA-12	66	female	166.6	75.6	27.24	36	97	108	0.90	60	37	166.33	83.00	11	10-15 Years	66-80	over 65	165-169 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Lehrabschluss
GSHA-13	82	female	159	65	25.71	36	89	99	0.90	52	36.6	128.00	71.50	11	10-15 Years	over 80	over 65	155-159 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Both sides	Lehrabschluss
GSHA-14	81	female	152	72	31.16	37	114	103	1.11	47	36.4	179.50	90.00	8	under 10 years	over 80	over 65	150-154 cm	obesity class I	obese, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Both sides	Volksschule/Hauptschule
GSHA-15	83	male	179.6	95.1	29.48	43	116	108	1.07	51	36.7	120.00	70.50	18	16-20 Years	over 80	over 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Right	FH/University
GSHA-16	57	male	171	113	38.64	44	128	115	1.11	59	36.8	135.50	95.50	11	10-15 Years	50-65	under 65	170-174 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-17	54	female	161.5	78	29.91	35	99	109	0.91	62	37.1	121.00	78.00	11	10-15 Years	50-65	under 65	160-164 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Lehrabschluss
GSHA-18	67	female	159	92	36.39	38	120	125	0.96	66	36.6	137.50	87.50	8	under 10 years	66-80	over 65	155-159 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Volksschule/Hauptschule
GSHA-19	70	male	166	67	24.31	39	92	101	0.91	54	36.9	168.67	85.67	19	16-20 Years	66-80	over 65	165-169 cm	normal	normal weight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	FH/University
GSHA-20	59	male	183.3	83	24.70	41	101	100	1.01	55	36.3	122.33	83.33	25	over 20 Years	50-65	under 65	180-184 cm	normal	normal weight, metabolically healthy	obese	overweight	obese	adipositas	under 60 cm	Right	FH/University
GSHA-21	78	female	159.5	51	20.05	33	80	92	0.87	47	36.4	162.33	90.33	20	16-20 Years	66-80	over 65	155-159 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-22	55	female	173	91	30.41	35	100	118	0.85	65	36.7	116.00	85.00	16	16-20 Years	50-65	under 65	170-174 cm	obesity class I	obese, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	FH/University
GSHA-23	73	male	175	68	22.20	38	87	97	0.90	49	36.5	170.33	106.00	19	16-20 Years	66-80	over 65	175-179 cm	normal	normal weight, metabolically abnormal	obese	overweight	normal	normal	under 60 cm	Right	FH/University
GSHA-24	62	female	163.5	55	20.57	33	72	94	0.77	53.5	36.4	154.50	87.00	12	10-15 Years	50-65	under 65	160-164 cm	normal	normal weight, metabolically healthy	obese	normal	normal	normal	under 60 cm	Right	Lehrabschluss
GSHA-25	55	male	177.9	94.3	29.80	41	105	110	0.95	55	36.7	148.33	92.67	11	10-15 Years	50-65	under 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Left	Volksschule/Hauptschule
GSHA-26	64	female	162.2	51.4	19.54	32.5	73.6	90	0.82	47	36.5	111.67	78.67	14	10-15 Years	50-65	under 65	160-164 cm	normal	normal weight, metabolically healthy	obese	normal	normal	overweight	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-27	48	female	154	46.4	19.56	30	66.6	89	0.75	48	37	140.33	94.00	16	16-20 Years	under 50	under 65	150-154 cm	normal	normal weight, metabolically healthy	normal	normal	normal	normal	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-28	76	female	165.3	64	23.42	33.5	86	100	0.86	53.5	36.2	139.00	82.50	10	10-15 Years	66-80	over 65	165-169 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-29	75	female	159.2	61.4	24.23	35.7	91	100	0.91	53	36.5	166.00	83.50	11	10-15 Years	66-80	over 65	155-159 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Lehrabschluss
GSHA-30	72	female	NA	NA	NA	NA	NA	NA	NA	NA	NA	132.33	81.00	10	10-15 Years	66-80	over 65	NA	NA	NA	NA	NA	NA	NA	NA	Right	Hohere Schule ohne Matura
GSHA-31	51	male	174.5	72	23.65	36.5	99	100	0.99	50	37.1	146.50	92.00	12	10-15 Years	50-65	under 65	170-174 cm	normal	normal weight, metabolically abnormal	normal	normal	obese	overweight	under 60 cm	Right	Lehrabschluss
GSHA-32	79	female	164.5	74	27.35	38	98.5	106	0.93	54.5	36.3	128.50	70.50	10	10-15 Years	66-80	over 65	160-164 cm	preobesity	overweight, metabolically healthy	obese	overweight	obese	adipositas	under 60 cm	Right	Volksschule/Hauptschule
GSHA-33	59	female	157	55	22.31	32	73	95	0.77	51	36.8	111.00	74.50	16	16-20 Years	50-65	under 65	155-159 cm	normal	normal weight, metabolically healthy	normal	normal	normal	normal	under 60 cm	Right	FH/University
GSHA-34	54	male	172.5	74	24.87	38	87	97	0.90	52	36.9	119.67	76.67	12	10-15 Years	50-65	under 65	170-174 cm	normal	normal weight, metabolically healthy	obese	overweight	normal	normal	under 60 cm	Right	Lehrabschluss
GSHA-35	73	male	164.6	73.1	26.98	39.5	105	100	1.05	50.5	37.1	146.50	93.00	24	over 20 Years	66-80	over 65	160-164 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Right	FH/University
GSHA-36	54	female	160.5	76.9	29.85	37	100	110	0.91	66	36.6	106.33	72.33	15	10-15 Years	50-65	under 65	160-164 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-37	69	male	172	72	24.34	40	90	97	0.93	50	36.7	155.33	91.00	19	16-20 Years	66-80	over 65	170-174 cm	normal	normal weight, metabolically healthy	obese	overweight	normal	overweight	under 60 cm	Right	FH/University
GSHA-38	76	female	173.9	70.1	23.18	35.5	88	102	0.86	56	36.8	137.00	88.33	10	10-15 Years	66-80	over 65	170-174 cm	normal	normal weight, metabolically healthy	obese	normal	obese	adipositas	under 60 cm	Both sides	Hohere Schule ohne Matura
GSHA-39	68	female	165.6	81.2	29.61	34.5	107	103	1.04	59	36.8	178.33	100.33	17	16-20 Years	66-80	over 65	165-169 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Matura
GSHA-40	55	female	152.5	71	30.53	34	93	104	0.89	60	37.2	140.00	98.50	11	10-15 Years	50-65	under 65	150-154 cm	obesity class I	obese, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura
GSHA-41	46	female	170.4	66.7	22.97	31.5	81	104	0.78	59	37	131.67	92.67	20	16-20 Years	under 50	under 65	170-174 cm	normal	normal weight, metabolically abnormal	normal	normal	normal	normal	under 60 cm	Right	FH/University
GSHA-42	48	female	160.9	57.9	22.36	33.5	84	96	0.88	54	36.9	116.00	80.50	12	10-15 Years	under 50	under 65	160-164 cm	normal	normal weight, metabolically healthy	obese	normal	obese	adipositas	under 60 cm	Right	Matura
GSHA-43	77	male	171	85	29.07	41	110	101	1.09	52	36.2	123.50	77.00	13	10-15 Years	66-80	over 65	170-174 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-44	54	male	182.4	87	26.15	40	100	105	0.95	57	36.6	152.33	100.00	13	10-15 Years	50-65	under 65	180-184 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	Matura
GSHA-45	66	female	153	66	28.19	37	98.5	92.5	1.06	51	37.1	168.00	93.50	11	10-15 Years	66-80	over 65	150-154 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Right	Lehrabschluss
GSHA-46	64	female	157.5	49.9	20.12	32	77	91	0.85	50	36.3	122.50	81.50	32	over 20 Years	50-65	under 65	155-159 cm	normal	normal weight, metabolically healthy	normal	normal	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-47	70	male	177.4	85.8	27.26	43	99.5	103	0.97	57.5	36.8	178.00	88.00	13	10-15 Years	66-80	over 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	overweight	under 60 cm	Right	Matura
GSHA-48	69	female	161.6	65	24.89	32.5	91.5	101	0.91	56	36.5	154.00	93.50	15	10-15 Years	66-80	over 65	160-164 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-49	71	female	165	66.5	24.43	35	99	103	0.96	54	36.4	140.67	80.33	13	10-15 Years	66-80	over 65	165-169 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-50	74	female	166	98.5	35.75	39	114	124	0.92	66	36.1	147.00	74.50	13	10-15 Years	66-80	over 65	165-169 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-51	61	female	161	75.8	29.24	35	92	108	0.85	62	36.4	132.00	73.50	12	10-15 Years	50-65	under 65	160-164 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Lehrabschluss
GSHA-52	66	female	158.3	63.9	25.50	33.5	81	103	0.79	60	36.4	129.67	75.33	8	under 10 years	66-80	over 65	155-159 cm	preobesity	overweight, metabolically abnormal	obese	normal	normal	normal	over 60 cm	Right	Volksschule/Hauptschule
GSHA-53	68	male	169.8	77.6	26.91	40	97	98	0.99	53	36.7	174.67	98.67	8	under 10 years	66-80	over 65	165-169 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	overweight	under 60 cm	Left	Volksschule/Hauptschule
GSHA-54	67	male	176.4	83	26.67	43.5	99	102	0.97	55.5	36.7	193.50	89.00	20	16-20 Years	66-80	over 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	overweight	under 60 cm	Right	FH/University
GSHA-55	70	female	160	86.5	33.79	40	109	118	0.92	63	36	139.33	87.33	13	10-15 Years	66-80	over 65	160-164 cm	obesity class I	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Matura
GSHA-56	68	female	167.3	67.3	24.04	34	85	102	0.83	56	36.9	137.00	85.00	11	10-15 Years	66-80	over 65	165-169 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	overweight	under 60 cm	Right	Lehrabschluss
GSHA-57	76	female	162.2	96.6	36.72	37.5	119	126	0.94	67	36.2	108.50	64.50	12	10-15 Years	66-80	over 65	160-164 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Both sides	Hohere Schule ohne Matura
GSHA-58	74	female	165.4	72	26.32	35.5	104	104	1.00	58.5	36.9	186.67	106.00	14	10-15 Years	66-80	over 65	165-169 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	FH/University
GSHA-59	68	male	184.4	91	26.76	43	101.5	104	0.98	57	36.5	138.00	76.50	16	16-20 Years	66-80	over 65	180-184 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	overweight	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-60	77	female	168.3	98.8	34.88	39	110	123	0.89	68	36.9	160.00	89.00	11	10-15 Years	66-80	over 65	165-169 cm	obesity class I	overweight, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura
GSHA-61	57	female	173.4	63.6	21.15	36.5	73.5	93.5	0.79	52	36.8	125.67	75.67	12	10-15 Years	50-65	under 65	170-174 cm	normal	normal weight, metabolically healthy	obese	normal	normal	normal	under 60 cm	Right	Volksschule/Hauptschule
GSHA-62	66	female	163.6	64.6	24.14	33	87	100	0.87	57	37	143.00	84.67	11	10-15 Years	66-80	over 65	160-164 cm	normal	normal weight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-63	55	female	172	76.5	25.86	34	81	103	0.79	63	36.5	119.67	79.00	12	10-15 Years	50-65	under 65	170-174 cm	preobesity	overweight, metabolically healthy	obese	normal	normal	normal	over 60 cm	Right	Hohere Schule ohne Matura
GSHA-64	56	male	181	82	25.03	38	90	101	0.89	51	36.6	132.00	86.67	13	10-15 Years	50-65	under 65	180-184 cm	preobesity	overweight, metabolically healthy	obese	overweight	normal	normal	under 60 cm	Right	Lehrabschluss
GSHA-65	54	male	193	103	27.65	43	108	114	0.95	61	36.5	115.33	75.33	9	under 10 years	50-65	under 65	NA	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	over 60 cm	Right	Volksschule/Hauptschule
GSHA-66	66	male	181	77.7	23.72	39	88	100	0.88	56.5	37.1	139.33	82.00	19	16-20 Years	66-80	over 65	180-184 cm	normal	normal weight, metabolically abnormal	obese	overweight	normal	normal	under 60 cm	Right	FH/University
GSHA-67	56	female	166	94	34.11	38	109	124	0.88	68	36.5	115.67	81.33	13	10-15 Years	50-65	under 65	165-169 cm	obesity class I	obese, metabolically normal	obese	overweight	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura
GSHA-68	68	female	160	92.9	36.29	38	107	121.5	0.88	69.5	36.9	144.50	95.50	14	10-15 Years	66-80	over 65	160-164 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Matura und Kolleg bzw. Akademie
GSHA-69	86	male	177	71	22.66	39	100	102	0.98	51	37.1	152.67	85.33	12	10-15 Years	over 80	over 65	175-179 cm	normal	normal weight, metabolically abnormal	obese	overweight	obese	overweight	under 60 cm	Right	Matura
GSHA-70	57	female	168	62	21.97	33	87	95	0.92	54	36.4	122.00	78.00	12	10-15 Years	50-65	under 65	165-169 cm	normal	normal weight, metabolically healthy	obese	normal	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-71	50	female	171	59	20.18	33	76	91	0.84	55	36.8	119.00	74.33	16	16-20 Years	50-65	under 65	170-174 cm	normal	normal weight, metabolically healthy	obese	normal	obese	overweight	under 60 cm	Right	FH/University
GSHA-72	66	female	154.7	86.5	36.14	39	119	112.5	1.06	58	36.5	150.00	97.00	15	10-15 Years	66-80	over 65	150-154 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Both sides	Matura und Kolleg bzw. Akademie
GSHA-73	65	female	158.9	76	30.10	36.5	105	107	0.98	60.5	36.8	145.67	89.67	12	10-15 Years	50-65	under 65	155-159 cm	obesity class I	obese, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Lehrabschluss
GSHA-74	51	female	162.5	83	31.43	35	97	114	0.85	63	36.5	133.00	83.00	13	10-15 Years	50-65	under 65	160-164 cm	obesity class I	obese, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura
GSHA-75	73	female	146	58.5	27.44	34	86	103	0.83	54	36.1	141.67	93.00	66	over 20 Years	66-80	over 65	NA	preobesity	overweight, metabolically abnormal	obese	normal	obese	overweight	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-76	79	male	170.2	74.8	25.82	43	90	100	0.90	50	36.7	185.00	99.67	14	10-15 Years	66-80	over 65	170-174 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	FH/University
GSHA-77	86	female	162.1	69.9	26.60	35.5	93	102	0.91	51.5	36.3	176.67	109.00	10	10-15 Years	over 80	over 65	160-164 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Lehrabschluss
GSHA-78	76	female	157.9	94	37.70	38.5	121	124	0.98	62	36.6	124.00	78.50	12	10-15 Years	66-80	over 65	155-159 cm	obesity class II	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Hohere Schule ohne Matura
GSHA-79	55	male	179.5	79	24.52	39	96.5	100	0.97	53.5	36.5	134.33	92.67	11	10-15 Years	50-65	under 65	175-179 cm	normal	normal weight, metabolically abnormal	obese	overweight	obese	overweight	under 60 cm	Right	Lehrabschluss
GSHA-80	76	female	162	78.5	29.91	36	93	110	0.85	60	36.6	181.00	102.00	13	10-15 Years	66-80	over 65	160-164 cm	preobesity	overweight, metabolically healthy	obese	normal	obese	adipositas	over 60 cm	Right	Lehrabschluss
GSHA-81	68	female	165.4	53.7	19.63	31	74	90	0.82	47	36.7	153.00	93.50	18	16-20 Years	66-80	over 65	165-169 cm	normal	normal weight, metabolically abnormal	normal	normal	normal	overweight	under 60 cm	Right	FH/University
GSHA-82	75	male	156.5	68	27.76	42	108	99	1.09	51	36.7	171.67	97.67	16	16-20 Years	66-80	over 65	155-159 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Left	FH/University
GSHA-83	76	male	178.2	86	27.08	42	100	105	0.95	54	36.6	135.00	79.00	13	10-15 Years	66-80	over 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-84	74	male	184.6	86	25.24	40	95.5	103	0.93	59	36.3	144.00	82.00	27	over 20 Years	66-80	over 65	180-184 cm	preobesity	overweight, metabolically abnormal	obese	overweight	normal	overweight	under 60 cm	Right	FH/University
GSHA-85	65	female	160.9	76	29.36	36	98	107	0.92	59	37.1	170.00	95.50	12	10-15 Years	50-65	under 65	160-164 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-86	62	male	173	76	25.39	39.5	88	99	0.89	52.5	36.4	138.00	86.00	12	10-15 Years	50-65	under 65	170-174 cm	preobesity	overweight, metabolically healthy	obese	overweight	normal	normal	under 60 cm	Right	Lehrabschluss
GSHA-87	72	female	161.1	103.9	40.03	42	124.5	131	0.95	62	36.7	128.50	86.00	9	under 10 years	66-80	over 65	160-164 cm	NA	obese, metabolically abnormal	obese	overweight	obese	adipositas	over 60 cm	Right	Volksschule/Hauptschule
GSHA-88	72	male	186.5	113	32.49	45	121	119	1.02	59	36.3	132.50	80.00	20	16-20 Years	66-80	over 65	NA	obesity class I	obese, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Right	FH/University
GSHA-89	72	male	176	83	26.79	42	104	101	1.03	52	36.8	133.50	78.50	15	10-15 Years	66-80	over 65	175-179 cm	preobesity	overweight, metabolically abnormal	obese	overweight	obese	adipositas	under 60 cm	Left	Matura und Kolleg bzw. Akademie
GSHA-90	61	female	155.2	72.9	30.27	35	102	108	0.94	60.5	36.3	129.33	81.00	19	16-20 Years	50-65	under 65	155-159 cm	obesity class I	obese, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	FH/University
GSHA-91	56	female	166.6	57.1	20.57	31.5	69	98	0.70	53	37	116.33	74.67	11	10-15 Years	50-65	under 65	165-169 cm	normal	normal weight, metabolically healthy	normal	normal	normal	normal	under 60 cm	Right	Hohere Schule ohne Matura
GSHA-92	76	male	181.6	66.7	20.23	36.5	81.5	98	0.83	51	36.7	168.67	111.67	17	16-20 Years	66-80	over 65	180-184 cm	normal	normal weight, metabolically healthy	normal	normal	normal	normal	under 60 cm	Right	FH/University
GSHA-93	69	female	152.5	61.2	26.32	35	93	97	0.96	51	36.7	129.50	84.00	13	10-15 Years	66-80	over 65	150-154 cm	preobesity	overweight, metabolically abnormal	obese	normal	obese	adipositas	under 60 cm	Right	Matura
GSHA-94	67	female	161.3	88	33.82	36.5	107.7	121.5	0.89	63	36.7	146.67	100.67	12	10-15 Years	66-80	over 65	160-164 cm	obesity class I	obese, metabolically abnormal	obese	normal	obese	adipositas	over 60 cm	Right	Matura

Table 16 ***Complete metadata (part 2)***

Sample	Cardiovascular risk factors	Hypertension	Hypercholesterolaemia	Cardiovascular Event	Hypertension ESC 2018	Effectively treated hypertension?	Smoker in last 12 months	Abdominal surgery	Appendectomy	Cholecystectomy	Hysterectomy	Intestinal polyp/Haemorrhoids	GERD	Hypothyroidism/Hashimoto	Tumour	PPI	Anticoagulation	Statin usage	Vit-D Substitution	Nutritional Supplements including homeopathics and herbal medicine	Nutritional supplements
GSHA-1	Yes	Yes	Yes	no	Hypertension I	poorly-treated	Non-smoker	No	No	No	No	no	no	No	No	No	No	Yes	No	No	No
GSHA-2	Yes	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	Haemorrhoids	no	No	No	No	Marcumar	No	No	Yes	Yes
GSHA-3	Yes	No	Yes	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	Yes	No	No	No
GSHA-4	Yes	Yes	Yes	Myocardial infarction	Hypertension II	poorly-treated	Non-smoker	No	No	No	No	no	no	No	No	No	ASS	Yes	No	Yes	Yes
GSHA-5	No	No	No	no	High-Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	Yes	No	No	No	No	No	No	No
GSHA-6	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-7	Yes	Yes	No	no	High-Normal	well-treated	Non-smoker	No	No	No	No	no	no	No	No	No	ASS	No	No	No	No
GSHA-8	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	No	No	No	no	no	No	Yes	No	No	No	No	No	No
GSHA-9	Yes	Yes	No	no	Normal	well-treated	Non-smoker	No	No	No	No	Diverticulitis	no	No	No	No	Marcumar	No	No	No	No
GSHA-10	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	Yes	No	no	no	No	No	Yes	ASS	No	No	No	No
GSHA-11	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	Yes	No	Yes	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-12	No	No	No	no	Hypertension II	no known hypertension	Smoker	Yes	No	No	Yes	no	no	No	No	No	ASS	No	Yes	Yes	Yes
GSHA-13	Yes	No	Yes	no	Normal	no known hypertension	Non-smoker	Yes	No	No	Yes	no	no	Yes	No	Yes	NOAK	Yes	Yes	Yes	Yes
GSHA-14	Yes	Yes	No	no	Hypertension III	poorly-treated	Non-smoker	Yes	Yes	No	No	no	yes	No	No	Yes	ASS	No	No	No	No
GSHA-15	Yes	No	Yes	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	Yes	No	No	No
GSHA-16	Yes	Yes	No	no	Hypertension I	poorly-treated	Non-smoker	Yes	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-17	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-18	Yes	Yes	Yes	no	High-Normal	well-treated	Non-smoker	Yes	No	No	No	no	yes	Yes	No	Yes	No	Yes	No	Yes	Yes
GSHA-19	No	No	No	no	Hypertension II	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	Yes	No	No	No	No	No	No
GSHA-20	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	Yes	No	No	No	No	No	No
GSHA-21	No	No	No	no	Hypertension II	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-22	Yes	Yes	No	no	High-Normal	well-treated	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-23	No	No	No	no	Hypertension II	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-24	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-25	No	No	No	no	Hypertension I	no known hypertension	Smoker	No	No	No	No	no	no	No	Yes	No	No	No	No	No	No
GSHA-26	Yes	No	Yes	Embolism	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	Yes	No	No	Clopidogrel	No	No	No	No
GSHA-27	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	Yes	No	No
GSHA-28	Yes	Yes	No	Stroke	High-Normal	well-treated	Non-smoker	Yes	Yes	Yes	Yes	no	no	No	Yes	No	ASS	No	Yes	Yes	Yes
GSHA-29	No	No	No	no	Hypertension II	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	Yes	No	No	No	No	Yes	Yes
GSHA-30	Yes	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	No	No	No	no	no	No	No	No	No	No	Yes	Yes	No
GSHA-31	Yes	Yes	No	no	Hypertension I	poorly-treated	Non-smoker	Yes	Yes	No	No	Diverticulitis	no	No	No	No	No	No	No	Yes	Yes
GSHA-32	Yes	Yes	Yes	no	Normal	well-treated	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	ASS	Yes	No	No	No
GSHA-33	No	No	No	no	Normal	no known hypertension	Smoker	No	No	No	No	no	no	No	No	No	No	No	Yes	No	No
GSHA-34	No	No	No	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-35	No	No	No	Thrombose	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-36	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-37	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-38	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	No	Yes	Yes	no	no	Yes	No	No	No	No	No	Yes	Yes
GSHA-39	No	No	No	no	Hypertension II	no known hypertension	Non-smoker	No	No	No	No	no	yes	Yes	No	No	No	No	No	No	No
GSHA-40	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	Yes	No	Yes	No	no	no	No	No	No	No	No	No	No	No
GSHA-41	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	Polyp	no	No	No	No	No	No	No	Yes	Yes
GSHA-42	No	No	No	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-43	Yes	Yes	Yes	Stroke	Normal	well-treated	Non-smoker	No	No	No	No	no	no	No	No	No	Clopidogrel	Yes	No	No	No
GSHA-44	No	No	No	no	Hypertension II	no known hypertension	Smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-45	Yes	Yes	No	no	Hypertension II	poorly-treated	Non-smoker	Yes	Yes	No	Yes	no	no	Yes	Yes	No	ASS	No	No	No	No
GSHA-46	No	No	No	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	Yes	No	No	No	No	Yes	Yes	No
GSHA-47	Yes	Yes	Yes	no	Hypertension II	poorly-treated	Non-smoker	No	No	No	No	no	no	No	No	No	ASS	Yes	No	Yes	Yes
GSHA-48	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-49	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-50	Yes	No	Yes	no	Hypertension I	no known hypertension	Non-smoker	Yes	No	No	Yes	Polyp	no	Hashimoto	No	No	NOAK	Yes	Yes	No	No
GSHA-51	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	No	No	Yes	no	no	No	Yes	No	No	No	No	No	No
GSHA-52	No	No	No	no	High-Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-53	Yes	Yes	Yes	no	Hypertension II	poorly-treated	Non-smoker	Yes	Yes	Yes	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-54	Yes	Yes	Yes	no	Hypertension III	poorly-treated	Non-smoker	No	No	No	No	no	no	Yes	No	No	ASS	Yes	No	No	No
GSHA-55	Yes	Yes	Yes	no	Hypertension I	poorly-treated	Non-smoker	Yes	Yes	Yes	No	no	no	Yes	No	No	Clopidogrel	No	Yes	Yes	No
GSHA-56	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	No	No	Yes	no	no	No	No	No	No	No	No	No	No
GSHA-57	Yes	Yes	No	PAE	Normal	well-treated	Non-smoker	Yes	Yes	No	No	no	no	No		No	No	No	Yes	No	No
GSHA-58	Yes	Yes	No	no	Hypertension III	poorly-treated	Non-smoker	Yes	Yes	Yes	No	no	no	No	No	No	No	No	No	Yes	No
GSHA-59	Yes	Yes	Yes	no	High-Normal	well-treated	Non-smoker	No	No	No	No	no	no	No	No	No	ASS	Yes	Yes	No	No
GSHA-60	Yes	Yes	No	no	Hypertension II	poorly-treated	Non-smoker	Yes	No	No	Yes	no	no	No	No	No	No	No	Yes	No	No
GSHA-61	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-62	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	Yes	No	No	Yes	no	no	No	Yes	No	No	No	Yes	No	No
GSHA-63	No	No	No	no	Normal	no known hypertension	Smoker	No	No	No	No	no	no	Yes	No	No	No	No	No	No	No
GSHA-64	No	No	No	no	High-Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-65	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	Haemorrhoids	no	No	No	No	No	No	No	No	No
GSHA-66	Yes	Yes	No	no	Hypertension I	poorly-treated	Non-smoker	No	No	No	No	no	no	Yes	No	No	No	No	No	Yes	Yes
GSHA-67	No	No	No	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	Yes	No	No	No	No	No	No	No
GSHA-68	Yes	Yes	No	no	Hypertension I	poorly-treated	Non-smoker	Yes	No	Yes	No	no	no	Hashimoto	No	No	ASS	No	Yes	Yes	Yes
GSHA-69	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	Yes	No	Yes	No	no	no	No	No	No	ASS	No	No	No	No
GSHA-70	No	No	No	no	Normal	no known hypertension	Non-smoker	Yes	Yes	No	Yes	no	no	No	No	No	No	No	Yes	No	No
GSHA-71	No	No	No	no	Normal	no known hypertension	Smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-72	Yes	Yes	No	no	Hypertension I	poorly-treated	Non-smoker	Yes	Yes	No	Yes	no	no	No	Yes	No	No	No	No	Yes	Yes
GSHA-73	Yes	No	Yes	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	Diverticulitis	no	Yes	No	No	ASS	Yes	Yes	Yes	No
GSHA-74	Yes	Yes	No	no	High-Normal	well-treated	Smoker	No	No	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-75	Yes	Yes	No	no	Hypertension I	poorly-treated	Non-smoker	Yes	No	No	No	no	no	No	No	Yes	No	No	Yes	No	No
GSHA-76	No	No	No	no	Hypertension III	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-77	Yes	Yes	Yes	no	Hypertension II	poorly-treated	Non-smoker	Yes	Yes	No	No	Haemorrhoids	no	No	No	No	No	No	No	No	No
GSHA-78	Yes	Yes	No	no	Normal	well-treated	Non-smoker	Yes	No	Yes	No	no	no	No	Yes	No	No	No	No	Yes	Yes
GSHA-79	No	No	No	no	Hypertension I	no known hypertension	Smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-80	No	No	No	no	Hypertension III	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-81	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	Yes	No	No	No	Polyp	no	Hashimoto	No	No	No	No	No	Yes	Yes
GSHA-82	Yes	Yes	No	no	Hypertension II	poorly-treated	Non-smoker	No	No	No	No	no	no	No	No	Yes	No	No	No	No	No
GSHA-83	No	No	No	no	High-Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	ASS	No	No	No	No
GSHA-84	No	No	No	no	Hypertension I	no known hypertension	Non-smoker	No	No	No	No	Haemorrhoids	no	No	Yes	No	No	No	No	No	No
GSHA-85	Yes	Yes	No	no	Hypertension II	poorly-treated	Non-smoker	Yes	No	No	Yes	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-86	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	No	No
GSHA-87	Yes	Yes	Yes	PAE	High-Normal	well-treated	Non-smoker	Yes	No	No	Yes	no	no	No	No	Yes	Marcumar	Yes	Yes	No	No
GSHA-88	Yes	Yes	Yes	no	High-Normal	well-treated	Non-smoker	No	No	No	No	no	no	No	No	No	ASS	Yes	Yes	Yes	No
GSHA-89	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	No	No	No	no	no	No	Yes	No	No	No	No	No	No
GSHA-90	No	No	No	no	High-Normal	no known hypertension	Non-smoker	Yes	Yes	No	No	no	no	No	No	No	No	No	No	Yes	Yes
GSHA-91	No	No	No	no	Normal	no known hypertension	Non-smoker	No	No	No	No	no	no	No	No	No	No	No	Yes	No	No
GSHA-92	Yes	Yes	Yes	Stroke	Hypertension III	poorly-treated	Non-smoker	Yes	No	Yes	No	no	no	No	No	No	ASS	Yes	No	No	No
GSHA-93	Yes	No	No	no	High-Normal	no known hypertension	Smoker	Yes	Yes	Yes	No	no	no	No	No	No	No	No	No	No	No
GSHA-94	Yes	Yes	No	no	Hypertension II	poorly-treated	Non-smoker	Yes	Yes	No	Yes	no	no	Hashimoto	No	No	No	No	Yes	Yes	Yes

Table 17 ***Complete metadata (part 3)***

Sample	SODIUM	POTASSIUM	CALCIUM	CHLORIDE	ALT	AP	AST	BILIRUBIN	CHOLINESTERASE	GGT	TOTAL PROTEIN	CRP	GLUCOSE	URIC ACID	UREA	CREATININE	LDH	CREATINEKINASE	CHOLESTEROL	LDL	HDL	TRIGLYCERIDE	VitD 1.25	VitD3	Aldosterone	Renin	C-Peptide	Insulin	Leptin	PTH	SHBG	Progesterone	Oestradiol	Free testosterone	Testosterone	TSH	fT3	fT4 (Thyroxine)
GSHA-1	142	4.1	2.17	103	20	50	24	0.64	9908	15	7	1.2	92	5	38	0.94	221	206	129	75	35	86	133	27.2	3.9	27.6	1.43	11.1	4.5	31.8	31.1	1.69	36.5	10.52	2.1	0.79	4.9	15.4
GSHA-2	142	4.5	2.38	103	20	86	21	0.59	9982	45	7.1	1.3	87	5.9	30	0.89	138	73	206	122	40	162	182	24.8	6.2	14.3	1.6	11.1	23.7	60.7	76.1	0.23	49.3	1.04	0.14	2.95	5.3	15.8
GSHA-3	140	4	2.43	101	16	73	18	0.37	7894	13	7.3	0.8	94	4	21	0.79	173	150	165	82	51	74	155	31.7	5.2	11.3	1.33	8.3	10.4	33.9	63.2	0.3	30.6	1.58	0.34	0.48	4.7	12.6
GSHA-4	141	3.6	2.48	97	35	98	31	0.76	8272	22	7.5	2.1	121	6.2	40	0.97	206	114	162	100	45	81	90	17.5	4.3	22.4	0.88	4.7	7.6	59.6	58.2	0.67	34.7	11.04	3.02	1.96	4.6	13.7
GSHA-5	140	3.9	2.21	104	17	55	21	0.47	7303	17	7.7	1.7	97	4.9	26	1.26	189	133	227	146	22	269	125	27.1	11.6	20.5	1.3	12.6	4.6	64.4	51.2	0.96	35.9	12.71	4.25	4.51	3.9	15.1
GSHA-6	141	3.9	2.37	101	25	47	36	1.14	8129	20	6.8	0.6	91	7.1	35	1.21	251	573	254	180	0	172	NA	20.2	NA	NA	1.58	11.4	NA	25.9	46.2	NA	NA	NA	2.38	4.08	5.3	13.3
GSHA-7	141	4	2.36	102	11	89	20	0.68	8191	14	7.1	3	78	5.7	35	0.81	109	59	327	216	85	72	96	15.3	6.5	9.3	0.63	6.8	13.2	68.6	115.2	0.19	20	0.77	0.09	2.54	4.6	14.8
GSHA-8	140	4.2	2.24	105	40	95	43	0.81	5571	103	6.9	1.7	87	5.2	56	1.06	192	265	143	85	48	46	60	15.3	0	9.4	0.88	5.4	4	88.2	115	0.96	30.4	5.88	4.61	0.93	4.9	13.5
GSHA-9	140	4.5	2.49	101	21	76	27	1.89	6207	54	7.4	1.5	94	6.2	43	0.98	192	97	154	90	40	103	42	21.4	15.2	13.9	1.58	14.4	15.1	24	59.9	0.79	38.3	8.06	2.51	0.38	4.7	13.8
GSHA-10	140	4.4	2.46	100	22	60	26	1.13	8058	26	7.3	1.8	94	4.5	46	0.92	181	38	250	156	72	98	41	34.2	6.8	16.1	1.57	13.2	35.8	80.1	53.2	0.35	26.8	1.64	0.43	1.17	4.4	14
GSHA-11	139	4.2	2.48	97	26	53	26	0.61	8891	56	7.5	10.2	108	4.7	22	0.9	199	56	181	93	61	132	239	23.2	0	12	3.26	27.1	17.3	49.7	51.3	0.27	41.2	8.03	2.24	1.62	4.4	9.7
GSHA-12	139	4.6	2.21	103	20	75	23	0.26	5941	17	7.3	1.4	97	6.1	37	0.99	203	87	245	148	74	87	60	30.4	0	30.4	1.27	6.5	15.3	41.5	72.4	0.09	32.5	1.12	0.21	3.54	4.2	12.5
GSHA-13	138	4.8	2.26	96	39	41	44	0.45	6413	49	7.5	2.2	131	5.9	21	0.68	207	254	161	95	39	158	129	23.1	8.8	14.7	5.19	73.5	18.9	53.8	14.2	1.55	0	0.89	0.07	4.82	4.4	10.7
GSHA-14	142	4.5	2.44	103	28	73	28	0.25	9162	66	7.5	2.9	118	7.6	48	1.04	226	147	254	176	56	108	22	21.5	0	28.7	2.76	23.1	32.4	62.3	62.5	0.18	20	1.89	0.3	1.6	4.7	14.2
GSHA-15	140	4.3	2.47	101	31	50	30	1.1	10023	37	7	0.6	105	5.6	40	0.86	178	82	132	72	23	147	169	25.5	104.5	13.6	3.98	30.3	21.8	52.9	20.7	0.82	31.9	10.53	1.85	0.77	4.9	14.4
GSHA-16	142	4.3	2.47	102	21	72	26	0.42	11230	37	7.1	4	110	7.2	48	1.18	203	119	200	118	56	76	45	14.5	0	85.2	3.04	25.8	34.5	55.6	46.1	0.7	47.2	14.79	3.92	1.62	5.1	14.6
GSHA-17	141	4.5	2.39	105	24	122	24	0.41	9684	16	7.1	2.6	85	5.3	23	0.71	177	79	172	105	45	94	45	18.5	8.7	60.2	1.48	15.3	21.8	53.6	155	0.26	20.3	1.21	0.43	0	7.5	21
GSHA-18	142	3.6	2.4	99	25	65	26	0.3	11089	40	7.5	1.4	115	5.5	31	0.68	238	221	167	109	44	67	160	25	10.7	84.6	1.99	20.4	55.7	77.8	65.8	0.31	33.2	1.19	0.44	1.62	4.4	15.8
GSHA-19	141	4.6	2.49	102	11	62	20	1.25	7855	35	7.6	1.4	95	6.8	29	1.22	145	90	225	149	54	78	129	43.7	5.9	5.1	1.17	7.5	3.3	60.9	36.6	0.96	37.2	11.66	3.37	2.26	5.5	16.5
GSHA-20	139	4	2.36	99	17	43	18	0.59	9215	16	7.3	0	95	6.6	48	1.06	167	81	232	137	65	69	146	24.3	6.6	33.2	2.49	20.1	19.4	58	40	1.48	33.5	9.56	2.51	2.68	5.4	12.5
GSHA-21	136	3.4	2.69	96	20	86	29	0.41	6850	12	7.3	1.4	78	4.5	16	0.54	251	189	226	127	82	66	146	32.9	12.1	3.7	1.16	11	7.4	61.2	165.9	1.01	34.9	0.89	0.32	2.47	4.5	14.9
GSHA-22	140	4.4	2.4	100	27	74	30	0.34	7649	21	7.2	5.2	75	6.6	47	1.04	182	165	186	110	54	66	71	32	4.8	11.8	1.49	12.8	35	60.6	62.8	0.19	26.9	0.63	0.17	1.65	4.9	11.7
GSHA-23	141	4.5	2.31	101	15	49	18	0.89	6101	28	7.2	0.7	87	5.1	58	1.03	188	105	216	122	77	58	112	17.6	4.4	8.1	1.11	6.3	4.2	74	74.9	0.63	43.3	15.14	4.82	2.99	4.3	13.7
GSHA-24	139	3.7	2.49	100	17	56	26	0.48	10372	18	7.2	0	79	5.9	25	0.9	185	87	283	172	91	56	163	24.4	0	2.2	0.89	5.2	6.4	58.6	74.2	0.29	0	0.82	0.09	1.4	4.5	12.7
GSHA-25	139	4.6	2.49	99	42	92	39	0.37	10105	45	8.4	0.7	86	6.1	27	1.08	234	79	320	229	58	181	53	18	9.9	22.8	1.64	11.6	11.6	54.6	52.4	0.93	41.7	19.55	4.35	0.71	4.3	12.6
GSHA-26	141	4	2.37	103	21	50	34	0.5	6116	19	6.3	0	89	3.8	31	0.66	217	259	191	59	124	49	141	38.1	0	14.7	0.67	3.5	3.9	43.7	92.2	0.31	20	0.77	0.22	1.17	4	21.9
GSHA-27	141	3.9	2.43	103	14	63	18	0.37	5710	8	6.6	0	82	4.1	25	0.75	160	62	214	130	73	51	248	27.1	3.7	16.8	1.17	8.1	17.4	44.8	155.1	0.43	153	1.5	0.11	0.97	4.4	15.7
GSHA-28	143	4	2.53	104	18	74	21	1.01	6427	15	7.2	4	91	4.5	28	0.82	178	83	202	109	60	145	96	24.8	0	25.7	1.32	12.6	33.7	66.8	83.5	0.32	34.8	1.98	0.21	2.86	4.5	13.3
GSHA-29	143	4.1	2.47	101	14	78	24	0.36	7869	15	7.5	2.5	76	4.6	31	1.01	211	104	230	150	60	72	96	29.2	8.4	14.7	1.1	7	31.1	50.4	120.8	0.2	27.3	2.59	0.41	0.93	4.1	17.9
GSHA-30	140	4.4	2.48	101	7	59	18	0.65	7050	11	7.2	0	88	4.4	24	0.81	183	53	237	154	39	216	144	32.6	6.5	34.1	1.46	11.2	26.9	73.2	73	0.27	0	1.06	0.13	2.06	5.3	16.1
GSHA-31	141	4.2	2.5	100	22	55	20	1.14	8634	29	7.3	0.7	87	6	21	0.96	158	82	190	123	48	74	147	27.7	4.8	48.6	1.19	6.4	8.3	35.7	29.7	1.1	20.4	10.6	3.03	1.53	5.4	14.4
GSHA-32	142	3.9	2.53	105	23	73	40	0.29	6495	8	5.9	0	98	4.2	45	1.22	162	124	201	126	54	98	26	21.9	0	38.5	1.78	8.2	49.4	44	93.2	0.16	22.5	1.24	0.25	2.13	3.8	11.8
GSHA-33	142	4	2.41	101	18	75	25	0.74	6159	16	7.1	0	91	3.5	21	0.7	165	84	191	105	72	69	53	34.8	15.3	28.5	1.04	5.1	9	37.4	122.5	0.57	24.5	1.09	0.22	0.62	5.2	18.3
GSHA-34	140	3.5	2.2	103	49	87	58	1.16	7390	17	7.3	2.1	78	6.5	56	0.91	329	667	184	103	66	41	150	66.8	0	39.7	0.65	2.3	2.9	48.3	125.2	1.43	47.2	14.8	6.68	2	4.1	13.4
GSHA-35	142	4	2.29	101	22	69	23	0.98	11723	26	7.7	2.6	91	7.6	27	1	189	105	268	165	69	157	100	16.5	5.6	23.4	1.39	9.3	4.6	101.3	66.2	0.82	34.7	19.88	5.39	4.03	4.4	15.4
GSHA-36	142	4.2	2.39	104	22	58	34	0.54	7444	23	7.2	0.9	77	5.5	25	0.82	254	141	226	140	57	151	124	36.3	0	9	1.86	12.4	54.5	34.6	50.1	0.28	29.3	2	0.34	1.82	4.5	10.9
GSHA-37	140	4.1	2.29	100	29	55	28	0.9	6275	30	7.1	0	97	4.1	34	0.91	191	102	255	184	47	114	55	32.4	6.6	8.3	1.56	9.9	8.4	53.6	93.1	0.81	56	14.33	7.86	1.43	4.6	15.7
GSHA-38	140	3.7	2.14	101	15	51	19	0.44	5846	14	6.5	0	89	3.8	20	0.75	218	110	193	93	90	43	79	19.9	4.2	0	1.09	5.8	17.6	61	82.9	0.19	66.5	1.46	0.31	0.2	3.7	20.1
GSHA-39	140	4.3	2.34	104	15	55	18	0.38	6861	20	7.3	0.7	113	6.2	34	0.83	236	106	274	204	40	111	50	25.2	3.9	17.1	1.88	14.9	42.2	43.5	39.1	0.21	49.9	2.58	0.23	1.92	4.5	15
GSHA-40	139	4.1	2.4	102	36	73	30	0.82	9934	15	7.5	3.6	93	5.3	27	0.84	261	82	233	180	46	120	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-41	140	4.2	2.32	102	17	32	15	0.39	11231	10	7.5	0.6	102	3.4	35	0.57	174	46	180	88	79	44	129	11.7	27.6	16.1	1.42	11	28.6	59.6	73.1	0.51	315	4.21	0.25	1.64	4.8	15.9
GSHA-42	138	3.6	2.35	101	14	57	17	0.38	7228	7	7.7	0	84	2.9	34	0.71	131	69	176	93	70	78	71	11.2	17	19.5	0.77	3.8	18.4	50.5	59.3	0.85	242	1.88	0.24	0.67	4.7	13.9
GSHA-43	140	4	2.32	102	35	59	27	0.4	10530	21	7.4	1.5	206	6.3	57	1.36	179	114	192	118	51	106	38	14.1	10.4	69.7	1.57	14.7	23.2	94	64	0.93	42.3	6.34	3.7	1.9	4.8	17
GSHA-44	141	3.7	2.5	104	25	49	31	0.38	7454	17	6.8	5.3	94	9.6	25	1.09	186	325	327	253	40	117	92	21.2	16.9	56.6	1.37	10.1	12.9	40.2	88.3	1.18	49.4	15.22	7.05	0.81	4	14.7
GSHA-45	139	4.2	2.34	101	22	99	21	2.12	11195	20	7.6	0	89	4.9	32	0.82	156	65	203	142	33	109	84	10.8	10.4	15	2.38	15.9	39	62.5	40.9	0.69	30.5	1.18	0.2	0.17	4.5	22.7
GSHA-46	141	4.2	2.33	102	19	52	24	0.63	8258	20	7.4	1.8	89	2.4	33	0.71	185	71	279	170	91	89	42	30.3	10.2	19.3	0.82	4.9	14.4	55.8	113	0	29.7	1.16	0.3	0.56	4.2	16.4
GSHA-47	141	3.7	2.36	105	29	63	27	0.83	8866	50	7.2	2.5	109	3.6	45	1	200	134	169	105	49	71	130	26.3	5.3	38.8	2.2	16.1	10.6	28.6	81	0.66	37.3	12.58	4.11	0.76	5.6	16.2
GSHA-48	139	4.2	2.4	99	16	76	21	0.46	8854	19	7.8	0.9	95	4.7	30	0.66	174	78	299	232	48	112	87	27.6	0	8.6	1.64	10.1	40.8	41.2	115.2	0.25	23.2	4.66	0.33	2.07	4.9	14.2
GSHA-49	144	4	2.39	103	14	54	16	1.05	8188	14	7.1	2.8	96	5.4	32	0.86	172	48	188	129	42	95	23	13.8	37.1	4.4	1.19	6.8	20	54.9	47.2	0.56	23.9	1.82	0.36	1.31	4.1	13.1
GSHA-50	142	4	2.3	106	19	73	23	0.37	8475	16	6.9	0	95	6.6	34	0.81	245	168	183	100	52	90	54	18.9	0	9.3	2.1	15.4	51.5	64.5	34.2	0.18	28.9	0.95	0.11	4.32	4.3	13
GSHA-51	144	4.2	2.41	104	21	63	23	0.56	8673	11	7.5	2.5	98	5.1	32	0.72	214	108	205	132	47	131	18	18.4	11.7	16	0.99	5.8	33.2	59.2	24.9	0.41	34	1.88	0.22	1.94	4.4	14.3
GSHA-52	140	4.1	2.4	102	22	62	27	0.53	6881	11	7.2	1.7	93	5.8	25	0.78	196	98	247	143	87	55	102	15.4	18.7	49.8	0.98	5.8	15.6	46	124.6	0.12	28.6	1.03	0.16	0.94	5.2	14.4
GSHA-53	139	4.1	2.35	99	13	55	22	1.17	7185	15	7	0	107	4.3	17	0.65	159	131	178	109	46	113	81	17.2	4	63.5	2.4	15.9	5.7	34.2	49.5	0.55	38.7	7.92	3.94	2.21	4.4	11.4
GSHA-54	141	4	2.33	106	25	43	33	0.86	7488	24	6.9	1.5	110	6.7	46	0.93	192	161	183	112	51	117	0	21.3	4.6	24.7	1.32	7.8	12.5	67.3	37.8	0.41	47.1	5.57	2.04	0.31	4.1	17.7
GSHA-55	144	3.9	2.5	104	30	48	29	0.54	9992	21	6.9	2.2	101	7.3	44	0.94	231	228	105	41	48	97	26	26	0	3.4	4.98	37.7	61.4	32.2	25.8	0.21	27.5	1.1	0.14	1.38	3.9	15.6
GSHA-56	137	3.9	2.43	99	15	69	23	1.2	7588	12	7.6	2.2	95	4.7	19	0.75	174	48	220	127	58	90	184	73.1	5.4	4.3	0.92	3.8	17.6	37.7	48	0.29	28.2	2.07	0.24	1.74	4.1	14.2
GSHA-57	140	4.2	2.52	103	17	58	21	0.59	7908	16	7.3	6	89	5.4	21	0.79	209	85	215	144	36	90	82	56.3	5.2	20.7	2.26	13.6	61.8	46	101.7	0.39	46.6	0.94	0.19	1.57	4.5	15.5
GSHA-58	143	3.8	2.39	102	14	86	22	0.43	10284	24	7.3	14.2	117	5.2	39	0.89	209	93	231	160	46	82	130	22.9	0	3.5	2.21	11.4	43.4	50.8	48.8	0.1	28.7	1.43	0.19	2.36	3.8	13.4
GSHA-59	135	5.2	2.45	97	17	30	14	1.37	8320	30	7.4	1.9	238	5.5	37	1	165	69	153	86	47	51	40	24.8	44.2	205.5	2	7.1	10.5	37.6	40.5	0.66	32.3	16.09	4.92	3.95	4.6	14.2
GSHA-60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-61	140	4	2.39	102	25	52	21	0.51	7179	14	7.2	0	81	3.9	38	0.88	232	267	238	108	95	60	29	31.2	0	15.7	0.71	4.2	3.5	35.4	102.6	0.27	20.4	1.32	0.45	0.69	4.2	13.8
GSHA-62	142	3.9	2.43	103	20	92	20	0.64	7375	14	7.7	1.6	94	4.3	43	0.75	186	106	318	229	72	80	198	30.5	13.2	14.5	1.92	10.6	19.3	24.7	67.2	0.63	52.1	1.24	0.16	1.73	4.4	13.7
GSHA-63	141	4.3	2.41	102	17	88	19	0.61	8177	18	7.7	1	85	5.3	36	0.77	184	117	232	117	101	58	134	64.8	11.6	17.9	1	4.5	46.9	27.6	68	0.48	55.6	1.95	0.32	1.42	4.2	15.2
GSHA-64	145	3.5	2.43	105	18	37	18	1.27	6422	18	7.3	0	81	7	45	0.97	148	139	208	113	80	75	94	33	4.5	7.6	0.88	3.4	2.7	43.4	66.3	1.36	21.1	13.91	4.03	2.37	4.6	15.8
GSHA-65	141	4.4	2.37	103	19	39	19	0.61	7295	22	7.4	1	85	4.3	41	0.97	172	72	217	155	36	111	20	28.8	10	13.4	1.78	8.4	9.4	56.6	56.4	1.91	40.6	11.62	5	3.52	3.8	11.3
GSHA-66	140	4.3	2.24	103	18	66	24	0.32	9150	16	7.4	2	88	6.6	22	0.94	175	139	130	76	42	42	51	33.1	0	4.6	1.6	7.3	2.1	35.1	44.4	0.41	22.4	8.8	3.55	2.27	5.4	17.3
GSHA-67	139	3.6	2.33	103	15	43	15	0.53	8914	17	7.3	3.6	95	4.3	36	0.82	211	90	233	137	78	70	24	16.5	0	5.7	1.21	8.4	40.6	31	50.6	0	0	1.04	0.15	0.94	3.9	19.4
GSHA-68	140	4.1	2.36	102	24	72	27	0.72	8901	39	7.5	10.3	111	6.5	35	0.78	172	41	181	122	43	84	98	31.8	0	198.3	2.99	21.2	47.6	47.6	59.9	0.38	53.7	2.05	0.31	0.02	4.4	15.8
GSHA-69	141	4.4	2.25	104	22	73	24	0.44	6339	31	7	1.2	91	4.8	45	0.72	180	93	176	118	46	56	55	12.1	4	3.6	0.89	3.9	7.4	45.2	98	0.58	49.8	10.32	7.06	0.62	3.5	15.1
GSHA-70	142	4.5	2.48	103	19	82	21	0.33	9472	14	7.4	0	83	3.9	25	0.77	199	81	230	129	88	36	66	26.6	0	11.6	1.33	9.4	19.3	48.1	65.5	0.25	26.4	1.18	0.16	0.54	5	13.5
GSHA-71	138	4.2	2.35	102	17	47	20	0.48	5983	16	7.3	0	83	3.9	23	0.91	184	168	221	162	46	55	20	27.5	19.9	20.3	0.64	3.7	4.7	53.1	109.2	0.47	114	1.44	0.18	1.13	3.7	12.7
GSHA-72	143	3.9	2.38	101	26	56	24	0.31	11475	32	7.4	1.5	139	6.8	31	0.63	187	118	250	150	37	332	76	22.5	11.7	7.2	2.9	23.9	58.8	62	25	< 0,09	20	2.1	0.2	1.97	5.2	16.9
GSHA-73	142	4.1	2.2	105	21	68	25	0.47	10314	21	7.1	1.4	88	5.6	43	0.76	274	139	214	144	49	92	37	22.9	8.6	4.1	1.24	8.7	33.6	63.4	40.9	0.2	82.9	1.78	0.23	1.67	4.3	17.6
GSHA-74	141	3.8	2.4	105	14	51	18	0.38	6174	12	7	5	99	5.6	38	0.7	172	71	210	153	40	59	56	18.4	0	4.3	1.42	9.9	24.6	27.9	82.6	0.26	24.5	1.24	0.2	3.3	4.8	16.2
GSHA-75	142	4.5	2.43	103	15	65	26	0.41	8118	13	7.2	1	97	7	48	0.94	269	132	210	111	74	84	68	15	7.5	147.9	2.52	12.6	51.7	66.4	65.6	< 0,09	22.1	0.55	0.08	1.42	5.1	13.1
GSHA-76	144	5.1	2.37	107	46	96	43	0.55	9168	27	7.8	0.9	95	5	58	1.39	199	320	231	157	42	144	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-77	140	4.2	2.47	100	25	58	32	0.72	10867	26	7.7	1.2	92	5.3	42	0.72	255	170	157	94	36	150	22	13.9	0	7.8	1.83	14.6	34.7	39.4	102.8	0.18	30.7	1.28	0.97	1.9	4.7	14.2
GSHA-78	143	4.4	2.33	104	23	54	19	0.45	10090	11	6.9	1.1	101	7.1	62	0.83	218	119	263	170	54	58	42	8.8	15.1	357.3	4	15.2	58.6	68.3	91.1	0.21	20	1.77	0.16	1.06	4.3	17.7
GSHA-79	140	4	2.23	104	23	41	23	0.51	8720	34	7	2.8	85	4.9	24	0.87	199	111	139	85	40	92	68	8.2	0	11.5	1.79	11.8	10	56	34.2	0.65	31.3	11.47	3.8	1.64	5.6	13.4
GSHA-80	140	4.1	2.49	101	28	61	38	0.91	9076	17	7.9	0	92	4.2	35	0.82	321	295	293	178	92	66	100	29.2	6	7.5	1.44	8.7	29.1	70.7	116	0	31.9	NA	0.37	1.46	5.1	14.4
GSHA-81	140	4	2.29	103	21	57	24	0.65	5980	13	6.8	1	88	4.4	28	0.74	208	134	232	146	70	69	147	28.5	0	14.9	1.37	11.8	7.3	68.4	66.1	0.45	0	0.88	0.14	1.72	4.5	12.8
GSHA-82	135	4.6	2.32	97	16	64	20	0.46	8023	30	7.2	1.4	93	7.1	36	1.03	192	78	201	118	55	131	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-83	140	4.6	2.21	101	15	37	22	0.41	6818	19	6.6	1.1	85	6.6	37	1.09	199	206	247	173	48	128	25	22.7	12.1	4.1	1.92	8.7	19.5	60.8	61.6	0.7	47.3	8.98	4.27	2.8	4.5	18
GSHA-84	143	4.1	2.31	106	20	56	31	0.61	8328	13	7.4	0.8	90	5.4	30	1.08	231	306	189	94	69	97	78	0	7.8	16.5	1.09	10	11.1	113.8	73.4	0.79	23.9	12.62	4.85	5.24	3.8	13.4
GSHA-85	141	3.6	2.49	99	9	70	23	0.63	9940	25	7.3	3.9	91	5	31	0.76	202	49	188	109	58	149	49	13.4	14.3	0	1.23	7.1	23.7	49.3	121	0.77	35.7	1.5	0.19	2.83	4.6	15.8
GSHA-86	139	4.1	2.41	101	17	51	21	1.27	8316	19	7.1	0	88	6.6	24	0.76	157	178	190	124	45	85	119	17.7	3.9	13.8	1.33	8.4	6	50.3	45.9	0.51	26.8	11.11	5.2	1.46	4.6	12.7
GSHA-87	143	4.3	2.34	104	23	46	17	0.42	8205	15	7	0	94	5.1	68	1.12	214	55	214	123	63	101	205	30.4	15.2	68.5	3.98	22.6	126.5	81.2	33.1	0	20	0.49	0	5.24	5.2	13.6
GSHA-88	142	4.3	2.38	104	33	63	21	0.95	7510	21	7.1	0	124	7.3	37	0.92	186	120	159	107	41	91	75	25.5	9.8	25.8	2.57	16.4	32.3	55	32.8	0.35	44	10.46	2.88	2.51	4	13.6
GSHA-89	142	4.1	2.23	105	26	78	25	0.45	11036	25	7	2.2	94	4.8	38	0.84	162	43	164	104	30	132	145	25.4	4.1	7.8	2.48	11	15.3	89.4	66.5	0.69	53.2	12.46	6.05	1.35	4.3	18
GSHA-90	139	3.9	2.43	102	17	50	30	0.61	7389	11	7.7	1	94	4.3	29	0.7	208	253	228	135	80	37	109	27.9	0	12.8	0.99	9.4	12.9	80.6	56.7	0.18	32.6	1.21	0.3	1.09	4.7	13.9
GSHA-91	140	4.2	2.59	101	18	47	17	0.24	7468	17	7.4	0	91	4.8	37	0.97	168	79	222	127	83	43	253	27.6	0	6.4	1.32	13.2	11.2	120.2	96.4	0.35	32.2	1.97	0.19	2.32	4.4	17.1
GSHA-92	143	4.2	2.38	102	21	62	31	0.73	5868	24	7.2	0	91	5.1	47	1.09	145	108	190	117	55	88	62	19.3	7.8	17.5	1.47	5.6	5.7	48.4	73.9	0.53	63.9	8.35	5.62	0.89	4.8	13.3
GSHA-93	144	4.3	2.34	103	14	89	20	0.38	8835	18	7.2	0	109	4	37	0.73	185	50	278	175	66	159	81	66.7	0	0	1.93	9.2	30.4	33.8	43.3	0.29	34.9	2.17	0.3	1.44	4.6	12.7
GSHA-94	141	3.9	2.38	102	19	77	16	0.4	6506	16	7.5	3	92	7.2	34	0.8	199	66	221	135	53	139	140	26.5	39	30.9	2.3	12.2	63	74.4	23.1	NA	NA	NA	0.34	1.36	3.6	12.8

Table 18 ***Complete metadata (part 4)***

Sample	Sodium	Potassium	Calcium	Chloride	ALT	AP	AST	Bilirubin	Cholinesterase	GGT	Total protein	CRP	Glucose	Uric acid	Urea	Creatinine	LDH	Creatininekinase	Cholesterol
GSHA-1	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal
GSHA-2	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	high	normal	normal	normal	normal	high
GSHA-3	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal
GSHA-4	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-5	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	high
GSHA-6	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	high	normal	high	high	high	high
GSHA-7	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	low	normal	high
GSHA-8	normal	normal	normal	normal	normal	high	normal	normal	normal	high	normal	normal	normal	normal	high	normal	normal	high	normal
GSHA-9	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-10	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	high
GSHA-11	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high	high	normal	normal	normal	normal	normal	normal
GSHA-12	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high
GSHA-13	normal	normal	normal	normal	high	high	normal	normal	normal	high	normal	normal	high	high	normal	normal	normal	high	normal
GSHA-14	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	high	high	high	high	normal	high	high
GSHA-15	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-16	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	high	normal	normal	normal	high
GSHA-17	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-18	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	high	normal	normal	normal	normal	high	normal
GSHA-19	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	high
GSHA-20	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	high
GSHA-21	normal	low	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	high
GSHA-22	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	high	normal	high	high	high	normal	high	normal
GSHA-23	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	high
GSHA-24	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high
GSHA-25	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	high
GSHA-26	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	low	normal	normal	normal	normal	normal	normal	high	normal
GSHA-27	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-28	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-29	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	high
GSHA-30	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-31	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-32	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	low	normal	normal	normal	normal	high	normal	normal	high
GSHA-33	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-34	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high	high	normal
GSHA-35	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high
GSHA-36	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high
GSHA-37	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-38	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-39	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	normal	normal	high
GSHA-40	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high
GSHA-41	normal	normal	normal	normal	normal	normal	low	normal	high	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-42	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-43	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high	high	normal	normal	normal
GSHA-44	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high	normal	normal	normal	high	high
GSHA-45	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-46	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-47	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-48	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-49	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-50	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	high	high	normal
GSHA-51	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-52	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high
GSHA-53	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-54	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high	normal	normal	high	normal
GSHA-55	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	normal	high	normal
GSHA-56	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-57	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	high
GSHA-58	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal	high
GSHA-59	normal	high	normal	normal	normal	normal	low	high	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-61	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high
GSHA-62	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-63	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-64	normal	normal	normal	normal	normal	normal	low	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-65	normal	normal	normal	normal	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-66	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-67	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-68	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high	high	high	normal	normal	normal	normal	normal
GSHA-69	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-70	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-71	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high
GSHA-72	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	high	high	normal	normal	normal	normal	high
GSHA-73	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	high
GSHA-74	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	high
GSHA-75	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	high	normal	high
GSHA-76	normal	high	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	high	high
GSHA-77	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal
GSHA-78	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	high	normal	normal	normal	high
GSHA-79	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-80	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	high
GSHA-81	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-82	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high
GSHA-83	normal	normal	normal	normal	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high
GSHA-84	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal
GSHA-85	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-86	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal
GSHA-87	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	high
GSHA-88	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal
GSHA-89	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-90	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	high
GSHA-91	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high
GSHA-92	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal
GSHA-93	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	high
GSHA-94	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	high

Sample	LDL	HDL	Triglycerides	VitD-1.25	VitD3	Aldosterone	Renin	C-Peptide	Insulin	Leptin	PTH	SHBG	Free testosterone	Testosterone	TSH	fT3	fT4 (Thyroxine)	LABOR hypothyroidism
GSHA-1	normal	low	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	low	normal	normal	normal	normal
GSHA-2	high	low	high	normal	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-3	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-4	high	normal	normal	normal	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-5	high	low	high	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	latent hypothyroidism
GSHA-6	high	low	high	NA	low	NA	NA	normal	normal	NA	normal	normal	NA	low	high	normal	normal	latent hypothyroidism
GSHA-7	high	normal	normal	normal	low	normal	normal	low	normal	high	high	normal	normal	low	normal	normal	normal	normal
GSHA-8	high	normal	normal	low	low	low	normal	normal	normal	normal	high	high	low	normal	normal	normal	normal	normal
GSHA-9	high	low	normal	low	low	normal	normal	normal	normal	high	normal	normal	low	normal	normal	normal	normal	normal
GSHA-10	high	normal	normal	low	normal	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-11	high	normal	normal	high	low	low	normal	high	high	high	normal	normal	low	low	normal	normal	normal	normal
GSHA-12	high	normal	normal	low	normal	low	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-13	normal	low	high	normal	low	normal	normal	high	high	high	normal	low	normal	low	high	normal	normal	latent hypothyroidism
GSHA-14	high	normal	normal	low	low	low	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-15	high	low	normal	normal	low	high	normal	high	high	high	normal	normal	normal	low	normal	normal	normal	normal
GSHA-16	high	normal	normal	low	low	low	normal	high	high	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-17	normal	normal	normal	low	low	normal	normal	normal	normal	high	normal	high	normal	normal	low	high	normal	manifest hyperthyroidism
GSHA-18	normal	normal	normal	normal	low	normal	normal	high	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-19	high	normal	normal	normal	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-20	high	normal	normal	normal	low	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-21	high	normal	normal	normal	normal	normal	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-22	normal	normal	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-23	high	normal	normal	normal	low	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal
GSHA-24	high	normal	normal	normal	low	low	low	normal	normal	normal	normal	normal	normal	low	normal	normal	normal	normal
GSHA-25	high	normal	high	low	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-26	high	normal	normal	normal	normal	low	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-27	high	normal	normal	high	low	normal	normal	normal	normal	high	normal	high	normal	low	normal	normal	normal	normal
GSHA-28	high	normal	normal	normal	low	low	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-29	high	normal	normal	normal	low	normal	normal	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-30	high	low	high	normal	normal	normal	normal	normal	normal	high	high	normal	normal	low	normal	normal	normal	normal
GSHA-31	high	normal	normal	normal	low	normal	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-32	high	normal	normal	low	low	low	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-33	normal	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-34	normal	normal	normal	normal	high	low	normal	low	low	normal	normal	high	normal	normal	normal	normal	normal	normal
GSHA-35	high	normal	high	normal	low	normal	normal	normal	normal	normal	high	normal	normal	normal	high	normal	normal	latent hypothyroidism
GSHA-36	high	normal	high	normal	normal	low	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-37	high	normal	normal	low	normal	normal	normal	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-38	normal	normal	normal	normal	low	normal	low	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-39	high	low	normal	low	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-40	high	normal	normal	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-41	normal	normal	normal	normal	low	normal	normal	normal	normal	high	normal	normal	high	normal	normal	normal	normal	normal
GSHA-42	normal	normal	normal	normal	low	normal	normal	low	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-43	high	normal	normal	low	low	normal	normal	normal	normal	high	high	normal	low	normal	normal	normal	normal	normal
GSHA-44	high	low	normal	normal	low	normal	normal	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-45	high	low	normal	normal	low	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-46	high	normal	normal	low	normal	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-47	high	normal	normal	normal	low	normal	normal	high	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-48	high	normal	normal	normal	low	low	normal	normal	normal	high	normal	normal	high	normal	normal	normal	normal	normal
GSHA-49	high	normal	normal	low	low	normal	low	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-50	high	normal	normal	low	low	low	normal	high	normal	high	normal	normal	normal	low	high	normal	normal	latent hypothyroidism
GSHA-51	high	normal	normal	low	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-52	high	normal	normal	normal	low	normal	normal	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-53	high	normal	normal	normal	low	normal	normal	high	normal	high	normal	normal	low	normal	normal	normal	normal	normal
GSHA-54	high	normal	normal	low	low	normal	normal	normal	normal	high	high	normal	low	low	normal	normal	normal	normal
GSHA-55	normal	normal	normal	low	low	low	low	high	high	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-56	high	normal	normal	normal	high	normal	low	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-57	high	low	normal	normal	normal	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-58	high	normal	normal	normal	low	low	low	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-59	normal	normal	normal	low	low	high	high	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-61	normal	normal	normal	low	normal	low	normal	low	normal	low	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-62	high	normal	normal	normal	normal	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-63	high	normal	normal	normal	high	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-64	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-65	high	low	normal	low	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-66	normal	normal	normal	low	normal	low	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-67	high	normal	normal	low	low	low	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-68	high	normal	normal	normal	normal	low	high	high	normal	high	normal	normal	normal	normal	low	normal	normal	latent hyperthyroidism
GSHA-69	high	normal	normal	low	low	normal	low	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-70	high	normal	normal	normal	low	low	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-71	high	normal	normal	low	low	normal	normal	low	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-72	high	low	high	normal	low	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-73	high	normal	normal	low	low	normal	low	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-74	high	low	normal	low	low	low	low	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-75	high	normal	normal	normal	low	normal	high	high	normal	high	high	normal	normal	low	normal	normal	normal	normal
GSHA-76	high	normal	normal	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-77	high	low	high	low	low	low	normal	normal	normal	high	normal	normal	normal	high	normal	normal	normal	normal
GSHA-78	high	normal	normal	low	low	normal	high	high	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-79	normal	low	normal	normal	low	low	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-80	high	normal	normal	normal	low	normal	normal	normal	normal	high	high	normal	NA	normal	normal	normal	normal	normal
GSHA-81	high	normal	normal	normal	low	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal
GSHA-82	high	normal	normal	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GSHA-83	high	normal	normal	low	low	normal	low	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-84	high	normal	normal	normal	low	normal	normal	normal	normal	high	high	normal	normal	normal	high	normal	normal	latent hypothyroidism
GSHA-85	high	normal	normal	low	low	normal	low	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal
GSHA-86	high	normal	normal	normal	low	normal	normal	normal	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-87	high	normal	normal	normal	normal	normal	normal	high	normal	high	high	normal	normal	low	high	normal	normal	latent hypothyroidism
GSHA-88	high	normal	normal	normal	low	normal	normal	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-89	high	low	normal	normal	low	normal	normal	high	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-90	high	normal	normal	normal	low	low	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-91	high	normal	normal	high	low	low	normal	normal	normal	high	high	normal	normal	normal	normal	normal	normal	normal
GSHA-92	high	normal	normal	low	low	normal	normal	normal	normal	high	normal	normal	low	normal	normal	normal	normal	normal
GSHA-93	high	normal	high	normal	high	low	low	high	normal	high	normal	normal	normal	normal	normal	normal	normal	normal
GSHA-94	high	normal	normal	normal	low	normal	normal	high	normal	high	high	normal	NA	normal	normal	normal	normal	normal