Diploma thesis

Predictive factors for a response to rifaximin in patients with irritable bowel syndrome

submitted by

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Affidavit

I hereby declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, 21th of August, 2017 Stefan Valentin Fürst eh

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Abstract

Introduction: Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder. The pathophysiology of the disease is insufficiently explored but alterations of intestinal microbiota including small intestinal bacterial overgrowth (SIBO) are thought to play a role in the development of IBS. SIBO can be diagnosed by lactulose breath test and treated with rifaximin, but studies investigating the efficacy of the non-absorbable antibiotic are controversial, as only a subset of IBS patients with SIBO and also SIBO-negative patients seem to benefit. Moreover, increased intestinal permeability indicated by elevated serum zonulin levels has been found in diarrhea-dominant IBS patients (IBS-D) but not in constipation-dominant IBS patients (IBS-C). Therefore, we investigated the predictive value of lactulose breath test for a response to rifaximin in IBS patients. Furthermore, we measured serum zonulin levels in our patients to find associations with IBS subtypes, symptom severity and alterations of lactulose breath test.

Material and Methods: IBS-D (n=11) and IBS-C (n=8) patients were included. Lactulose breath test (25 g lactulose), measuring both hydrogen and methane every 30 min for 3 h, was performed before and after 10-day rifaximin intake (1200mg/daily) Symptom severity was assessed by a validated questionnaire, the IBS severity scoring system (IBS-SSS). Serum zonulin concentrations were measured before as well as after antibiotic treatment.

Results: Overall, 44% of IBS patients symptomatically improved after rifaximin intake. This improvement was mainly seen in IBS-D patients, who improved significantly (p=0.003), whereas IBS-C patients remained unchanged. IBS-D patients showed a trend for having lower, but not significant, hydrogen values compared to IBS-C patients. Patients with 90 min hydrogen increase < 20 ppm improved significantly (p=0.028), but no significant difference was observed when analyzing absolute hydrogen values between patients with and without improvement. IBS-D patients had significantly higher (p=0.009) serum zonulin values compared to IBS-C patients. Serum zonulin was not associated with symptom severity. Patients with IBS-C and low zonulin levels showed significantly higher (p=0.037) total 3 h H2 excretion. Further, serum zonulin of patients who improved decreased significantly (p=0.046) after rifaximin.

Conclusion: In summary, IBS-D patients improved after a therapy with rifaximin. Even though low hydrogen levels in expiratory air seem to identify patients who will benefit from a therapy with rifaximin, the lactulose breath test did not reveal significant predictive values. We could confirm abnormal high serum zonulin levels in IBS-D patients. Symptom improvement could be associated with an improvement of intestinal permeability indicated by

a decrease of serum zonulin. The positive effect of rifaximin in IBS-D patients may be due to its impact on altered microbiota composition, which is not detectable by lactulose breath test.

Zusammenfassung

Einführung: Reizdarmsyndrom (RDS) ist die häufigste funktionelle gastrointestinale Erkrankung. Die Pathophysiologie dieser Erkrankung ist unzureichend erforscht, jedoch wird angenommen, dass Veränderungen des intestinalen Mikrobioms einschließlich einer Dünndarmfehlbesiedelung (SIBO) eine Rolle in der Entstehung von RDS spielen. SIBO kann durch einen Lactulose Atemtest diagnostiziert und mit Rifaximin behandelt werden. Allerdings sind Studien über die Wirksamkeit des nicht-absorbierbaren Antibiotikums kontrovers, da nur ein Teil der RDS PatientInnen mit SIBO und auch SIBO negative PatientInnen davon profitieren. Darüber hinaus wurde eine erhöhte Darmpermeabilität, angezeigt durch erhöhte Zonulinwerte im Serum, bei Diarrhö-dominanten (RDS-D), nicht aber Obstipations-dominanten (RDS-O) RDS PatientInnen gefunden. Wir untersuchten den prädiktiven Wert des Lactulose Atemtests für ein Therapieansprechen auf Rifaximin bei RDS PatientInnen. Des Weiteren haben wir Zonulinwerte im Serum von unseren PatientInnen gemessen um Zusammenhänge mit dem RDS Subtyp, Schweregrad der Symptome und Veränderungen des Lactulose Atemtests zu finden.

Material und Methoden: PatientInnen mit RDS-D (n=11) und RDS-O (n=8) wurden eingeschlossen. Der Lactulose Atemtest (25 g Lactulose), bei welchem 3 h lang alle 30 min Wasserstoff- und Methanwerte gemessen wurden, wurde vor und nach 10-tägiger Rifaximineinnahme (1200mg/täglich) durchgeführt. Der Schweregrad der Symptome wurde durch einen validierten Fragebogen erhoben. Zonulinwerte im Serum wurden sowohl vor als auch nach antibiotischer Therapie gemessen.

Ergebnisse: Insgesamt haben sich 44% der RDS-PatientInnen symptomatisch durch Rifaximin verbessert. Diese Verbesserung wurde hauptsächlich bei RDS-D PatientInnen beobachtet, welche sich signifikant verbessert haben ($p=0.003$), wohingegen RDS-O PatientInnen unverändert blieben. RDS-D PatientInnen zeigten tendenziell niedrigere, jedoch nicht signifikante, Wasserstoffwerte im Vergleich zu RDS-O PatientInnen. PatientInnen mit Wasserstoffanstiegen < 20 ppm innerhalb von 90 min verbesserten sich signifikant ($p=0.028$), allerdings fand sich kein signifikanter Unterschied bei den Wasserstoffanstiegen zwischen PatientInnen mit und ohne Symptom-Verbesserung. RDS-D PatientInnen hatten signifikant höhere ($p=0.009$) Zonulinwerte im Vergleich zu IBS-O PatientInnen. Es gab keinen Zusammenhang zwischen Zonulinwerten im Serum und Schweregrad der Symptome. PatientInnen mit IBS-C und niedrigen Zonulinwerten zeigten eine signifikant höhere ($p=0.037$) totale H2 Ausscheidung. Zonulinwerte von PatientInnen, welche sich verbessert haben, haben sich nach Rifaximin signifikant verringert ($p=0.046$).

Schlussfolgerung: Zusammenfassend profitieren RDS-D PatientInnen von einer Therapie mit Rifaximin. Obwohl es den Anschein hat, als würden niedrige Wasserstoffwerte PatientInnen kennzeichnen, welche von einer Therapie mit Rifaximin profitieren, fanden wir keine signifikanten prädiktiven Werte im Lactulose Atemtest. Wir konnten abnormal hohe Zonulinwerte im Serum von RDS-D PatientInnen bestätigen. Eine symptomatische Verbesserung könnte mit einer Verbesserung der intestinalen Permeabilität zusammenhängen, welche durch eine Verringerung von Zonulin im Serum angezeigt wird. Die positive Wirkung von Rifaximin könnte durch seine Wirkung auf die veränderte Zusammensetzung des Mikrobioms von RDS-D PatientInnen bedingt sein, welche aber durch den Lactulose Atemtest nicht nachweisbar ist.

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Abbreviations

5

5-HT 5-Hydroxytryptophan

A

ACTH ..... Adrenocorticotropic-hormone

AJ.....Adherens junction

ANS .....Autonomic nervous system

Asymp. sign. ..... Asymptotic significance

AUC.....Area under the curve

B

BSC.....Bristol stool scale

C

CFU .....Colony forming units

CH4..... Methane

CI.. .....Confidence interval

CNS .....Central nervous system

CRF..... Corticotropin-releasing-factor

E

EGFR.....Epidermal growth factor receptor

ELISA…… enzyme linked immunosorbent assay

ENS.....Enteric nervous system

F

FGID..Functional gastrointestinal disorders

FMT.....Fecal microbiota transplantation

FODMAPS….....Fermentable oligo-di-monomonosaracchides and polyols

G

g…. ..... Gram

GHBT .....Glucose hydrogen breath test

GI.....Gastrointestinal

H

H2. ..... Hydrogen

H_2S..... Hydrogen sulfide

HPA .....Hypothalamus-pituitary-axis

I

IBS..... Irritable bowel syndrome

IBS-C.....IBS with constipation

IBS-D..... IBS with diarrhea

IBS-M.....Mixed IBS

IBS-SSS……Irritable bowel syndrome - severity scoring system

IBS-U..... Unclassified IBS

IQR .....Interquartile range

J

JAM ..... Junction adhesion molecule

K

kDa ..... Kilodalton

L

LHBT..... Lactulose hydrogen breath test

M

Max……...Maximum

mg…….Milligram

Min…….Minimum

ml. ..... Millilitre

MLC ..... Myosin light chain

mm..... Millimeter

µl..... Microlitre

N

NCGS .....Non-celiac gluten sensitivity

nm.....Nanometer

O

O2. .....Oxygen

OR .....Odds ratio

P

PAR ..... Protease activating receptor

PI-IBS……..Post-infectious irritbale bowel syndrome

PPI ..... Proton-pump inhibitor

ppm .....Parts per million

R

rpm.....Rounds per minute

S

SIBO…… Small intestinal bacterial overgrowth

SD ..... Standard deviation

SV. ..... Study visit

T

TJ.. .....Tight junction

TMB ..... Tetramethylbenzidine

Z

ZO. .....Zonula occludens protein

Zot.....Zonula occludens toxin

1 Introduction

1.1 The Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder. It is considered to be a disorder of gut-brain interactions and typically characterized by altered bowel habits, discomfort and pain, but symptoms and their severity show a vast difference among affected people. The pathophysiology of IBS is multifactorial and complex and – despite excessive studies on this subject - still poorly understood. Several mechanisms which might play a role in the development of IBS symptoms are an altered gut-brain axis, visceral hypersensitivity, low-grade intestinal inflammation, psychological factors, epigenetic and genetic factors, altered motility, impaired intestinal permeability, dysbiosis and bacterial overgrowth. IBS is associated with numerous other visceral, somatic and psychosocial comorbidities. Hence, a bio-psycho-social point of view is essential for the understanding and management of this medical condition. IBS has a huge impact on the patient's quality of life and is a major task for both, the physician and the patient itself. Due to the high prevalence, IBS induces a major economic burden for the health care system. The diagnosis IBS is made with the help of criteria for functional gastrointestinal disorders based on clinical symptoms and the exclusion of other gastrointestinal diseases such as inflammatory bowel disease or celiac disease. So far, the treatment of IBS usually consists of psychological support in form of a solid physician-patient relationship, dietary interventions and symptom based pharmacological agents, which are often very unsatisfying (1–4).

1.1.1 Epidemiology of IBS: Prevalence and Incidence

The prevalence of IBS varies among countries and depends on the criteria used to define it. Therefore, the world wide prevalence based on population studies ranges from 1.1% to 45%, leading to a pooled prevalence of 11.2%. (95% CI, 9.8%-12.8%) (5). Most European countries are reporting a prevalence of 5-10%. Women are more often affected than men (OR, 1.67; 95% CI, 1.53-1.82), and individuals older than 50 years are less often affected than individuals younger than 50 (OR, 0.75; 95% CI, 0.62-0.92) (5). However, 50% of IBS patients have had their first symptoms before the age of 35 (6). Canavan et al argue that this might indicate spontaneous remissions and that IBS might not be a life-long condition,

as symptoms sometimes seem to improve or even remit over time, otherwise the prevalence would remain constant or even increase (1,7).

The exact incidence of IBS is difficult to determine, due to frequent issues to diagnose this disorder. Affected people are often not under medical care, which results in a discrepancy between the first onset of IBS symptoms and the diagnosis of the disease (7–9). A 12-year population-based study in the United States estimates an annual incidence of 1-2% (10), which might be very similar in central Europe. IBS is neither associated with the socioeconomic status nor with an increased mortality risk (4,5,7,11).

1.1.2 Clinical features and diagnostic aspects

1.1.2.1 Gastrointestinal symptoms

The irritable bowel syndrome is characterized by abdominal pain or discomfort, which is associated with altered bowel habits, e.g. a change in stool frequency and consistency. Beside those main characteristics, IBS can show various other gastro, - and extraintestinal symptoms (12).

IBS is diagnosed according to the Rome criteria, which were first published in 1994 and have been regularly updated since then over the last decades. The newest version, Rome IV, was published in May 2016 and brought some changes of the diagnostic criterion compared to Rome III. However, patients in this prospective study were screened according to Rome III, because the trial started prior to the release of Rome IV (3).

1.1.2.2 Rome criteria

The diagnostic criterion for IBS according to Rome III* are:

*"Recurrent abdominal pain or discomfort** at least 3days/month in the last 3 months associated with two or more of the following:*

- *Improvement with defecation*
- *Onset associated with a change in frequency of stool*
- *Onset associated with a change in form (appearance) of stool*

** Criterion fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis*

*** "Discomfort" means an uncomfortable sensation not described as pain. In pathophysiology research and clinical trials, a pain/discomfort frequency of at least 2 days a week during screening evaluation is recommended for subject eligibility"*

[Directly adopted from (13)]

Depending on the predominant stool form, there are four different IBS subtypes: diarrhea-dominant (IBS-D), constipation-dominant (IBS-C), mixed-type (IBS-M) and unclassified (IBS-U). The subtypes are defined according to Rome III:

Subtype	Bowel habits
IBS-D	>25% loose or watery stool and <25% hard or lumpy tool
IBS-C	>25% hard or lumpy stool and <25% loose or water stool
IBS-M	>25% loose or watery stool and >25% hard or lumpy stool
IBS-U	<25% loose or watery stool and <25% hard or lumpy stool

Table 1: IBS subtypes adapted from (14)

1.1.2.3 Bristol stool scale

The Bristol stool scale (BSC) is a medical tool to asses and classify bowel movements. It was first published in 1997 by Lewis S. and Heaton K. and it helps to monitor the stool appearance, especially of IBS patients (15). BSC categorizes bowel movements into seven different types, whereas type 1 and 2 indicate constipation, type 5, 6 and 7 indicate diarrhea and type 3 and 4 indicate the ideal stool form (14–16).

BSC	Definition	Stool form
Type 1	Separate hard lumps	constipation
Type 2	Lumpy and sausage like	
Type 3	A sausage shape with cracks in the surface	normal
Type 4	Like a smooth, soft sausage or snake	
Type 5	Soft blobs with clear-cut edges	diarrhea
Type 6	Mushy consistency with ragged edges	
Type 7	Liquid consistency with no solid pieces	

Table 2: Bristol stool scale. Adapted from (14).

Beside the above mentioned symptoms to diagnose IBS, there are several other gastro-intestinal symptoms that are non-specific for IBS. Often-described symptoms are bloating with flatulence, the feeling of incomplete evacuation, excessive straining during defecation, urgency of defecation, discharge of mucus with bowel movements and postprandial worsening of symptoms (17,18).

Moreover, some IBS patients show symptoms which are overlapping with other functional gastrointestinal disorders (FGID) like dyspepsia, upper abdominal pain, dysphagia, nausea, gastro-esophageal reflux, early satiety, food intolerance or other organic disorders (13).

1.1.2.4 Extra-intestinal symptoms

IBS is also linked to other functional non-gastrointestinal symptoms like chronic fatigue, fibromyalgia, sleep disorder, muscle and joint pain and uro-gynaecological symptoms, e.g. chronic pelvic pain (19,20). Further, it shows a high comorbidity with psychological disorders such as anxiety and depression (21).

1.1.3 Pathophysiology of IBS and functional gastrointestinal disorders

As already mentioned, IBS belongs to the group of functional gastrointestinal disorders. FGID have previously been defined as a group of disorders with gastrointestinal symptoms, for whom no organic or biochemical cause could be found. With growing scientific knowledge about the pathophysiology of FGID and therefore IBS, the definition according to Rome IV was changed. FGID are considered to be disorders of gut-brain interactions which are characterized by gastrointestinal symptoms. IBS is considered to be of complex origin and shows connections with altered intestinal motility, visceral hypersensitivity, increased intestinal permeability, altered immune function, low-grade inflammation, altered central nervous system (CNS) processing and altered composition of gut microbiota. It is considered to result from interactions between psychosocial factors and altered gut physiology via the gut-brain axis. Given the huge variety of possible underlying causes and connections of IBS, a biopsychosocial approach is essential for the understanding and successful management (3).

1.1.4 Treatment of IBS

Due to the heterogeneous nature of the disease, there is no standard therapy for IBS. Key elements for a successful management are a biopsychosocial point of view and a solid physician-patient-relationship. The therapy is symptom orientated and every patient receives his or her individual treatment plan. This plan may involve dietary interventions, stress management, drug therapy, and psychosomatic, -psychotherapeutic therapy (22).

Patients with diarrhea-dominant IBS may benefit from a high-fiber diet, loperamide, cholestyramine and tricyclic antidepressants. Treatment of pain my include spasmolytics,

phytotherapeutics, probiotics and antidepressants. The basic therapy of constipation-dominant IBS involves increased physical activity, high-fiber diet and adequate hydration. Laxatives such as bisacodyl and macrogol, followed by the prokinetics prucalopride and linaclotide are the next steps in the treatment of IBS-C. Psychotherapy and probiotics are generally recommended for all IBS subtypes (22,23). Antibiotics such as rifaximin are beneficial for a subset of IBS patients and will be discussed later. Further, hypnosis focusing on the abdomen offers a treatment method with no side effects and shows high evidence for a long-term success (22). Some patients might also benefit from complementary or alternative therapies like acupuncture, traditional chinese medicine and homeopathy (23).

1.1.5 The gut-brain-microbiota axis

The term gut-brain-microbiota axis describes the bidirectional communication between the gut, brain and the intestinal microbiota. It is the neuroanatomic and neurophysiological substrate for the biopsychosocial model, which is very essential for the understanding of IBS (24). This communication axis includes the central nervous system (CNS) with the hypothalamus-pituitary-axis (HPA), the sympathetic and parasympathetic nerves of the autonomic nervous system (ANS), the neuroendocrine and neuroimmune system, the enteric nervous system (ENS) and the intestinal microbiota. This network allows reciprocal interactions between the gut and the brain, which is called “top-down” or “bottom-up”, respectively (25). On the one hand, emotions and psychosocial stressors operating through HPA axis and the ANS can influence the intestinal barrier (26–28), motor,- and secretory functions (29) and can even alter microbiota composition in the gut (30), possibly leading to IBS symptoms (25). On the other hand, visceral messages from the gastrointestinal tract can influence mental functions and brain structures (25), which may lead to greater pain and the development of anxiety and depression. Further, even alterations of the gut microbiota can influence brain functioning and vice versa. (1–3,24,31) *(Figure 1)*

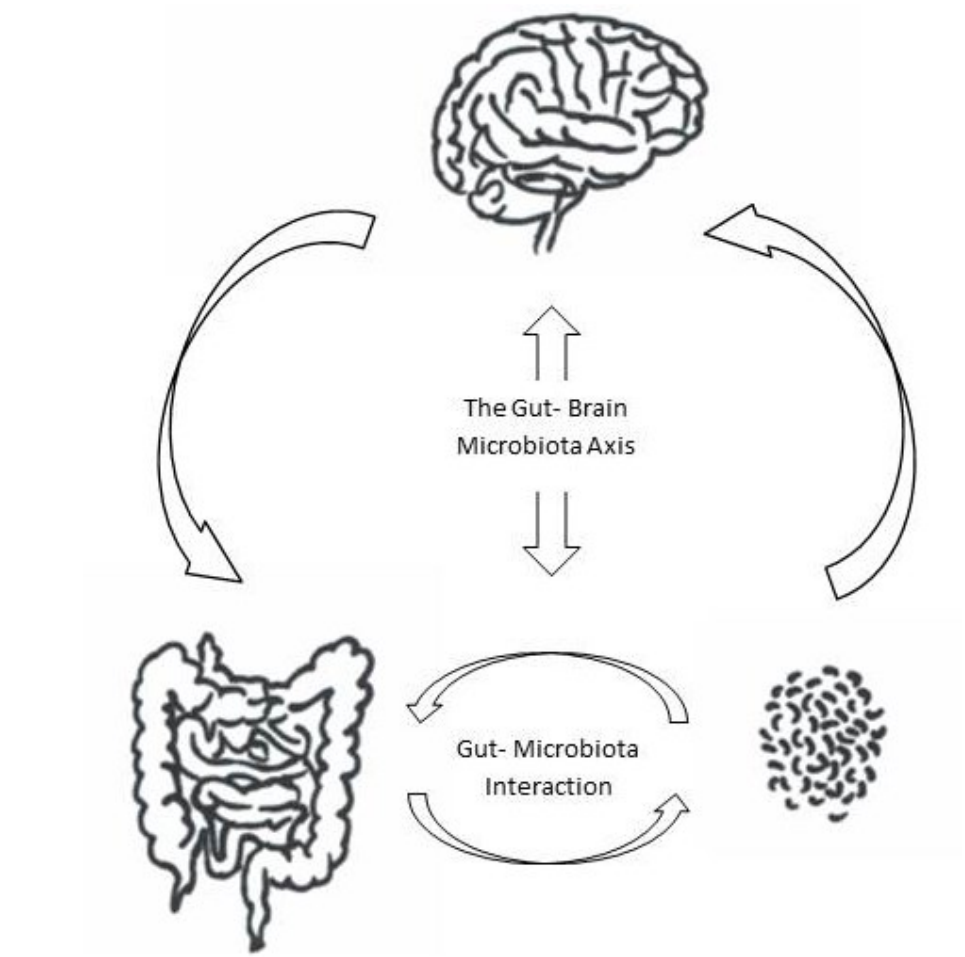

Figure 1: The gut-brain-microbiota axis: On the one hand, emotions and psychosocial stressors operating through the central nervous system, HPA axis, ANS and ENS can influence gastrointestinal functions. On the other hand, visceral messages from the gastrointestinal tract can influence mental functions and even brain structures, which may lead to greater pain and the development of anxiety and depression. Further, even alterations of the gut microbiota can influence brain functioning and vice versa. Figure adapted from (206).

1.1.5.1 Hypothalamus-pituitary-axis

The hypothalamus-pituitary-axis (HPA) is a neuroendocrine regulatory circuit which might play an important role in the development of IBS symptoms. In response to psychological stress, corticotropin-releasing-factor (CRF) is released from the paraventricular nucleus of the hypothalamus and stimulates the pituitary gland to release adrenocorticotropic-hormone (ACTH), which further stimulates the adrenal cortex to release glucocorticoids, in particular cortisol (31). In the following, cortisol inhibits the central CRF production via negative feedback. But this regulatory circuit seems to be impaired in IBS patients, which is leading to hypersecretion of CRF (32,33). CRF then is suspected to induce changes in colonic motility and perception (34). If IBS patients receive non-selective CRF antagonists, which block the CRF receptor, the visceral pain and the exaggerated colonic motility is decreased. On the other hand, CRF administration induces IBS-like symptoms

in healthy individuals and aggravates symptoms in IBS patients, which shows the importance of CRF in gut-brain interactions and its influence on the development of IBS symptoms (34–36).

The influences of stress and activation of the HPA axis on the gastrointestinal tract has been shown in several other studies. For example, chronic stress in rats leads to increased corticosterone release, which subsequently induces intestinal inflammation and mucosal barrier dysfunction (37–41).

The influence of psychosocial stress, especially early life stress, on the gastrointestinal tract was also impressively demonstrated in mice studies with an experiment of maternal separation. Rat pups were removed from the mother's cage for 3 hours a day from post-natal day 2 to 12, which resulted in an enhanced stress response (42), alterations in the pain modulating system via the serotonin system (43),visceral hypersensitivity (42), increased colonic transit, increased intestinal permeability (44), mucosal inflammation and altered composition of microbiota (25,31,42). The reciprocity of this axis was demonstrated by Sudo et al in 2004: mild restrained stress induced an increased release of ACTH and cortisol in germ-free mice, compared to specific pathogen-free controls. The increase of stress hormones was partially reversed by recolonization of fecal matter from the control group and fully reversed by colonization with *Bifidobacterium infantis*. This shows the major influence of the intestinal microbiota on the HPA axis and provides hypothetical opportunities for therapeutic interventions (45).

Important mechanisms for the communication between intestinal bacteria and the brain include the vagus nerve (46) and the influence of bacteria on neurotransmitters. For example, Desbonnet et al described an elevation of plasma tryptophan levels, which is a precursor to the neurotransmitter serotonin (5-HT), induced by a bacterium called *Bifidobacterium infantis 35624* (47).

1.1.6 Visceral hypersensitivity and mast cells

In some patients with IBS, a greater pain sensation in the gastrointestinal tract triggered by distension or bloating is suspected to play an important role in the pathophysiology of the disease. The concept of visceral hypersensitivity is an often-described feature of IBS and has been demonstrated in several studies (48,49). One way to measure visceral hypersensitivity is by using balloon insufflation. Balloons were placed in the rectum of IBS patients and healthy controls and were then inflated to assess the sensory thresholds of both

groups. IBS patients showed a significantly lower threshold which was even worse in periods of psychological stress (49,50). A possible explanation of visceral hypersensitivity might be impaired neurological structures responsible for central pain processing in those patients. More precisely, the anterior cingulate cortex, which has rational cognitive functions and is linked to motivation and emotional response (3), was found to be structurally changed (51,52) and over-activated in IBS patients with colon distension (53).
An important association has been found between mast cell function and visceral hypersensitivity (54–57). Mast cells are more numerous and also more active than usual in the intestinal mucosa of IBS-D patients (58,59). Further, mast cells were found to be closer to nerve endings of afferent enteric nerves in IBS patients compared to healthy individuals (60). Different factors like food antigens or stress can lead to mast cell activation, which then release mediators such as histamine, serine protease, cytokines and prostaglandins (61). It is thought that the protease stimulates the sensory nerve endings and thus contributes to visceral hypersensitivity and further to IBS symptoms (62). Barbara G. et al showed in 2004 that colonic mast cell infiltration and mediator release in proximity to mucosal nerves correlate with abdominal pain and contribute to pain perception (62). Moreover, increased intestinal secretion of water and electrolytes, a feature which is found especially in IBS-D patients, is associated with increased intestinal mast cell activity. Released mast cell mediators such as histamine, chymase and prostaglandin D2 stimulate the intestinal epithelial cells to secrete water and chloride, which alters the stool form (63–65). Beside their influence on the intestinal sensory and secretory function, mast cells are also associated with an altered gut barrier (66). Human studies demonstrated that mast cell mediators increase the intestinal permeability, whereas mast cell stabilizers cause the opposite, namely a decrease in intestinal permeability (67).

1.1.7 Intestinal permeability and tight junction proteins

The intestinal epithelium has an enormous surface of approximately 400m^2 and separates the external environment from the inner milieu. The intestinal barrier has two major functions: on the one hand, it enables the absorption of nutrients, water and electrolytes. On the other hand, it prevents the entry of luminal antigens and microorganism. To maintain the selective permeability, the integrity of the complex working barrier is inevitable. If this barrier is impaired, the intestinal permeability is increased and potential noxious molecules in the gut lumen can penetrate the mucosa and reach the circulation,

leading to immune-mediated diseases. Increased intestinal permeability is suspected to play an important role in the pathophysiology of inflammatory bowel disease, coeliac disease, type 1 diabetes, multiple sclerosis and in IBS patients with diarrhea predominant subtype (26,68–70).

1.1.7.1 Intestinal epithelial structure

The intestinal wall is composed of four different layers: the mucosa, submucosa, muscularis and serosa (26). When talking about the intestinal barrier, we have to take a closer look at the most inner layer, the mucosa. The mucosa itself consists of the lamina epithelialis, followed by the lamina propria and the lamina muscularis mucosae (71). Once molecules from the luminal side cross the epithelial layer or the mucosa, respectively, they enter the submucosal side and get in contact with the inner milieu (26). The single layer of epithelial cells on the luminal side of the mucosa forms the main physical barrier between those two sides (26). Apart from that, the intestinal barrier has other physical as well as immunological and biochemical components and is modulated by the intestinal microbiota (26,72).

1.1.7.2 Intestinal cells and their functions

The intestinal epithelium forms villi alternating with crypts to increase the absorptive surface necessary for nutrient uptake (73). Intestinal epithelial cells live for approximately 5 days and are extruded into the lumen after apoptosis (74). Stem cells located in the crypts of the villi can differentiate into 4 different cell types, namely goblet cells, Paneth cells, enteroendocrine cells and enterocytes (75). Goblet cells produce mucus, which physically prevents intestinal microbes to come to close to the epithelium (26). Paneth cells are found in the crypts and produce antimicrobial factors like alpha-defensins and lysozyms and thus contribute to the chemical barrier function (76,77). Enteroendocrine cells represent less than 1% of mucosal cells and are spread throughout the intestinal tract. They secrete a variety of hormones including serotonin, cholecystokinin, gastrin, ghrelin and somatostatin (78).

Enterocytes represent with 80% the majority of the intestinal epithelial cells. Their main function is the regulation of nutrient-uptake via specific channels, receptors and transporters (79). Therefore, two different pathways can be differentiated: On the one hand, there is an energy dependent transcellular pathway, which is facilitated by selective

transporters for amino acids, sugars, short chain fatty acids and electrolytes (80–82). Another way for substance uptake is the paracellular pathway between neighboring enterocytes. The paracellular pathway is less selective and the substance uptake results from diffusion and osmosis (83). It is regulated by different intercellular connections, especially the so-called tight junctions, which seal the space between adjacent cells (84–87).

1.1.7.2.1 Junctional complexes

There are three different intercellular connections between intestinal epithelial cells, namely tight junctions, adherens junctions and desmosomes. The most luminal junction complexes are tight junctions (TJ), which encircle the apical end of the intercellular space. They are the actual modulators of the selective paracellular permeability and regulate the paracellular flow of water ions and small molecules (88). Below the TJ are the adherens junctions (AJ), followed by the subjacent desmosomes which both act as mechanical linkages between adjacent cells. AJ are important for cell-cell signaling and epithelial restitution and desmosomes support epithelial stability (26).

1.1.7.2.2 Molecular structure of tight junctions

Tight junctions consist of various proteins and the exact composition of the complex depends on the specific function of a particular intestinal region and localization. Important components are the transmembrane proteins occludin, different members of the claudin family and junction adhesion molecules (JAM). The extracellular domains of the transmembrane proteins are connected with the ends of transmembrane proteins of neighboring cells and thus build the paracellular barrier. These anastomoses can either be between identical proteins (homophilic) or non-identical proteins (heterophilic) (68). Claudins are mainly responsible for the intestinal barrier function, whereas occludin and JAM have a regulatory function (79).

The other important group of proteins in the composition of TJ are intracellular proteins, called zonula occludens proteins (ZO-1, ZO-2, ZO-3).(26) These scaffolding proteins are located on the cytoplasmic side of the junctional complex and act as an anchorage, linking the intracellular terminal of the transmembrane proteins with the actin cytoskeleton (89). There, the actomyosin cytoskeleton forms a circular structure - the contractible actomyosin ring - which forms the so-called cytoplasmic plaque or perijunctional actomyosin complex

(90,91). An opening of the intercellular space precedes a contraction of the actomyosin complex. An important step is the phosphorylation of so-called myosin light chains (MLC), a regulatory component of myosin (69). This leads to a structural change of myosin, which increases the tension of the cytoskeleton and further results in the disassembly of TJ proteins and therefore induces paracellular leakage (92,93).

1.1.7.3 Proteases and their role in IBS

There are various mediators known to regulate TJ opening and thus affect the paracellular permeability, such as growth factors, proteases, cytokines, intestinal microbiota and dietary components (69). Regarding IBS, proteases require a closer consideration. One the one hand, proteases are enzymes that hydrolyze peptide bonds and are therefore able to degrade extracellular matrix, mucosal proteins and even bacteria (94). There are several mechanisms to prevent an excessive proteolytic activity, such as the synthesis of inactive precursors (zymogens) or the inhibition by endogenous antiproteases (95).

On the other hand, proteases act as signaling proteins on specific receptors, called protease activating receptors (PAR1-4). PARs in the gut are found in epithelial cells, endothelial cells, mast cells, smooth muscle cells, neurons, inflammatory cells and fibroblast. This indicates that proteases play a role in the regulation of GI processes such as motility, cell proliferation, neurogenic inflammation, pain and permeability (96).

There are different groups of proteases, of which serine proteases are the largest group (97,98). Further, most proteases which activate PAR belong to the serine group (99).

IBS-D patients were found to have 3-fold higher fecal serine protease activity (95,100) but the origin is not entirely clear. It could be due to increased endogenous protease secretion (e.g. from the pancreas), increased presence of microbial proteases or insufficient degradation of endogenous proteases by altered intestinal bacteria (101). The latter theory is supported by the fact that *bifidobacteria*, which produce serine protease inhibitors (102), are found less numerous in fecal and mucosal samples of IBS patients (103,104). However, because oral antibiotic treatment in mice decreased fecal protease activity, the intestinal bacteria are suspected to play an important role maintaining the equilibrium between proteases and their inhibitors (105).

1.1.7.4 Tight junction modulator

In 2000, Fasano et al (106) discovered zonulin, a 47-kDa (107) serine protease analogue and first-known endogenous tight junction modulator. Zonulin is similar to zonula occludens toxin (Zot), which has a similar effect on permeability and was previously discovered in Vibrio cholera (108). It is also known as pre-haptoglobin 2, because it acts as a hemoglobin scavenger in its cleaved form. In its intact single chain form (i.e. Zonulin) it regulates intestinal permeability. Zonulin transactivates epidermal growth factor receptor (EGFR) through PAR2, which starts an intracellular pathway leading to the disengagement of zonula occludens protein (ZO1), resulting in the disassembly of tight junctions (109). *(Figure 2)* According to Fasano et al, zonulin is only secreted in the small intestine as a result of two, so far known, triggers: first, luminal exposure to enteric bacteria. Fasano et al argued, that zonulin-induced hyperpermeability might be a defense mechanism of the small intestine against bacterial overgrowth (109). The zonulin induced opening of the paracellular pathway leads to an increased luminal water secretion, whereby bacteria are flushed out (110,111). Secondly, gliadin, which is found in wheat, triggers the release of zonulin by binding at the chemokine receptor CXCR3, which is overexpressed in celiac patients (112). Patients with celiac disease (106) and recently also with non-celiac gluten sensitivity (NCGS) (113) were found to have increased intestinal permeability and higher serum zonulin levels compared to controls. Zonulin concentrations can be measured in serum samples and have found to be elevated in patients with various immune–mediated diseases such as type 1 diabetes (114). In 2015, Barbaro et al found out that serum zonulin concentrations in IBS-D patients are higher compared to healthy controls, possibly leading to an increased intestinal permeability (113). These findings underline the results of Martinez et al, who described ultrastructural abnormalities of jejunal intercellular connections in IBS-D patients. These alterations correlated with mast cell activity and clinical symptoms (70).

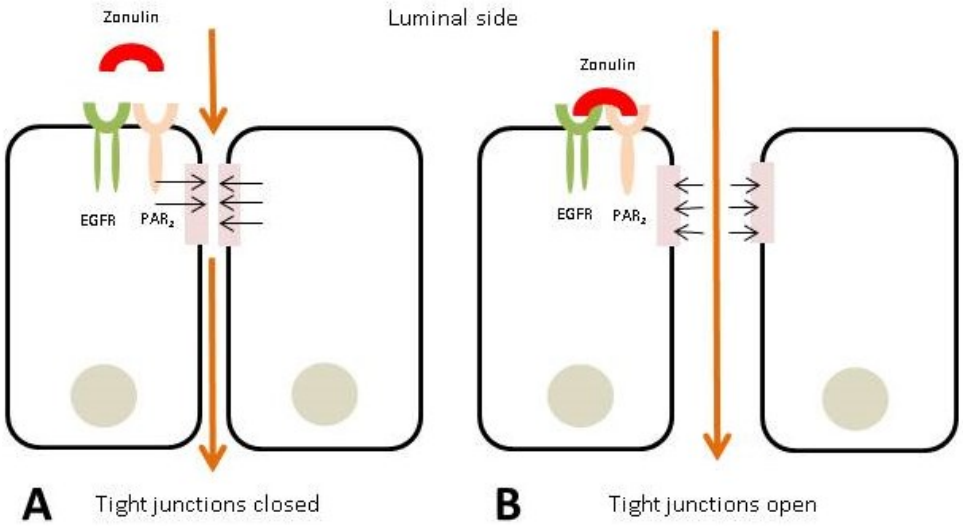

Figure 2: Schematic presentation of zonulin mechanism. A) During the resting state, tight junctions are closed and seal the paracellular space. B) Zonulin binds on EGRF and PAR2 and thus activates an intracellular cascade, leading to the disengagement of TJ proteins. As a result, the paracellular space is no longer sealed by TJ and the intestinal permeability is increased. Figure adopted from (109)

1.1.8 Intestinal microbiota

The intestinal microbiota, by many described as the "neglected organ"(115), consists of 500 to 1000 different bacterial species per subject (116) with 10^{14} bacterial cells, which outnumber the cells of all other organs in the human body combined ten times (117). The intestinal microbiota mainly consists of bacteria but also includes viruses, protozoa, fungi and archaea (31,118,119). Commensal bacteria have a vast amount of different functions such as the production of enzymes and metabolites which are important for digestion of nutrients (120,121). Intestinal bacteria are essential for the development and function of the intestinal immune system (115,116) and further contribute to the maintenance of gut homeostasis by preventing the colonization of pathogenic microorganisms through various defense mechanisms (124–129). As previously explained, the intestinal microbiota also play an important role in the bidirectional communication between the gut and the brain (30). As Mayer et al summarize (30), they are able to influence the intestinal permeability (130), the mucosal immune response (131), the ENS (132), HPA axis (45) and intestinal pain sensation (133). Therefore, a change in the composition of intestinal microbiota is thought to contribute to IBS symptoms and their development (30).

So far, more than 50 phyla have been identified in the human intestinal tract (134). The two largest phyla colonizing the gut are *Bacteroidetes* and *Firmicutes*, followed by *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* (128,129).

A change in the composition of the intestinal microbiota is termed as "dysbiosis" (124) and was found to be present in IBS patients (137–139). However, studies about microbial alterations in IBS patients are not consistent (30,124) For example, studies report an increased number of *Firmicutes* and a decreased number of *Bacteroidetes* in the majority of IBS patients (30,140). Further, the two genera *Bifidobacterium* and *Lactobacillus* are often found less numerous in fecal samples of IBS patients compared to healthy controls (30). It is still unclear, whether these alterations are primary or secondary (1,4,141).

1.1.8.1 Bacterial metabolites

Another important function of the intestinal microbiota involves the degradation of non-digestible dietary ingredients (142). So-called FODMAPS, fermentable oligo-di-monosaracchides and polyols, are only poorly absorbed in the small intestine and are fermented by bacteria in the large intestine (143). The main fermentative end-products of FODMAP's are short chain fatty acids (SCFA's) such as butyrate, propionate and acetate (30). As Bennet et al summarized (124), butyrate acts as an important energy source for gastrointestinal epithelial cells and has an anti-inflammatory effect (144). Further, butyrate regulates tight junction proteins (145) and colon epithelial mucin production (146) and thus improves the intestinal barrier function (147,148). Reduced levels of butyrate-producing bacteria were found in IBS patients (103,149–152) and might additionally explain symptom development in a subset of IBS patients (124). However, because a high intake of non-absorbable carbohydrates is associated with IBS-like symptoms such as bloating and flatulence (153), a diet low in FODMAP's can reduce symptoms (143) and is often recommended for IBS patients (143,154).

Another metabolite is methane, a gas that is generated by Methanogens (Archaea) through conversion of hydrogen, which is produced through fermentation. Methane was found to slow down gut transit (155,156), which might explain symptoms of IBS-C patients. Patients suffering from constipation-dominant IBS were found to have higher volumes of methane and Methanogens (157–159), predominantly *Methanobrevibactersmithii* (159), which converts 4 atoms of hydrogen to 1 atom of methane (160). *Methanobrevibactersmithii* is found in the gut flora in 15% to 30% of all humans (160).

The connection between microbial alterations and symptoms of IBS was further shown in an impressive animal model: the microbiota of IBS-D patients, showing an increased number of sulfate-reducing bacteria and *Enterobacteriaceae* and less *Bifidobacteria*, was

transferred to rats via fecal microbiota transplantation and resulted in increased colonic sensitivity in those animals (161). The microbiota-induced hypersensitivity was linked to an increased number of sulfate-reducing bacteria and their metabolic product hydrogen sulfide (H_2S), which was also previously found to be higher in IBS-C patients (151). H_2S is considered to be a gaseous neurotransmitter that facilitates visceral pain and plays an important pro-nociceptive role (162).

Other bacterial metabolites which are thought to play a role in a subset of IBS patients are secondary bile acids. Primary bile acids are synthesized in the liver and secreted in the intestine, were some of them are converted into secondary bile acids by intestinal microbiota. It is possible, that a change in microbiota composition leads to a different bile acid pool in the large intestines with a shift towards bile acids that induce IBS symptoms (27,163).

1.1.8.2 Post infectious IBS

Post infectious IBS (PI-IBS) is most likely the strongest evidence for the involvement of microbiota in the pathophysiology of IBS.(124) PI-IBS describes the development of IBS following a bacterial, viral or parasitic infection of the gastrointestinal tract.(164,165) Risk factors for PI-IBS include the severity and duration of the gastroenteritis, young age, female sex and psychological conditions such as anxiety and depression.(164–166) Most PI-IBS follow bacterial infections with an incidence ranging from 4% to 32%, with a mean of 10%.(167) The underlying pathophysiology is not entirely clear but involves an impaired intestinal permeability in connection with a persistent mucosal inflammation and infection-induced dysbiosis.(4)

1.1.8.3 Small intestinal bacterial overgrowth

Small intestinal bacterial overgrowth (SIBO) describes a condition, where an increased number of bacteria are found in the small bowel (168). As Ghoshal UC et al summarize (169), affected patients can present with symptoms such as bloating, flatulence, abdominal pain and loose bowel movements (170,171) due to fermentation of carbohydrates and the resulting excessive gas production (172,173). It is suggested that SIBO plays an important role in the development of IBS, but this relationship is seen controversially. The frequency of SIBO ranges from 4% to 78% among IBS patients and from 1% to 40% among healthy controls. The large discrepancies of the prevalence mainly result from different methods

and criteria to diagnose SIBO (169). SIBO is associated with different factors among IBS patients, such as female gender, older age and diarrheal subtype (168,172.(170,174) Further, it was shown that the stool form in IBS patients with SIBO depends on the amount of bacteria in the small bowel: the more colony forming units (CFU), the looser the bowel movements (175). Several studies suggested that PPI-intake might be a risk factor for the development of SIBO (170,176). However, a recent study (177) showed that PPI-intake did not influence the prevalence of SIBO in IBS-patients.

1.1.8.3.1 Diagnosis of SIBO

Normally, the small intestine harbors 10^0 to 10^3 colony forming units per ml (CFU/ml) in the duodenum and jejunum (178), which mainly consist of gram-positive aerobic bacteria. The ileum already harbors a denser bacterial environment with 10^5 to 10^8 CFU/ml of colonic-type bacteria, mainly consisting of gram-negative aerobes, obligate anaerobes and enterococci. The majority of intestinal bacteria, namely 70%, resides in the colon (135), where mainly gram-negative obligate anaerobes and facultative anaerobes (116,179) are found in a density of 10^{10} to 10^{12} CFU/ml (178).

A quantitative culture of jejunal aspirat is considered the gold standard to diagnose SIBO. The diagnosis is made when more than 10^5 CFU/ml of colonic-type bacteria are detected (180). However, this method is quite invasive and difficult to perform, hence culture-independent hydrogen breath tests are often used because they are cheap and non-invasive alternatives (169). The breath tests work according to the following theoretical principle: bacterial fermentation of carbohydrates produces hydrogen and methane, of which 80% are eliminated with flatus. The remaining 20% diffuse into systemic circulation and are exhaled by the lungs, which is measureable by breath test devices using gas chromatography (169,181). *(Figure 3)* Two substrates are used for the detection of SIBO, either glucose or lactulose. Usually, glucose is absorbed in the proximal small intestine and does not result in significant gas production. If a patient has SIBO with higher bacterial counts in the proximal part of the intestines expiratory hydrogen concentrations become detectable. Lactulose, on the other hand, is non-absorbable and naturally fermented by colonic bacteria; therefore a hydrogen rise in expiratory air is expected once lactulose arrives in the caecum. An early hydrogen peak should indicate a pre-colonic fermentation due to SIBO (182–184).

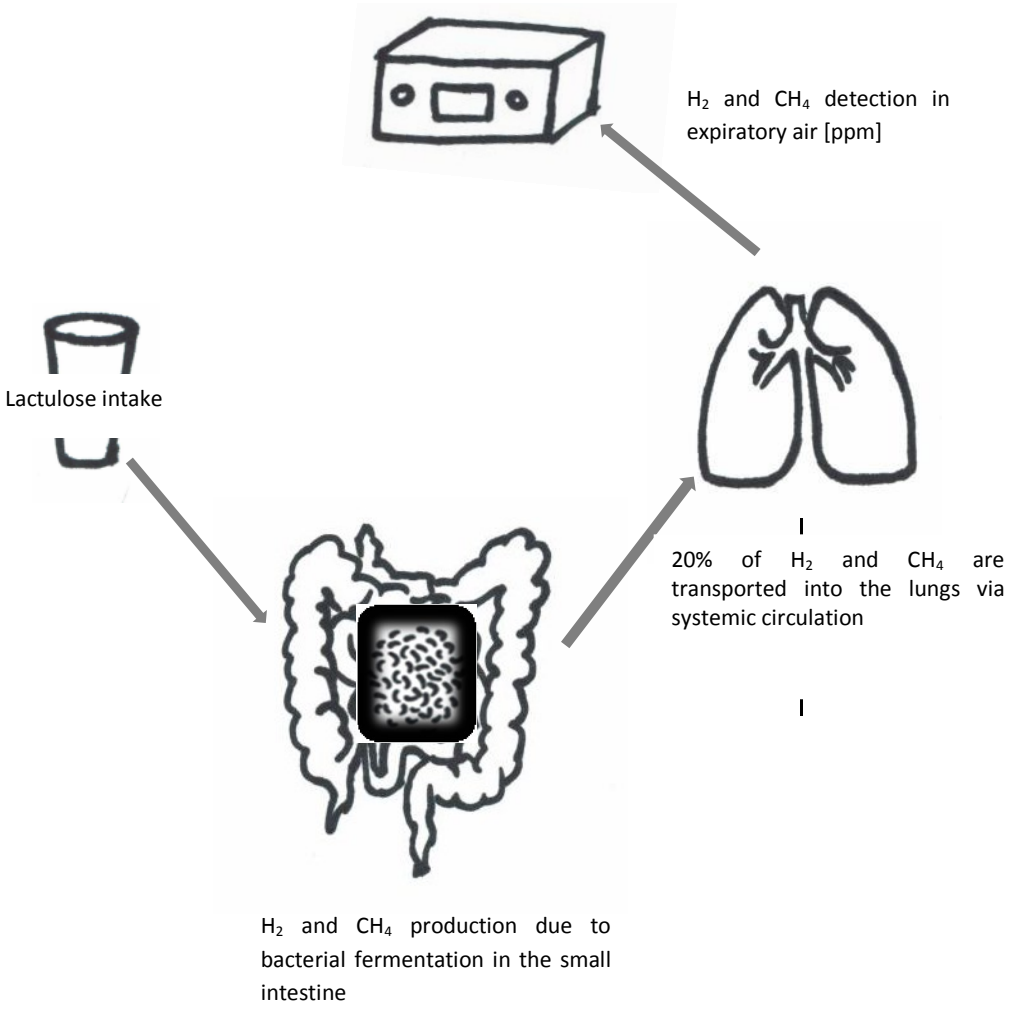

Figure 3: Theoretical principle of breath tests: Bacterial fermentation of the ingested substrate (lactulose) in the small and large intestine results in H2 and CH4 production. Parts of these gases diffuse into the systemic circulation and are expired by the lungs, where they can be measured using gas chromatography. Figure adopted from (185)

1.1.8.3.2 Diagnostic criteria for SIBO

Besides lack of standardized performance of SIBO tests, diagnostic criteria for SIBO vary widely amongst different authors which leads to significantly different prevalence rates (169,181). For glucose hydrogen breath tests (GHBT), most authors consider a rise of breath hydrogen of 12 ppm or more above basal levels indicative of SIBO. The amount of glucose administered to the patient ranges between 50 and 100 g glucose (185). Ghoshal U.C.et al summarize in a review, that the GHBT is highly specific (78% to 97%) but lacks sensitivity (15.7% to 62%) (169).

Lactulose hydrogen breath tests (LHBT) are usually performed with 10 to 25 g of lactulose (181). Initial trials conducted by Pimentel et al (186) suggested hydrogen increases of 20 ppm or more above basal levels within 90 minutes to be diagnostic of SIBO but turned out

to create false positive results with a specifity of 65% to 97.9%. The sensitivity of LHBT ranges from 31% to 68% (169). Further, high basal breath hydrogen values greater than 10 ppm or 12 ppm, depending on the author, are also suggested to indicate SIBO, especially of the proximal small intestine (187–189). Previously, oro-caecal transit time was assumed to be longer than 90 minutes, which is obviously not the case in most patients, as reviewed by Ghoshal et al and Aziz et al. This leads to the limitations of LHBT and with-it the overestimation of the prevalence SIBO (169,181).

Beside hydrogen, expiratory methane values are also used to diagnose SIBO in LHBT. The idea is to detect patients with methane producing gut bacteria, who would otherwise be SIBO negative because of low breath hydrogen levels (185). According to a new consensus from 2017, methane values above 10 ppm, which was previously 3 ppm, regardless of the time point is suggested to be indicative of SIBO (190).

1.1.8.3.3 Therapy of SIBO

Various broad-spectrum antibiotics have been used over the last decades to treat SIBO, of which rifaximin turned out to be the most effective and safest (191). Rifaximin is a broad-spectrum antibiotic that covers gram-positive and gram-negative, both aerobic and anaerobic bacteria (191,192). Chemically, rifaximin is a nonsystemic derivate of rifamycin that holds an extra pyrido-imidazole ring. This structural difference minimizes the systemic absorption of the substance in the gut (193), leading to a high solubility in the small intestine which allows rifaximin to act mainly locally (192). As Gatta L. et al summarize, the recommended dosage of rifaximin varies between studies and ranges from 600 mg/day to 1600 mg/day, for a duration between 5 and 28 days (191).

Ghoshal et al summarize, that SIBO is eradicated by rifaximin in 40.8% to 64.1% of cases and thus improves symptoms (169). Further, rifaximin has shown to relief global IBS symptoms and bloating in IBS-D patients (169,181,192). Rifaximin is already approved for therapeutic use in adult diarrhea-predominant IBS patients in the United States (181), but is not yet approved in Europe (1). In their study, Pimentel et al showed that a two-week treatment of IBS-D patients with rifaximin achieves symptom improvement for more than 12 weeks (192). The exact mechanisms for improvement of symptoms in IBS patients due to rifaximin are not entirely revealed; SIBO might only play a role in some cases (192). According to Pimentel, the antibiotic effect of rifaximin might lead to a microbial resetting, which reduces bacterial fermentation and thus contributes to symptom

improvement. Furthermore, a positive effect is suggested to result from modulation of gut motility and host inflammatory responses (1,4,192).

1.1.8.3.4 Controversy

However, there are controversial and contradicting results on the predictive value of the LHBT for a successful treatment with rifaximin (162,186). Most studies report high hydrogen values to be predictive for SIBO and with-it, for a successful response to a therapy with rifaximin (162,184,187). However, a recent study by Kasir et al introduced different diagnostic criteria for SIBO which showed better predictive values regarding symptom improvement after therapy with rifaximin and therefore, emphasizes current problems with diagnostic criteria (194). Further, not all IBS patients with SIBO improve and show a normalization of breath test values after antibiotic treatment with rifaximin (192). For example, a meta-analysis reported an overall normalization rate of breath tests of 49% (196). On the other hand, even a subset of SIBO-negative IBS patients symptomatically improve after rifaximin (188,197).

1.2 Hypothesis and aim

We have had two primary objectives in this clinical trial. On the one hand, our aim was to evaluate the predictive value of LHBT for symptom improvement after a ten-day treatment with rifaximin in IBS patients of different subtypes. Here, we hypothesize that symptom improvement in IBS patients with pathologic LHBT (indicative of SIBO) will be more pronounced than in patients with normal LHBT since in patients with extensive bacterial colonization of proximal parts of the intestines, reduction of bacterial numbers will lead to decreased bacterial production of gas and consequently to an improvement of intestinal symptoms. Furthermore, we hypothesize that symptom improvement will be accompanied by improvement in LHBT values.

Our second aim was to study the prevalence of elevated serum zonulin levels indicating increased intestinal permeability in our IBS patients and to correlate zonulin values with IBS subtypes, symptom severity and LHBT findings. We hypothesize that IBS symptoms are influenced by an increased permeability; therefore we expect a positive correlation between symptom severity and serum zonulin levels. Further, we wanted to study whether an administration of oral antibiotic rifaximin leads to a change in intestinal permeability measured by the zonulin serum levels.

2 Material and Methods

2.1 Overview

This diploma thesis is part of a prospective, single-center, randomized and controlled double-blind pilot study conducted at the Clinical Division of Gastroenterology and Hepatology of the Medical University of Graz. The pilot study investigates the effect of fecal microbiota transplantation (FMT) in patients with irritable bowel.

In the first part of the study, IBS patients are performing a lactulose breath test, followed by a 10-day intake of an oral non-absorbable antibiotic drug (rifaximin, 400mg three times daily), after which a second lactulose breath test is performed. Rifaximin was given to patients as a preparation for the following FMT.

This diploma thesis is an interim analysis of the data gathered during the first part to evaluate the effect of a 10-day intercourse of rifaximin on symptom severity, lactulose breath test and serum zonulin levels in IBS patients.

2.1.1 Study design

Consecutive patients with IBS were enrolled. After signing informed consent at the first study visit, patients were asked to complete a validated German symptom severity score for IBS, the IBS-SSS. Furthermore, blood samples were taken to measure serum zonulin levels and patients had to perform the lactulose breath test. The first study visit was followed by a 10-day intake of rifaximin, an oral non-absorbable antibiotic drug, which was administered in a dosage of 400mg three times daily for a total dosage of 1200 mg per day. At the second study visit after the antibiotic treatment, a re-evaluation of the IBS-SSS, a second blood collection for zonulin measurements as well as a lactulose breath test took place. *(Figure 4)*

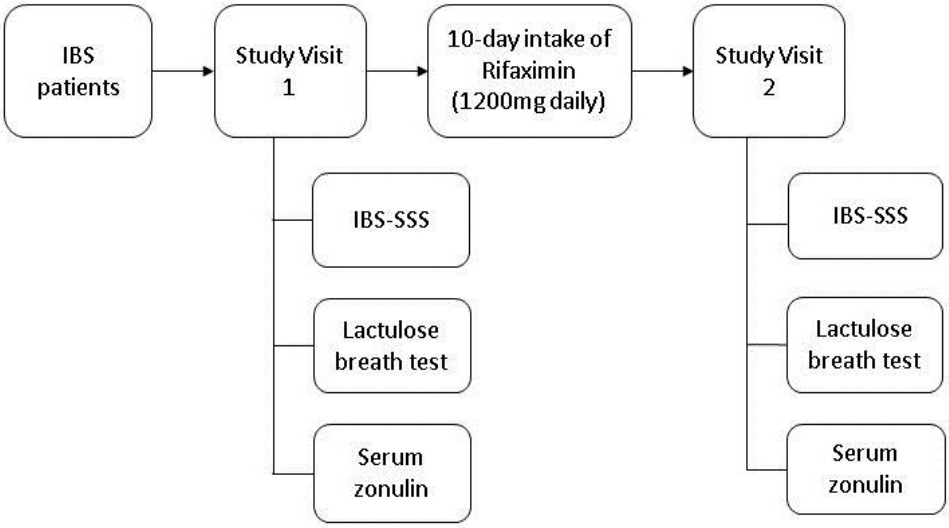

Figure 4: Study design

2.1.2 Control Group

To compare the measured serum zonulin levels of the IBS patients with healthy individuals, an age-matched control-group was included in this study. Healthy controls were recruited and serum zonulin was measured in 2015 in the context of a previous clinical trial conducted by Ass. Prof.Vanessa Stadlbauer-Köllner and Mag.rer.nat. Angela Horvath, PhD. et al. at the Medical University of Graz, Department of Gastroenterology and Hepatology (198).

2.1.3 In- and exclusion criteria

The study participants were screened as outpatients at the Clinical Division of Gastroenterology and Hepatology of the Medical University of Graz and have been selected according to the following in- and exclusion criteria. *(Table 3 and 4)*

Nr.	Inclusion criteria
1.	Adult patients with age > 18
2.	Informed consent
3.	Diagnosed IBS according to ROME III criteria
4.	Anti-tissue transglutaminase antibodies negative
5.	Colonoscopy and gastroscopy - including histology – without significant pathological findings
6.	Stool calprotectin<300 µg/g

Table 3: Inclusion criteria of the interim analysis of the prospective study.

Nr.	Exclusion criteria
1.	Pregnancy
2.	Lactation
3.	Coagulation disorder
4.	Oral anticoagulant drugs
5.	Severe chronic disease
6.	Secondary motility disorder (e.g. Parkinson's Disease)
7.	Major abdominal surgeries (e.g. Hemicolectomy)
8.	Participation in another clinical trial
9.	Unclassified or mixed IBS subtype

Table 4: Exclusion criteria of the interim analysis of the prospective study. Our aim was to include 20 patients of either IBS-D or IBS-O subtype for the interim analysis; hence IBS patients with unclassified or mixed subtype were exlcuded from the analysis but are still included in the prospective FMT study.

2.2 Irritable bowel syndrome – severity scoring system

The irritable bowel syndrome – severity scoring system (IBS-SSS) is a tool to evaluate the severity of IBS symptoms. It was first validated in 1997 by Francis et al. in its English version, showing a very high sensitivity as well as a high reproducibility and sensitivity to assess changes in symptom severity.(199) In 2012, the IBS-SSS was validated by C. Betz et al. in its German version, making the scoring system a valuable instrument to monitor the severity of irritable bowel syndrome.(200)

The IBS-SSS is a questionnaire consisting of five questions. Each question generates a maximum score of 100 points, leading to a total possible IBS-SSS of 500 points. Those five questions include pain, distension, bowel habits and quality of life. The first two questions target pain, both severity and duration. The former is assessed by asking the patient about the severity of pain, the latter is assessed by the number of days pain usually occurs in 10 days, where e.g. 4 out of 10 equals 40 points. The next question targets distension by asking about abdominal tightness or bloating. Question four rates satisfaction of the patient's bowel habits. The last question assesses global well-being by asking about how IBS symptoms are interfering with the patient's life in general.

All questions, except the one about pain duration, are answered by using a visual analog scale from 0% to 100%, whereas 0% means no symptom and 100% means very severe.

As Francis et al. state in their study, a mild IBS is indicated by a score of 75 to 175, a moderate by 175 to 300 and a severe IBS by a score above 300. A patient with a score below 75 is considered to be in remission. *(Table 5)* Further, a change of 50 points reliably

indicates symptom improvement (199). *(See "Appendix" for a validated German version of IBS-SSS)*

Patients in our prospective study completed the IBS questionnaire at the first study visit as well as at the second study visit after the 10-day antibiotic treatment, to assess a possible change in the severity of their symptoms.

IBS-SSS	Clinical graduation
< 75	Remission
75 – 175	Mild
175 – 300	Moderate
> 300	severe

Table 5: Clinical severity classification of IBS based on IBS-SSS

2.3 Zonulin measurement

Twelve-hour fasting venous blood was drawn from the patients at study visit 1 as well as at study visit 2.The blood was collected in serum tubes (VACUETTE®, Greiner Bio-One International) and centrifuged for 15 min with 1500 rpm at 4 °C. The serum was then frozen at -80 °C until subsequent use.

To measure zonulin levels in serum we used the IDK® zonulin enzyme linked immunosorbent assay Kit (K5601) from Immundiagnostik (Benheim, Germany) according to the manual provided. This assay is based on the method of competitive ELISA.

Before we started, all reagents and samples reached room temperature (15-30° C) and were well mixed. As a first preparation step, we diluted 25 µl of our serum samples with 475 µl dilution buffer, resulting in a dilution factor of 20. We then added 150 µl tracer (biotinylated zonulin) to 150 µl of the diluted sample standards and controls. In the next step, 100 µl of samples, standards and controls were transferred into microtiter plate wells, which were coated with polyclonal anti-zonulin antibodies. During the following incubation period, the free antigen in our samples competes with the tracer (biotinylated zonulin) for the binding with the antibodies (anti-zonulin antibodies) in the plate well. After one hour incubation on a horizontal shaker with 550 rpm with an orbit of 2 mm at room temperature, we decanted the content of each well and washed the microtiter plate 5 times with a washing buffer. Thereby the unbound components were removed. Next, we added 100 µl peroxidase-labeled streptavidin, which binds to the biotinylated zonulin, and

incubated again for one hour on the horizontal shaker with the above-mentioned settings. After a second washing step with washing buffer, we added the peroxidase substrate tetramethylbenzidine (TMB) and incubated again for 15 minutes at room temperature, until the color changed distinctively and visibly from transparent to blue, which furthermore turned into a yellow color after adding acidic stop solution. The intensity of the yellow color is inverse proportional to the zonulin concentration in our samples. As a last step, the absorbance of the enzymatic reaction was immediately measured at 450 nm using a photometer.

2.4 Lactulose breath test

Lactulose breath tests were performed at study visit 1 and 2, using the GastroCH4ECK® ("Bedfont Scientific Ltd.") to measure both, the concentration of H2 (Hydrogen) and CH4 (Methane), in parts per million (ppm) in the expiratory air. The measurements were done after a 12-hour fasting period prior to testing and according to the user manual provided. Patients were instructed not to eat or drink anything on the day of the breath test, except water without gas. Furthermore, patients were asked to abstain from cigarette smoking and not to use toothpaste on the day of the test. After measurement of baseline H2, CH4 and O2 (as a correcting factor) concentrations in expiratory air, patients were given 25g lactulose dissolved in 250 ml water. Subsequently, repeated measurements were performed every 30 minutes for the following 180 minutes and results in parts per million were recorded by the patients in provided data sheets.

Due to a technical defect, a second breath test device had to be used in 5 out of 19 patients, which was the "EC60 Gastrolyzer 2®, Bedfont Scientific Ltd". This device slightly differed from the first device and measured only H2 and O2 concentration in expiratory air; hence CH4 concentrations in expiratory air were not available in 5 of 19 patients. *(Figure 5)*

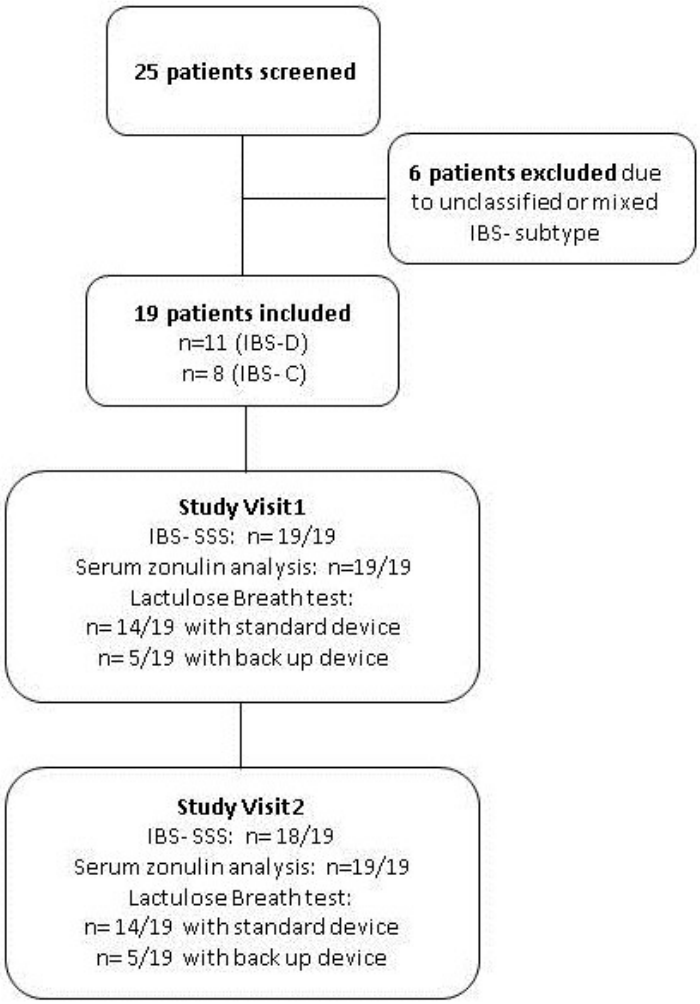

Figure 5: Workflow.

2.4.1 Breath test interpretation

Hydrogen absolute values as well as increases above basal levels at all time points were analyzed in connection with subtype, symptom improvement and serum zonulin levels. Early hydrogen rises, especially above 20 ppm in 90 min, were evaluated in detail, as this represents the most used diagnostic criterion for SIBO in LHBT.

Similar analyses were performed regarding methane, whereas values above 3 ppm or above10 ppm, depending on the authors, were regarded indicative of SIBO.

2.5 Statistical analysis

Microsoft Excel 2017 was used for data collection. Further statistical analysis was performed by using IBM SPSS statistics 23. Normal distribution was tested by using the Kolmorogov-Smirnov test. Mean and standard deviation (SD) was used to describe

normal-distributed variables, median and interquartile range (IQR) was used to describe non-normal distributed variables. If variables were normally distributed, differences between two groups were assessed by using student's t-test for independent samples and paired samples, respectively. The equality of variances was assessed by using Levene's test. Welch's test was applied if unequal variances were present. Differences between two groups which were not normally distributed were assessed by using the Mann-Whitney U test for independent samples and Wilcoxon signed-rank test for paired samples. Chi square or Fisher's exact test was used, depending on whether the assumption was violated or not, to assess a significant correlation between two nominal scaled variables. Further, we used Phi to measure the strength of the correlation.

Pearson's correlation test was used to identify correlations between two normally distributed variables. If variables were not normally distributed, Spearman's rho was used.

The area under the curve (AUC) was calculated based on absolute H2 values at all time points as measure of overall H2 excretion. A p-value < 0,05 was considered to be statistically significant. P-values are presented as asymptotic significances.

2.5.1 Graphics - Boxplots

Outlier are presented by * and °. The top whisker shows the highest case within 1.5 times IQR. The top of the box shows the 3rd quartile. The line in the middle of the box shows the median value. The bottom of the box shows the 1st quartile. The bottom whisker shows the lowest case within 1.5 times IQR.

2.6 Ethical considerations

An application for the pilot study has been filed at the Ethics Committee of the Medical University of Graz. Informed consent was obtained in accordance with the declaration of Helsinki for all participants included in the study.

3 Results

3.1 Study cohort

Between January 2016 and March 2017, 25 consecutive patients were evaluated for inclusion, of whom 6 patients were excluded for this analysis and 19 were enrolled. Furthermore, 10 healthy controls were included in the zonulin control group, to compare the measured serum zonulin levels of IBS patients with the zonulin levels of healthy controls.

3.1.1 Demography

From 19 IBS patients, 10 were male and 9 were female. The mean age in male and female patients was 37 (±9) and 39 (±15), respectively. The average age of all 19 patients was 38 (±12). Concerning the IBS subtype, 11 patients belonged to the diarrhea and 8 to the constipation group. *(Table 6 and 7)*

Gender	Count	Mean age	SD	Min	Max
male	10	37	±9	24	48
female	9	39	±15	24	68

Table 6: Demographic data of IBS patients

Gender	IBS-D	IBS-C	Total
male	5	5	10
female	6	3	9
Total	11	8	19

Table 7: IBS subtype in relation to gender

3.2 Analysis of IBS-SSS

3.2.1 IBS-SSS and subtypes

The IBS-SSS was completed by 19 patients prior and 18 patients after antibiotic treatment; hence the score and its change could be analyzed in 18 patients.

The mean IBS-SSS at baseline was 320 (±59) in IBS-D patients, compared to 283 (±87) in IBS-C patients, which did not differ significantly (p=0.258). After antibiotic treatment, the mean score in IBS-D patients was 220 (±851), compared to 291 (±88) in IBS-C patients.

Again, the post-antibiotic IBS-SSS did not differ significantly (p=0.104) between the subgroups. However, patients in the diarrhea group improved significantly (p=0.003) on average by -100 (± 78) points, whereas the score of patients in the constipation group did non-significantly (p=0.801) change on average by +8 (± 85). The mean improvement of the IBS-SSS was significantly higher (p=0.012) in IBS-D patients compared to IBS-C patients. *(Table 8 and figure 6)*

	Before rifaximin			**After rifaximin**			**Change of IBS-SSS**		
	All patients	***IBS-D n=10***	***IBS-C n=8***	***All patients***	***IBS-D n=10***	***IBS-C n=8***	***All patients***	***IBS-D n=10***	***IBS-C n=8***
Mean	304	320	283	252	220	291	-52	-100	8
SD	±73	±59	±87	92	±86	±88	96	±77	±85
Median	310	326	271	261	225	314	-35	-77	-12
Min	171	211	171	113	113	146	-215	-215	-112
Max	415	415	384	394	394	378	150	-8	150
IQR	121	86	177	189	117	165	119	145	124
Asymp. Sign (2-tailed)		p=0.285			p=0.104		p=0.034*	p=0.012*	
								p=0.003*	p=0.801

Table 8: IBS-SSS of IBS patients. IBS-SSS before and after rifaximin for the total patient cohort (IBS-D and IBS-C) and for separated subtypes are shown. Statistical analysis showed no significant difference of IBS-SSS between IBS-D and IBS-C patients before (p=0.285) or after (p=0.104) rifaximin intake. Overall, IBS patients improved significantly (p=0.034) by a mean change of -52 points. This significant results is based on the change of IBS-D patients, who improved significantly (p=0.003) on average by -100 points, whereas IBS-O patients changed non-significantly (p=0.801) by an average of +8. The mean changes/improvements differed significantly between the subtypes (p=0.012). (Significant p-values are marked with *)

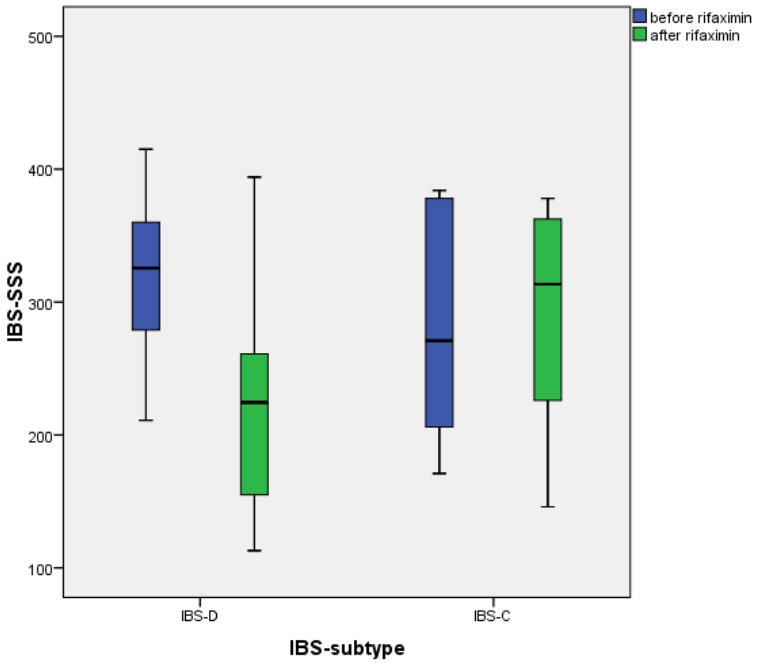

Figure 6: IBS-SSS before and after rifaximin in IBS subtypes. Left two box plots show the significant improvement of IBS-SSS in IBS-D (n=10) patients (p=0.003) after rifaximin with an average reduction of -100 (± 78) points. IBS-SSS in IBS-C patients (n=8) did not change significantly, which is shown in the right two box plots (p=0.801, mean change +8 points).

1.2.2. Categorical analysis of symptom improvement

We categorized our patients in terms of symptom improvement in two groups, depending on whether their IBS-SSS change exceeded -50 points or not. The IBS-SSS of 8 patients (44%) improved by 50 points or more; whereas the IBS-SSS of 10 patients (56%) improved by less than 50 points or even worsened. The group of people who improved by 50 points or more consisted of 7 IBS-D patients and only 1 IBS-C patient. The other group without improvement consisted of 7 IBS-C patients and 3 IBS-D patients. *(Table 9 and figure 7)* Altogether, symptoms from 7 out of 10 (70%) IBS-D patients improved, compared to only 1 out of 8 (13%) IBS-C patients. We found here a significant association between subtype and symptom improvement (Chi-Square; Fisher's exact test: p=0.025), which is of medium strength (Phi: 0.575; p=0.015).

Categorical symptom improvement			
Subtype	< 50	≥ 50	total
IBS-D	3	7	10
IBS-C	7	1	8
Total	10	8	18

Table 9: IBS subtype in relation to categorical symptom improvement

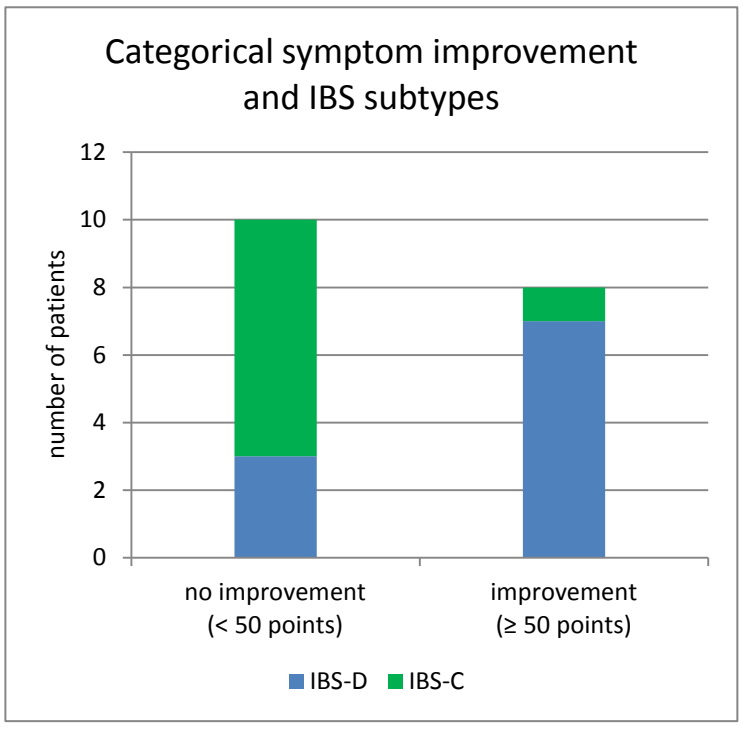

Figure 7: Categorical *s*ymptom improvement and IBS subtypes. A change in IBS-SSS of -50 points or more was considered as symptom improvement. The group of patients who improved after rifaximin mainly consists of IBS-D patients (7/8). IBS subtype and symptom improvement after therapy correlated significantly (p=0.025).

3.2.2 Clinical severity based on IBS-SSS

Among the diarrhea subgroup, 6 patients were classified as severe, 4 as moderate and 0 as mild at the baseline of the study. After antibiotic treatment with rifaximin, only 1 patient was classified as severe, 5 as moderate and 4 as mild. Among patients with constipation, 3 were classified as severe, 4 as moderate and 1 as mild prior to rifaximin intake. Afterwards, 4 were classified as severe, 3 as moderate and 1 as mild. No patient went into remission (i.e. below 75 points). IBS-D patients showed a strong trend (Chi-Square; McNemar: p=0.063) for improvement from severe to non-severe (i.e. mild or moderate). *(Table 10 and 11)*

IBS-D patients		
	before	after
Mild	0	4
Moderate	4	5
Severe	6	1
Total	10	11

Table 10: Clinical severity of IBS-D

IBS- C patients		
	before	after
Mild	1	1
Moderate	4	3
Severe	3	4
Total	8	8

Table 11: Clinical severity of IBS-C

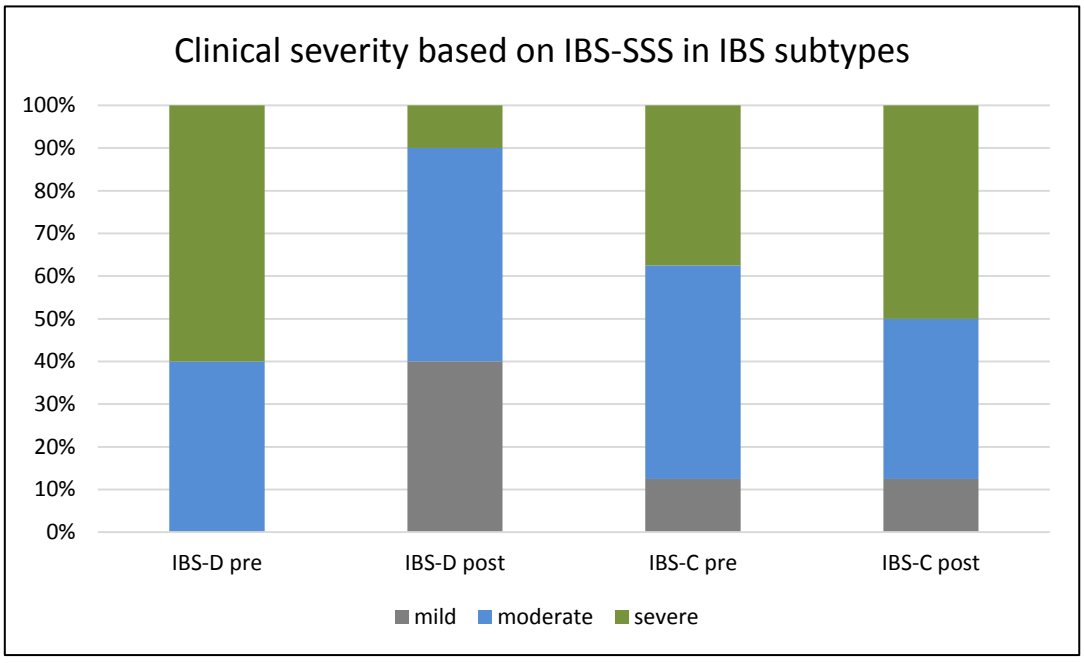

Figure 8: Clinical severity based on IBS-SSS in IBS subtypes before (pre) and after (post) rifaximin treatment. No IBS-D patient was classified as having a mild IBS prior to antibiotic treatment. IBS-D patients showed a trend for an improvement from severe IBS to non-severe IBS (i.e. mild or moderate).

3.3 Analysis of lactulose breath test

Due to a technical issue, a different breath test device had to be used in five patients, either at study visit 1 or 2 or at both time points. To avoid falsification of gathered data, hydrogen values of 14 patients measured by the standard device at both time points were included in the main analysis, whereas values measured by the second device were analyzed separately. The second device only measured hydrogen. Hence, methane values are available in 14 patients.

One patient measured by the standard device showed noticeable low breath hydrogen values at all time points. In synopsis with high corresponding methane values, the patient turned out to be a methane producer. However, hydrogen values of this patient were included in the main analysis (n=14), as results from statistical analysis with or without this patient did only differ minimally.

3.3.1 Hydrogen (H2) analysis in IBS-D and IBS-C patients

3.3.1.1 Basal breath hydrogen

Basal hydrogen values did not differ significantly between IBS-D (n=9) and IBS-C (n=5) patients, neither before (p=0.285) nor after (p=0.350) rifaximin intake. IBS-D patients had a median basal H2 excretion at study visit 1 and 2 of 3 (±16) and 5 (±8), respectively. Median basal H2 values in IBS-C patients at study visit 1 and 2 were 13 (±14) and 5 (±8). The changes of H2 basal values between before and after rifaximin were neither significant in the diarrhea (p=0.889) nor in the constipation (p=1.000) group.

3.3.1.2 Hydrogen increase at time point 60-min

Because some authors share the opinion that 90 minutes after lactulose ingestion might be too late to assess the hydrogen rise above basal levels due to a possible interfering peak caused by physiologic colonic metabolization, we analyzed hydrogens rises above basal levels after 60 minutes. At study visit 1, IBS-D patients showed a mean increase of 17 (±25) compared to 15 (±26) in IBS-C patients; the difference in-between the two groups was not statistically significant (p= 0.504). After rifaximin intake, IBS-D patients showed a mean increase of 14 (±14), compared to 13 (±15) in IBS-C patients. The 60-min H2 increases above basal levels did neither change significantly in IBS-D patients (p=0.602)

nor IBS-C patients (p=1.000). The change of the 60-minute hydrogen increases between the two subgroups did not differ significantly (p=0.841). *(Table 12)*

	Before rifaximin		**After rifaximin**		**Change of 60 min rise**	
	IBS-D n=9	***IBS-C n=5***	***IBS-D n=9***	***IBS-C n=5***	***IBS-D n=9***	***IBS-C n=5***
Mean	17	15	14	13	-2	-2
SD	±25	±26	±14	±15	±14	±17
Median	3	1.00	17	19	0	3
Min	-12	-2	-1	-5	-22	-29
Max	61	60	42	31	14	18
IQR	41	39	22	27	28	28
Asymp. Sign (2-tailed)	p=0.504		p=0.946		p=0.602	p=1.000
					p=0.841	

Table 10: 60 min H2 increase in relation to IBS subtype. Statistical analyses of 60 min H2 increases did not reveal any significant results. There were no significant differences in 60 min H2 increase between IBS- D and IBS- C patients before (p=0.504) or after (p=0.946) rifaximin treatment. Further, there was no significant difference of H2 increase between before and after treatment within subgroups (IBS-D p=0.602, IBS-C p=1.000).
(Significant p-values are marked with *)

3.3.1.3 Hydrogen increase at time point 90 min

The mean hydrogen increase of IBS-D patients was slightly lower, but not statistically significant (p=0.386), with 31 (±29) compared to 46 (±48) in IBS-C patients. After rifaximin intake, IBS-D patients had a mean increase above basal level of 32 (±28) compared to IBS-C patients with 44 (±27), which again did not differ significantly (p=0.640). The 90 min H2 increases above basal levels did neither change significantly in IBS-D patients (p=0.889) nor IBS-C patients (p=0.500). The change of the 90-minute hydrogen increases between the two subgroups did not differ significantly (p=0.109). *(Table 13 and figure 9)*

	Before rifaximin		After rifaximin		Change of 90 min rise	
	IBS-D n=9	***IBS-C n=5***	***IBS-D n=9***	***IBS-C n=5***	***IBS-D n=9***	***IBS-C n=5***
Mean	31	46	32	44	12	-3
SD	±29	±48	±28	±27	±14	±44
Median	18	58	27	55	0	-22
Min	4	-20	4	-0	-20	-29
Max	77	90	78	68	27	75
IQR	52	93	54	47	20	57
Asymp. Sign (2-tailed)	p=0.386		p=0.640		p=0.889	p=0.500
					p=0.109	

Table 11: 90 min H2 increase in relation to IBS subtype. Statistical analyses of 90 min H2 increases did not reveal any significant results. There were no significant differences in 90 min H2 increase between IBS- D and IBS- C patients before (p=0.386) or after (p=0.640) rifaximin treatment. Further, there was no significant difference of H2 rise between before and after treatment within subgroups (IBS-D p=0.889, IBS-C p=0.500).
(Significant p-values are marked with *)

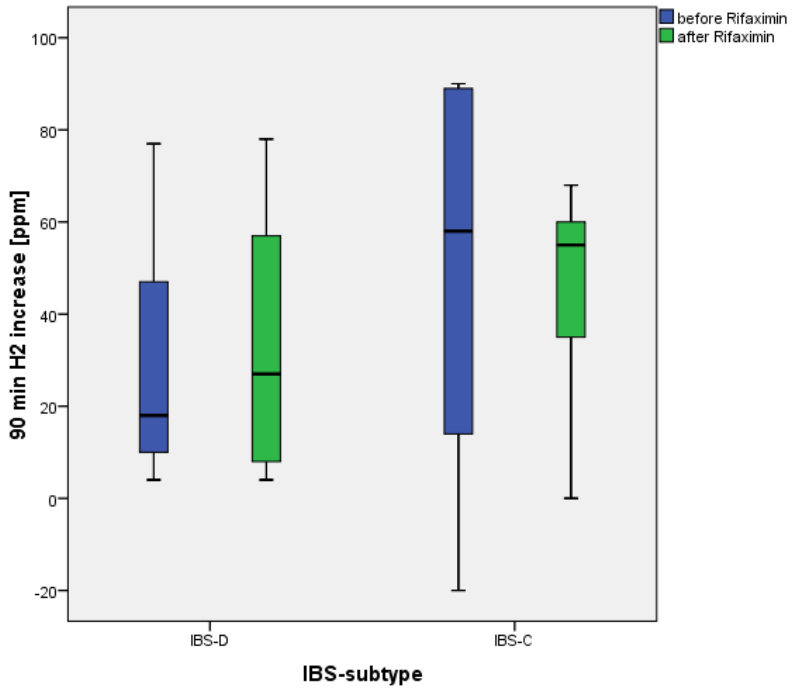

Figure 9: 90 min H2 increase in relation to IBS subtypes. 90 min H2 increase did not differ significantly between IBS-D (n=9) and IBS-C (n=5) patients, neither before (p=0.386) nor after (p=0.640) rifaximin intake. The box plots show that there is no significant difference of 90 min H2 increase between before and after rifaximin treatment within subgroups (IBS-D p=0.889, IBS-C p=0.500).

3.3.1.3.1 90 minute hydrogen rise < or ≥ 20 ppm

At study visit 1, 7 out of 14 patients showed hydrogen values exceeding 20 ppm above basal levels after 90 minutes, indicating presence of SIBO according to guidelines. (190) Four out of those 7 were IBS-D patients (44% of measured IBS-D patients), 3 were IBS-C patients (60% of measured IBS-C patients). After rifaximin intake at study visit 2, even 9 out of 14 patients showed a rise in 90 min breath hydrogen of 20 ppm or more above basal levels. This time, 5 were IBS-D (56% of measured IBS-D patients) and 4 were IBS-C patients (80% of measured IBS-C patients). The group of patients with 90 min H2 < 20 ppm consisted predominantly of IBS-D patients. *(Table 14)*

90 min hydrogen rise (< or ≥ 20 ppm) in IBS subtypes					
subtype	*Before rifaximin*		*After rifaximin*		
	< 20 ppm	≥ 20 ppm	< 20 ppm	≥ 20 ppm	Total
IBS-D	5	4	4	5	9
IBS-C	2	3	1	4	5
Total	7	7	5	9	14

Table 12: 90 min H2 increase < or ≥ 20 ppm in relation to IBS subtype. Patients are divided into two groups depending on the 90 min H2 increase. A 90 min H2 rise ≥ 20ppm is suggested to be diagnostic of SIBO. Before rifaximin intake, half of patients (7/14) had 90 min H2 rises < 20ppm, the other half showed rises ≥ 20ppm. Five out of 7 patients with H2 rises < 20 ppm were IBS-D patients. After rifaximin treatment, even 9 patients showed 90 min H2 rises ≥ 20ppm.

3.3.1.4 Hydrogen increases at all time points

To assess possible other differences, we analyzed the hydrogen increase at all time points between IBS subtypes. We did not observe significant differences of hydrogen increases at 30 min (p=0.107), 60 min (p= 0.504), 90 min (p=0.386), 120 min (p=0.083), 150 min (p=0.073) or 180 min (p=0.096) between IBS-D and IBS-C patients. However, we observed a trend towards statistical significance at 120 and 150 min. As seen in figure 10, mean hydrogen values of IBS-D patients peak after 120 minutes, compared to 150 minutes in IBS-C patients. *(Figure 10)*

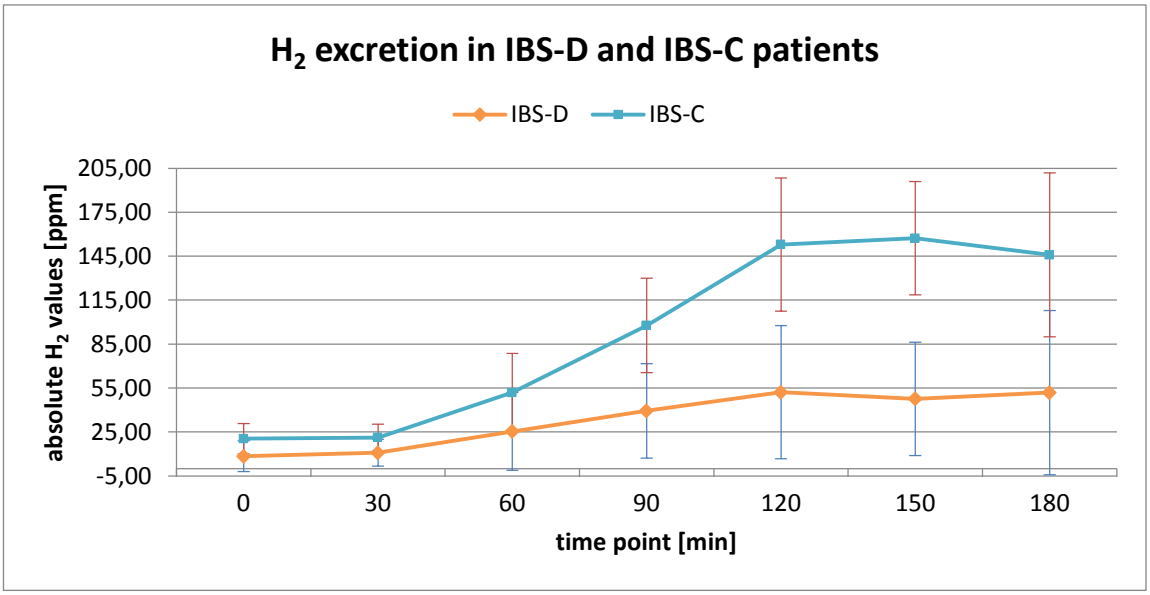

Figure 10: H2 excretion in IBS-D and IBS-C patients. Shown is a comparison of mean values ± standard deviation of absolute H2 values of IBS-D (n=9) and IBS-C (n=5) patients during 3 h lactulose breath test. Even though no significant differences were observed at any time point during 3h test period, H2 values at 120 and 150 min showed a trend towards statistical significance.(120min p=0.083; 150min P=0.073).

3.3.2 Categorical analysis of breath hydrogen in relation to IBS-SSS

3.3.2.1 Basal hydrogen values (< or ≥ 10 ppm)

The IBS-SSS did neither differ significantly before (p=0.619) nor after (p=0.773) rifaximin intake between patients with basal hydrogen values < and ≥ 10 ppm. Patients with basal values ≥ 10 ppm (n=6) improved on average by -66 (±114), which was not significant (p=0.218). However, patients with basal hydrogen values < 10 ppm (n=7) improved significantly (p=0.049) on average by -67 (±72) points. The changes of IBS-SSS did not differ significantly (p=0.984) between the two groups. *(Figure 11)*

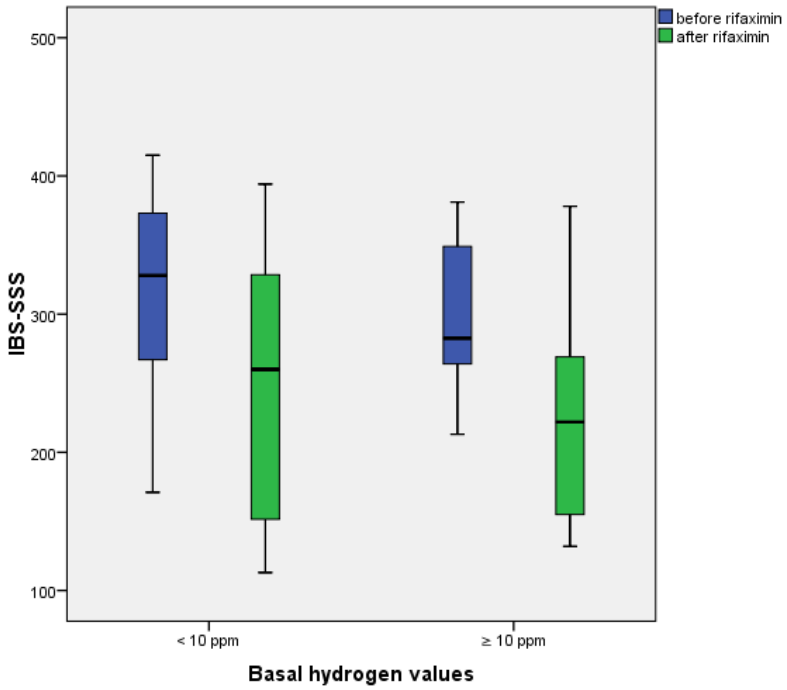

Figure 11: Basal H2 values < or ≥ 10ppm and IBS-SSS. Patients with basal H2 values < 10 ppm (n=7) improved significantly (p=0.049) after rifaximin, whereas patients with levels ≥ 10 ppm (n=6) showed no significant change of IBS-SSS (p=0.218). Additionally, there was no significant difference of IBS-SSS changes between the two groups (p=0.984).

3.3.2.2 60-minute hydrogen rise (< or ≥ 20 ppm)

Patients with a 60-minute hydrogen rise below 20 ppm (n=9) showed a trend (p=0.068) towards symptom improvement: they improved on average by -70 (±99) from a mean pre-antibiotic IBS-SSS of 300 (±88) to a post-antibiotic IBS-SSS of 230 (±104). The IBS-SSS of patients with 60 min H2 rise ≥ 20 ppm changed non-significantly (p=0.144) by -59 from 316(±45) to 257(±45).

3.3.2.3 90 minute hydrogen rise (< or ≥ 20 ppm)

Patients, whose 90 minute hydrogen rise exceeded 20 ppm above basal levels at study visit 1 (n=7) showed a higher, but not statistically significant (p=0.370) IBS-SSS with an average of 326 (±51), compared to patients with a 90-minute increase below 20 ppm (n=6), who had a mean IBS-SSS of 281 (±96).

When analyzing the change of IBS-SSS and therefore the symptom improvement between study visit 1 and 2, patients with 90-minute hydrogen increase of 20 ppm or more above basal levels improved on average by 460, (±91; p=0.230), whereas patients below 20 ppm improved significantly on average by 90 (±90; p=0.028). The mean change of IBS-SSS

between patients < or ≥ 20 ppm, did not differ significantly (p=0.403). *(Figure 12 and Table 15)*

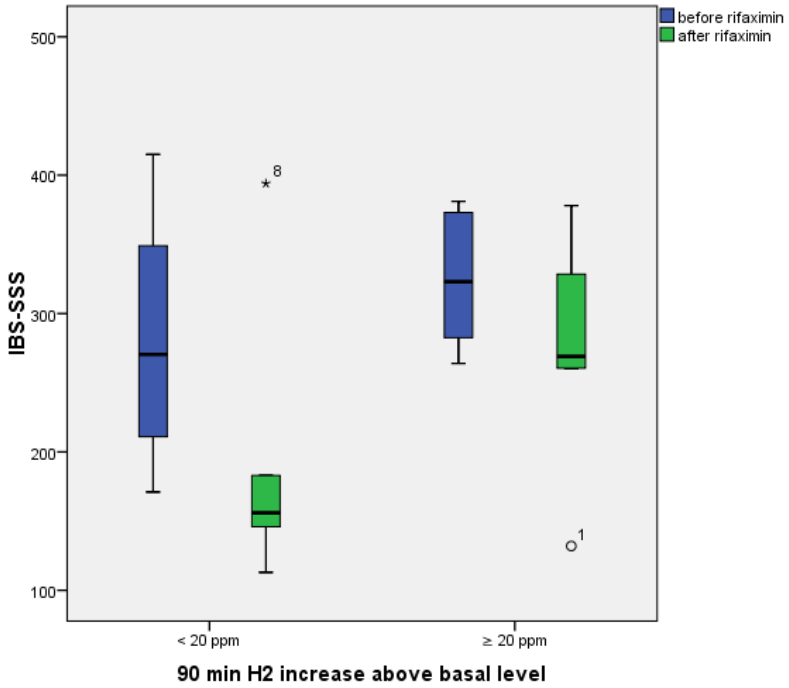

Figure 12: 90 min H2 < or ≥ 20 ppm and IBS-SSS: Patients with 90 min H2 increases < 20 ppm improved significantly (p=0.028) after rifaximin (left box plots). The IBS-SSS of patients with 90 min H2 increases ≥ 20ppm remained symptomatically unchanged (p=0.403).

	Before rifaximin		**After rifaximin**		**Change of IBS-SSS**	
	< 20 ppm n=6	***≥ 20 ppm n=7***	***< 20 ppm n=6***	***≥ 20 ppm n=7***	***< 20 ppm n=6***	***≥20 ppm n=7***
Mean	281	326	191	280	-90	-46
SD	±96.	±51	±102	±83	±90	±91
Median	270	323	156	269	-42	-63
Min	171	264	113	132	-215	-164
Max	415	381	394	378	-21	114
IQR	165	106	98	116	175	113
Asymp. Sign (2-tailed)	p=0.370		p=0.153		p=0.028*	p=0.230
					p=0.403	

Table 13: IBS-SSS in relation to 90 min H2 increase < or ≥ 20 ppm. Patients are divided into two groups depending on the 90 min H2 increase. A 90 min H2 rise ≥ 20ppm is suggested to be diagnostic of SIBO There were no significant differences of IBS-SSS between the groups, neither before (p=0.370) nor after (p=0.153) rifaximin intake. However, patients with 90 min H2 < 20ppm improved significantly (p=0.028), whereas patients ≥ 20ppm remained unchanged (p=0.403).
(Significant p-values are marked with *)

3.3.2.4 90-min hydrogen rise (< or ≥ 30 ppm)

Due to the fact, that we used a relatively high amount of lactulose, we set a hypothetical, higher 90 minute hydrogen threshold value to diagnose SIBO. Therefore, we set the value from 20 ppm to 30 ppm in 90 minutes after ingestion and analyzed patients according to it.

Patients with 90-min H2 ≥ 30 ppm (n=6) had a mean IBS-SSS of 318 (±51), compared to 294.00 (±94) in patients with hydrogen increases less than 30 ppm (n=7), which did not differ significantly (p=0.648). Patients with a 90-minute increase of 30 ppm or more improved on average by -389 (±97; p=0.376), whereas patients with an increase less than 30 ppm improved significantly by -90 (±82; p=0.027). The change of IBS-SSS did not differ significantly between the two groups (p=0.325).

Further, 90-minute hydrogen rise greater or lower than 30ppm did not correlate with symptom improvement change of IBS-SSS of -50 points or more; Chi Square: Fisher's exact test: p=1.000) *(Figure 13)*

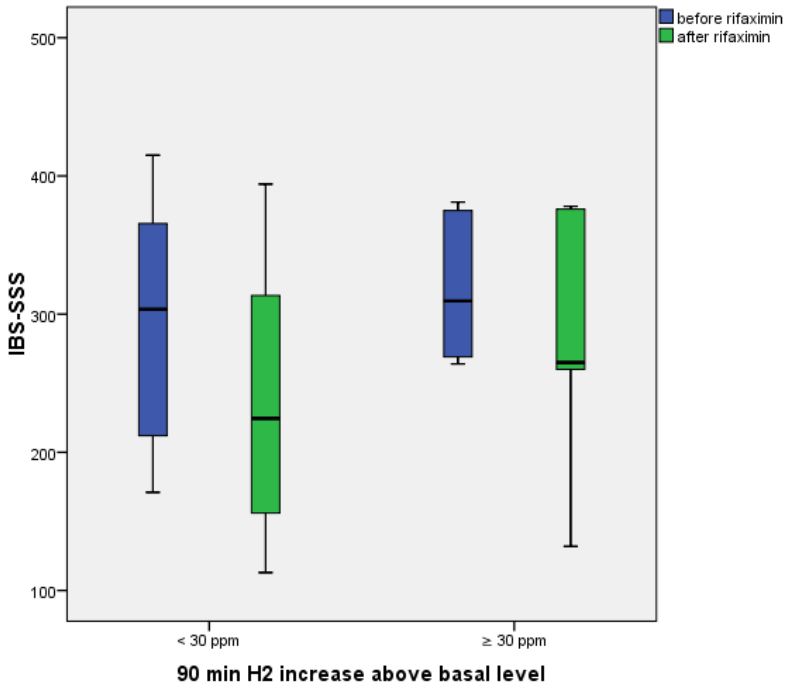

Figure 13: 90 min H2 < or ≥ 30 ppm and IBS-SSS: A hypothetical higher threshold to diagnose SIBO was set to H2 increase < or ≥ 30 ppm in 90 min Patients with 90 min H2 increases < 30 ppm (n=7) improved significantly (p=0.027) after rifaximin, whereas patients in the other group (n=6) remained unchanged (p=0.376).

3.3.3 Categorical analysis of improvement in relation to hydrogen values

3.3.3.1 Definition:

We categorized our patients in terms of symptom improvement into two groups. Patients with a change of IBS-SSS of -50 points or more symptomically improved (n=7). Patients with a change of IBS-SSS less than -50 points did not improve or even worsened (n=6).

3.3.3.2 Basal hydrogen values

Basal hydrogen values of patients who improved (9±11) did not differ significantly (p=0.770) from basal values of patients who did not improve (11±9).

3.3.3.3 60-minute hydrogen increase

At study visit 1, patients who improved had a mean 60-minute increase of 14 (±25), compared to patients who did not improve with a mean increase of 21 (±28). However, the pre-antibiotic hydrogen increases did not differ significantly between the groups (p=0.640). The rise of hydrogen in 60 minutes before and after rifaximin did neither change significantly in patients who improved (p=0.625) nor in patients who did not improve (p=0.221). After rifaximin intake, patients who improved had a mean 60-minute hydrogen increase of 17 (±13), which did not differ significantly (p=0.202) compared to the other group with 13 (±16). *(Table 16)*

	Before rifaximin		**After rifaxmin**		**H2 change of 60 min rise**	
	<50points n=6	***≥ 50points n=7***	***< 50 points n=6***	***≥ 50 points n=7***	***< 50 points n=6***	***≥ 50 points n=7***
Mean	21	14	13	17	-8	3
SD	±28	±25	±16	±13	±14	±15
Median	9	3	11	17	-2	7
Min	-2	-12	-5	2	-29	-19
Max	60	61	31	42	4	18
IQR	55	31	31	15	27	29
Asymp. Sign (2-tailed)	p=0.640		p=0.638		p=0.221	p=0.625
					p=0.202	

Table 14: 60 min hydrogen increase in relation to symptom improvement: Statistical analysis of 60 min H2 rises between patients with (n=7) and without (n=7) improvement did not reveal any significant results. 60 min H2 rises did neither differ significantly before (p=0.640) nor after rifaximin (p=0.638) between patient w/o improvement.
(Significant p-values are marked with *)

3.3.3.4 90-min hydrogen increase

At study visit 1, patients who improved showed a mean 90-minute hydrogen increase above basal levels of 38.86 (±33), compared to patients in the no-improvement group with a mean increase of 38.17 (±43). There was no significant difference between the two groups (p=0.975). Patients who improved 50 points or more showed a mean 90-minute increase at study visit 2 of 41 (±30), compared to 35.83(±26) in the other group, which again did not differ significantly (p=0.749). The 90 minute hydrogen increase before and after rifaximin did neither change significantly in patients who improved (p=0.749) nor who did not improve (p=0.889). *(Table 17)*

	Before rifaximin		**After rifaximin**		**Change of 90 min rise**	
	< 50 points n=6	***≥ 50 points n=7***	***< 50 points n=6***	***≥ 50 points n=7***	***< 50 points n=6***	***≥ 50 points n=7***
Mean	38	39	36	41	-2	2
SD	±43	±33	±26	±30	±39	±17
Median	36	27	45	37	-17	2
Min	-20	4	0	4	-29	-22
Max	89	90	60	78	75	27
IQR	77	66	52	60	41	32
Asymp. Sign. (2-tailed)	p=0.975		p=0.749		p=0.889	p=0.749
					p=0.174	

Table 15: 90 min hydrogen increase in relation to symptom improvement: Statistical analysis of 90 min H2 rises between patients with (n=7) and without (n=7) improvement did not reveal any significant results. 90 min H2 rises did neither differ significantly before (p=0.975) nor after rifaximin (p=0.749) between patient w/o improvement. (Significant p-values are marked with *)

3.3.3.4.1 90 minute hydrogen rise (< or ≥ 20 ppm) and symptom improvement

We analyzed and compared patients with pre-antibiotic 90 minute hydrogen greater or equal 20 ppm on the one side, and patients with symptom improvement assessed by a change greater or equal 50 points on the other side. No significant correlation was found between the two variables (Chi-Square: p=1.000). Seven patients showed hydrogen rises greater or equal 20 ppm at study visit 1, of whom 4 improved and 3 did not. The other 6 patients had 90-minute hydrogen values lower than 20 ppm, of whom 3 improved and 3 remained unchanged or worsened. *(Figure 14)*

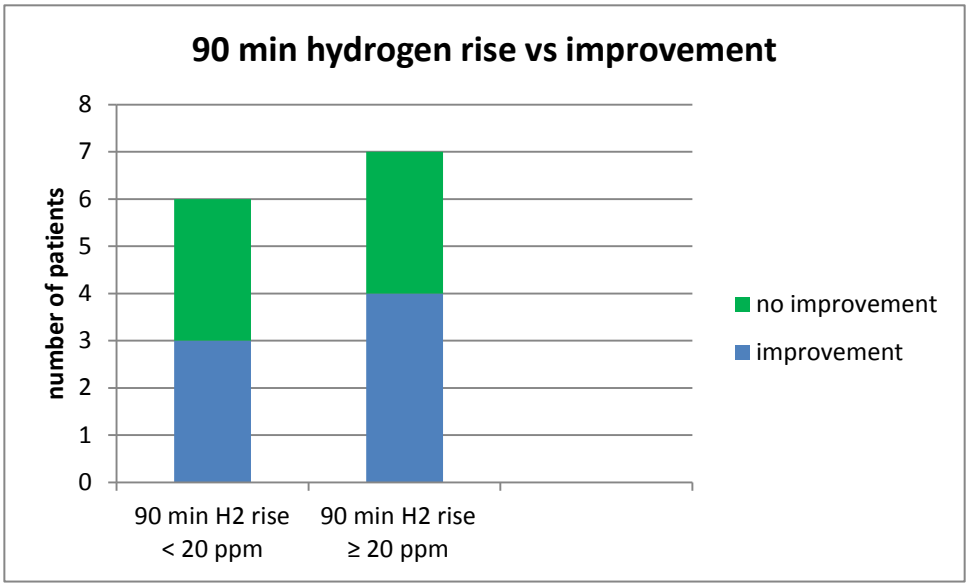

Figure 14: 90 min H2 increase and categorical symptom improvement. Categorical analysis of 90 min H2 increase in relation to symptom improvement did not show a significant correlation (p=1.000). Seven patients showed hydrogen rises greater or equal 20 ppm at study visit 1, of whom 4 improved and 3 did not. The other 6 patients had 90-minute hydrogen values lower than 20 ppm, of whom 3 improved and 3 remained unchanged or worsened.

3.3.3.5 Hydrogen increases at all time points

We analyzed the hydrogen rises at all time points between patients who improved and patients who did not improve. Although absolute H2 values of patients who improved appear to be lower, no significant difference was found at 30 min (p=0.785), 60 min (p=0.640), 90 min (p=0.975), 120 min (p=0.710), 150 min (p=0.757) and 180 min (p=0.622). H2 values of patients who improved peaked at time point 180 min, compared to 120 min in patients who did not improve. *(Figure 15)*

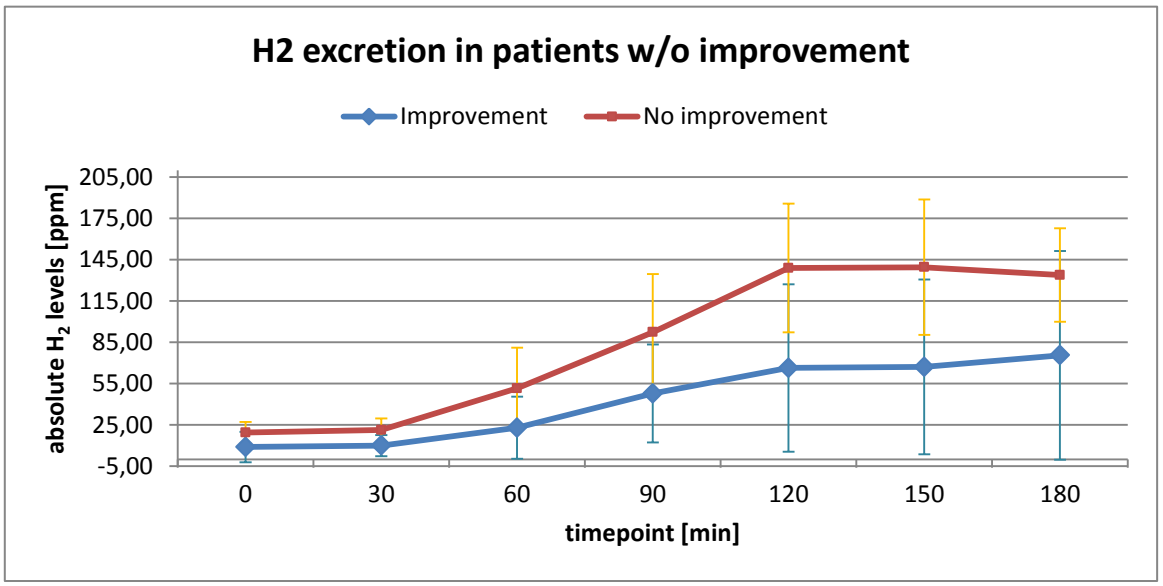

Figure 15: H2 excretion in patients w/o improvement. Shown is a comparison of mean values and standard deviation of absolute H2 values of patients with (n=7) and without (n=6) improvement during 3h lactulose breath test. No significant differences were observed at any time point.

3.3.4 Correlation between change of IBS-SSS and hydrogen values

To assess possible predictive hydrogen values at certain time points, we analyzed the hydrogen rise at all time points in connection with symptom improvement assessed by the change of IBS-SSS. We did not observe a significant correlation between the change of IBS-SSS and basal hydrogen values (Pearson: -0.093, p=0.762). Further, no significant correlation was found with hydrogen increases above basal levels at 30 min (Pearson: 0.012; p=0.969), 60 min (Spearman's rho: 0.083; p=0.789), 90 min (Pearson: 0.215; p=0.480), 120 min (Pearson: 0.126; p=0.681), 150 min (Pearson: 0.170; p=0.579) or 180 min (Pearson: -0.141; p=0.645).

3.3.5 Global expiratory breath hydrogen: AUC

Global hydrogen excretion during the 3-hour test period was assessed by calculating the area under the curve (AUC). No significant correlation was observed between hydrogen AUC and IBS-SSS (Pearson: 0.157; p=0.593), change of IBS-SSS (Pearson: 0.094; p=0.759) and change of serum zonulin (Pearson: -0.243; p=0.402). However, we found a significant negative correlation between global H2 excretion and serum zonulin concentrations, which was of high strength (Pearson: -0.709; p=0.005). In other words, low serum zonulin levels significantly correlated with high global H2 values.

The global H2 excretion did not differ significantly between patients who improved and who did not improve (p=0.668). Further, no significant difference of H2 AUC was observed between subtypes (p=0.162) or gender (p=0.439).

3.3.5.1 60-minute AUC

To evaluate early H2 excretion as an expression of early small bowel fermentation, AUC of hydrogen values within 60 minutes was calculated: no significant correlation was observed between 60 min-AUC and IBS-SSS (Pearson: 0.027; p=0.928), change of IBS-SSS (Pearson: 0.013; p=0.965), serum zonulin concentration (Pearson: -0.322; p=0.261) and change of serum zonulin (Pearson: -0.492; p=0.074). Again, no difference in 60 min-AUC was observed between patients who improved and who did not (p=0.632), between subtypes (p=0.866) and between gender (p=0.957).

3.3.5.2 Categorical analysis of AUC

We evaluated high/low AUC values in terms of symptom improvement assessed by a change of IBS-SSS; therefore we set a hypothetical threshold of AUC at 250. Patients with lower 3h H2-AUC < 250 (n=5) improved on average by -81.00 (±79.85), compared to patients with higher H2-AUC ≥ 250 (n=8), who improved on average by -57.00 (±99.29). The change of IBS-SSS did not differ significantly (p=0.661) between the two groups.

3.3.6 Total hydrogen excretion: H2 and 4 –times CH4

3.3.6.1 Definition

As explained in "Introduction", methane-producing bacteria are able to convert 4 atoms hydrogen into 1 atom methane. To include and evaluate H2 values of patients with predominant methane-producing gut flora, we calculated total breath H2 by the following formula: total H2 = H2 + 4 x CH4. Above-mentioned analysis were also calculated using combined H2 values (H2 + 4xCH4) to evaluate whether total H2 values lead to more predictive values than H2 alone.

In summary, calculations with total hydrogen excretions did not show more convincing or predictive results. Analysis and correlations are described in detail below.

3.3.6.2 Total hydrogen excretion: subtypes

No statistical significant difference of total H2 was observed between IBS-D and IBS-C patients at baseline (p=0.205) or at increases at 30 min (p=0.504), 60 min (p=0.947), 90 min (p=0.739), 120 min (p=0.286), 150 min (p=0.205), 180 min (p=0.463).

3.3.6.3 Total hydrogen excretion: categorical improvement

We did not observe statistical significant differences of total H2 excretion between patients who improved and who did not at baseline (p=1.000) or at increases at 30 min (p=0.283), 60 min (p=0.283), 90 min (p=0.317), 120 min (p=0.721), 150 min (p=0.775), 180 min (p=0.352).

3.3.6.4 Total hydrogen excretion: correlation with change of IBS-SSS

Spearman's Rho was used to assess all of the following correlations. No significant correlation was found between change of IBS-SSS and total H2 excretion at baseline (-0.217; p=0.476). Further, no significant correlation was found with total hydrogen increases at 30 min (-0.085; p=0.782), 60 min (-0.022; p=0.943), 90 min (0.137; p=0.655), 120 min (0.083; p=0.789), 150 min (0.137; p=0.655) or 180 min (-0.025; p=0.936).

3.3.6.5 AUC of total hydrogen excretion

Spearman's Rho was used to assess all of the following correlations. No significant correlation was observed between AUC of total H2 and IBS-SSS (-0.222; p=0.446), change of IBS-SSS (-0.011; p=0.972), serum zonulin (-0.213; p=0.464) and change of serum zonulin (-0.240; p=0.409). AUC of total H2 excretion did neither differ significantly between patients who improved and who did not improve (p=0.568) nor between subtypes (p=0.317) or gender (p=0.156).

3.3.7 Methane (CH4) analysis

3.3.7.1 Categorical analysis: methane and subtypes

Breath methane values at both time points were available from 14 out of 19 patients. Patients were divided into 2 groups, depending on methane level < and ≥ 3 ppm regardless of time point.

At study visit 1, 8 patients showed methane levels greater or equal 3 ppm, of whom 7 were IBS-D patients (78% of measured IBS-D patients) and only 1 was IBS-C patient (20% of measured IBS-C patients). At study visit 2, 10 patients had methane levels greater or equal 3 ppm, of whom 7 were IBS-D and 3 were IBS-C patients. *(Figure 16)*

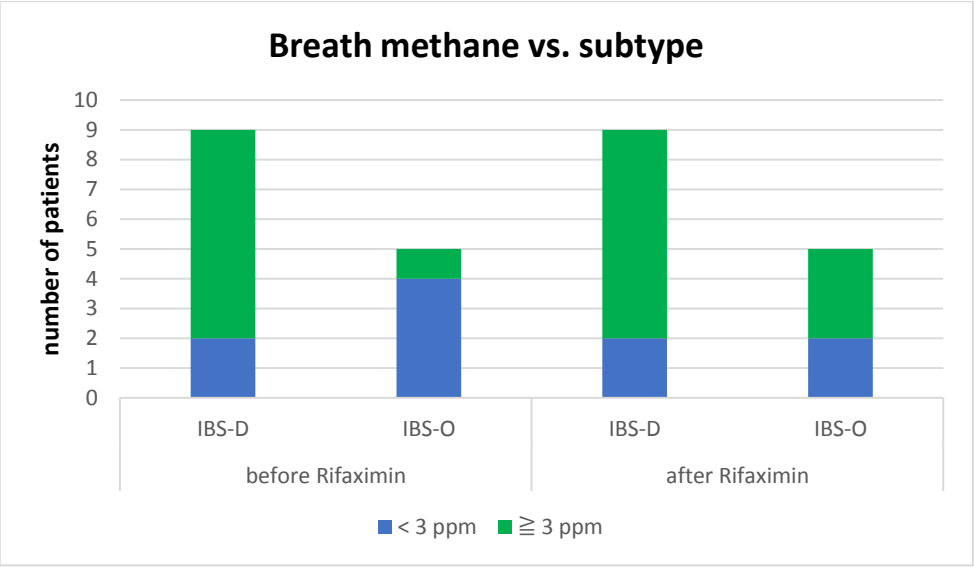

Figure 16: Methane levels in IBS subtypes. Patients were divided into 2 groups, depending on methane level < and ≥ 3 ppm regardless of time point. Former guidelines suggested methane levels ≥ 3 ppm regardless of time point to be diagnostic of SIBO. The majority of IBS-D patients (7/9) showed methane levels ≥ 3 ppm at both time points.

3.3.7.2 Categorical analysis: methane and categorical symptom improvement

Methane levels ≥ 3 ppm at study visit 1 were analyzed regarding their relation to symptom improvement assessed by a change of IBS-SSS of 50 or more. Seven patients had pre-antibiotic breath methane levels below 3 ppm, of whom 6 remained symptomatically unchanged and 1 improved. The other group of 7 patients with breath methane greater or equal 3 consists of 1 patient who did not improve compared to 6 patients who improved. However, among patients who improved, 5 still showed breath methane values ≥ 3 ppm at study visit 2. We observed a significant correlation between symptom improvement and pre-antibiotic breath methane greater or equal 3 ppm (Chi-Square; Fisher's exact test: p=0.029), which is of high strength (Phi: correlation coefficient: 0.714; p=0.008). *(Figure 17)*

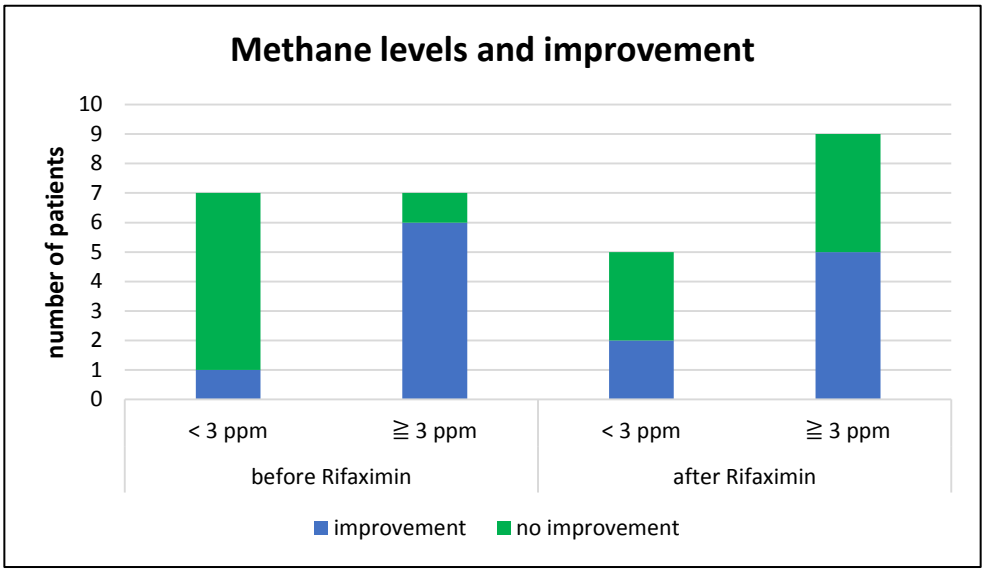

Figure 17: Methane levels and categorical symptom improvement. Methane levels < and ≥ 3 ppm regardless of time point before treatment were analyzed regarding their relation to symptom improvement assessed by a change of IBS-SSS of 50 or more. The majority of patients who improved (6/7) showed methane values ≥ 3 ppm at study visit 1. We found a significant correlation of high strength between categorical symptom improvement and methane ≥ 3 ppm (p=0.029).

3.3.7.3 Categorical methane analysis in relation to symptom improvement

The IBS-SSS of patients with methane levels ≥ 3 ppm at study visit 1 changed significantly (p=0.007) compared to patients, who had methane levels lower than 3 ppm. Patients with breath methane ≥ 3 ppm improved significantly (p=0.006) from 315 (±61) with an average of -120 (±76) to 195 (±72). The IBS-SSS of patients with methane < 3 ppm changed non-significantly (p=0.879) on average by -4 (±61) from 293 (±95) to 290 (±108) *(Figure 18)*

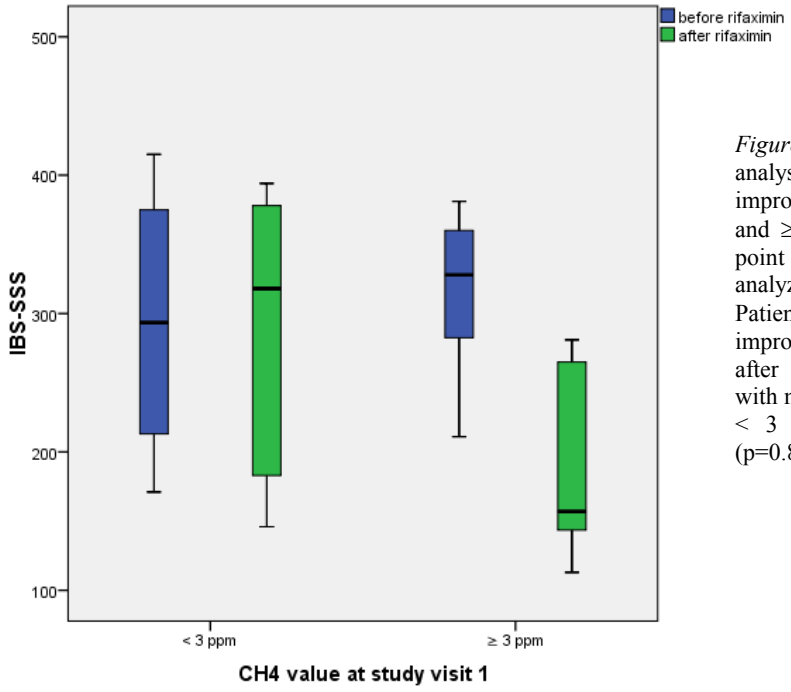

Figure 18: Categorical methane analysis in relation to symptom improvement: Methane levels < and ≥ 3 ppm regardless of time point before treatment were analyzed in relation to IBS-SSS. Patients with methane ≥ 3 ppm improved significantly (p=0.007) after rifaximin, whereas patients with methane < 3 ppm remained unchanged (p=0.879).

3.3.7.4 Categorical methane analysis in relation to serum zonulin

Patients with methane ≥ 3 ppm at study visit 1 had higher, but not statistically significant (p=0.202) serum zonulin levels with a mean of 44.29 (±13.51) compared to the group < 3 ppm with 36.82 (±6.90). Serum zonulin in the methane ≥ 3 ppm group changed significantly (p=0.041) on average by -1.80 (±2.04) to 42.48 (±12.70), although the absolute numbers were low.

3.3.7.5 Methane ≥ 10 ppm

Due to the recent suggestions concerning the diagnostic threshold of methane values (190), patients with levels above 10 ppm regardless of the time-point were analyzed. Two out of 14 patients showed breath methane levels of 10 ppm or more at study visit 1 as well as at study visit 2 after rifaximin intake. One of them was classified as IBS-D, the other as IBS-C. Both showed extremely high breath methane values compared to the other IBS patients, which did not change with the intake of rifaximin. The IBS-SSS of both "methane producers" improved, in one patient by -112 and in the other by -54. *(Table 18)*

Variables	Patient 1	Patient 2
Subtype	IBS-C	IBS-D
Age	28	68
Gender	female	female
Highest CH4 at SV1	56	200
Highest CH4 at SV2	127	200
IBS-SSS at SV1	381	211
IBS-SSS at SV2	269	157
Change of IBS-SSS	-112	-54
Zonulin at SV1	19.07	59.29
Zonulin at SV2	20.66	59.00

Table 18: Data from patients with methane ≥ 10 ppm: Shown are demographic data, CH4 values, IBS-SSS and serum zonulin levels of 2 methane producing patients. One of them was classified as IBS-D, the other as IBS-C. Both showed extremely high breath methane values compared to the other IBS patients, which did not change after the intake of rifaximin.

3.3.8 Compound diagnostic criteria for SIBO

When applying SIBO criteria on the 14 patients measured with the standard device (hydrogen rise ≥ 20 ppm in 90 minutes), 7 out of 14 patients (50%) were diagnosed for having SIBO at study visit 1. However, after antibiotic treatment, even 9 out of 14 (64,3%) patients were SIBO positive.

When applying the new SIBO criteria from the North American Consensus 2017 (hydrogen rise ≥ 20 ppm in 90 minutes; methane ≥ 10 ppm), 8 out of 14 (57,1%) were SIBO positive at study visit 1, compared to 10 out of 14 (71,4%) at study visit 2.

3.4 Serum zonulin analysis

3.4.1 Demographical analysis: IBS patients and control group

Serum zonulin concentrations of 19 IBS patients and 10 healthy control patients were analyzed. From 19 IBS patients, 10 were male and 9 were female. The average age of all 19 patients was 38.05 (±12.09). The mean age in male and female patients was 36.78 (±8.91) and 39.20 (±14.79), respectively. We could observe a trend towards a significant correlation between zonulin levels and the age of patients in our study cohort (Pearson: 0.349; p=0.064).

The control group consisted of 10 people, of whom 5 were male and 5 female. The mean age of all 10 individuals was 41.2 (±10.65). They mean age of male and female controls was 41.4 (±8.36) and 41.0 (±12.52), respectively. No correlation was found between zonulin levels and the age of healthy controls (Spearman's rho: -0.043; p=0.907).

The unit for all serum zonulin values is ng/ml.

3.4.2 Serum zonulin in relation to IBS subtype

Serum zonulin concentration of IBS-D patients prior to rifaximin intake were significantly higher (p=0.009) with a median of 45.81. (±12.08) compared to IBS-C patients with 31.63 (±8.03). After rifaximin intake, IBS-D patients again showed significantly higher (p=0.029) serum zonulin levels. Serum zonulin concentrations before and after rifaximin intake did neither change significantly in the diarrhea (p=0.105), nor in the constipation (p=0.575) subgroup. IBS-D patients showed a mean change of -1.39 (±2.58), compared to -0.71 (±3.80) in IBS-C patients. The mean changes of serum zonulin concentration did not differ significantly (p=0.648) between the two subgroups. *(Figure 19)*

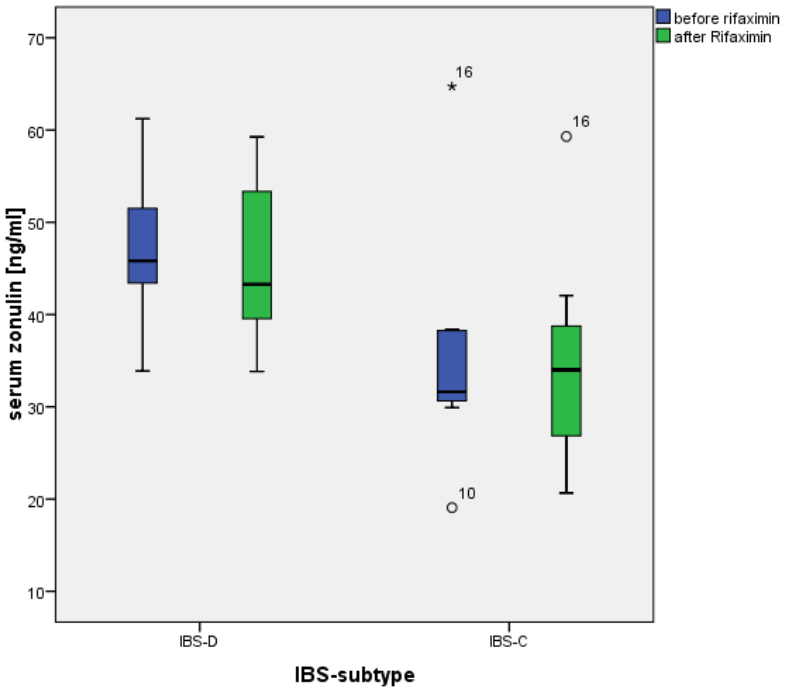

Figure 19: Serum zonulin levels in relation to IBS subtype. IBS-D (n=11) patients showed significantly higher serum zonulin concentrations before (p=0.009) and after (p=0.029) rifaximin intake. Serum zonulin levels did neither change significantly in IBS-D (p=0.105) nor IBS-C (n=8) patients (p=0.575) after rifaximin.

3.4.3 Serum zonulin in relation to improvement

Serum zonulin concentrations did neither correlate with the IBS-SSS before (Pearson: 0.108; p=0.659) nor after (Pearson: -0.278; p=0.264) rifaximin intake. We did not find a relationship between serum zonulin concentrations and change of IBS-SSS (Spearman: -0.339; p=0.169). The change of IBS-SSS did not correlate with the change of serum zonulin concentration (Pearson: -0.032; p=0.900). Serum zonulin concentrations before rifaximin intake did not differ significantly (p=0.661) between the groups improvement (≥ 50 points change) and non-improvement (< 50 points change). However, serum zonulin concentration of patients, whose symptoms improved, changed significantly (p=0.046) on average by -1.50 (±1.75) from 43.76 (±13.52) to 42.27 (±12.72). Patients, who did not improve, showed a non-significant (p=0.621) change of serum zonulin of -0.65 (±3.98). The changes of zonulin did not differ significantly (p=0.555) between the two groups. *(Figure 20)*

Because IBS-D patients showed significantly higher zonulin concentrations, they were analyzed separately concerning IBS-SSS and improvement: serum zonulin concentrations of IBS-D patients did not correlate with IBS-SSS (Pearson: -0.322; p=0.335). Further, the

change of IBS-SSS among IBS-D patients did not correlate with their change of serum zonulin concentrations. (Pearson: 0.293; p=0.411).

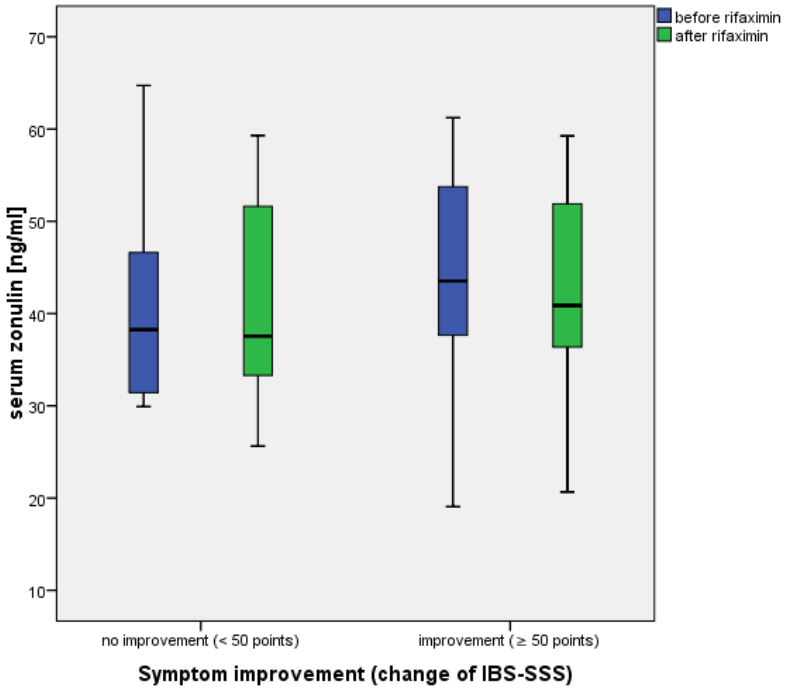

Figure 20: Serum zonulin levels in patients w/o improvement. Serum zonulin levels before rifaximin intake did not differ significantly (p=0.661) between the groups improvement (≥ 50 points change) and non-improvement (< 50 points change). Serum zonulin concentration of patients, whose symptoms improved (n=8) changed significantly (p=0.046) on average by -1.50 (±1.75). Patients, who did not improve (n=10), showed a non-significant (p=0.621) change of serum zonulin of -0.65 (±3.98). The changes of zonulin levels did not differ significantly (p=0.555) between the two groups.

3.4.4 Breath hydrogen in relation to serum zonulin levels

Based on the assumption of Fasano et al (2012) that zonulin release may present a defense mechanism against bacterial colonization in the small intestine, we analyzed serum zonulin concentrations in connection with breath hydrogen values. As above-mentioned, we found a significant negative correlation between global H2 excretion (AUC) and serum zonulin concentrations, which was of high strength (Pearson: -0.709; p=0.005). In other words, low zonulin levels correlated significantly with high global H2 values. When analyzing subtypes, H2-AUC did not significantly correlate (Spearman's rho: -0.500; p=0.170) with serum zonulin in IBS-D patients (n=9), but correlated significantly in IBS-C patients (n=5) (Spearman's rho: -0.900; p=0.037). No significant correlations were found between zonulin levels and basal hydrogen excretion (Pearson: -0.300, p=0.298), hydrogen increases at 30-minutes (Pearson: 0.167; p=0.569) and 60-minutes (Spearman's rho: -0.037; p=0.899). However, we found a significant negative correlation (p=0.020) between

serum zonulin concentrations and the 90-minute hydrogen rise, which was of moderate strength (Pearson: -0.611). In connection with this finding, we analyzed zonulin concentrations in relation to 90-min hydrogen < or ≥ 20 ppm: Patients with 90-minute hydrogen rises < 20 ppm had significantly higher (p=0.016) serum zonulin concentration with an average of 48.23 (±9.12), compared to patients with hydrogen rises ≥ 20 ppm, who had mean zonulin levels of 34.70 (±8.88). *(Figure 21)* Further, significant negative correlations were also found between zonulin levels and hydrogen rises at 120 minutes (Pearson: -0.675; p=0.008), 150 minutes (Pearson: -0.793; p=0.001) and 180 minutes (Pearson: -0.654; p=0.011)

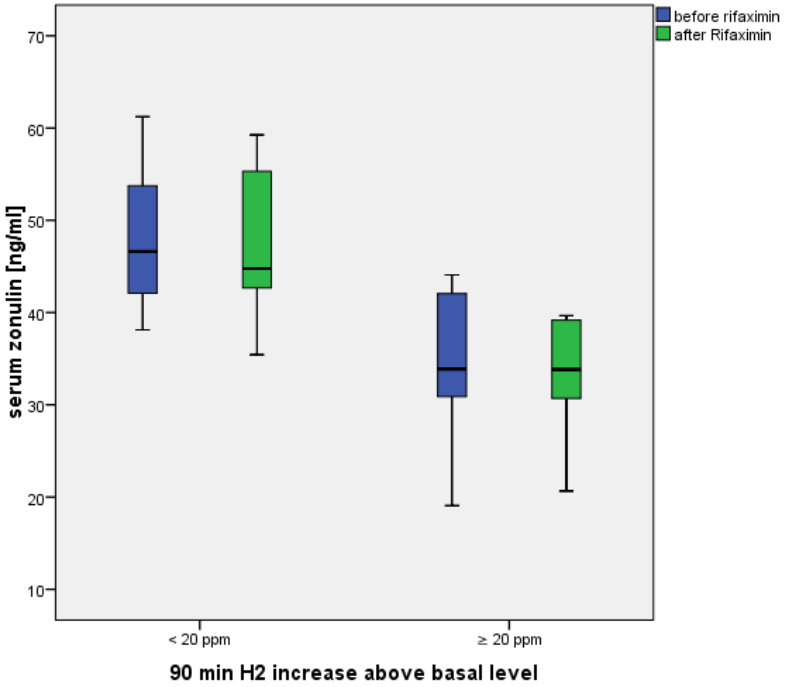

Figure 21: Serum zonulin levels in relation to 90 min H2 increase. Patients with 90 min H2 increases < 20 ppm (n=7) had significantly higher (p=0.016) zonulin levels with an average of 48.23 (±9.12), compared to patients with hydrogen rises ≥ 20 ppm (n=7), who had mean zonulin levels of 34.70 (±8.88).

3.4.5 Control group compared to IBS patients

The median serum zonulin concentration in the control group was 52.89 (±7.92), which was significantly higher (p=0.012) compared to the median zonulin concentration of IBS patients at study visit 1 (42.49±16.35) When analyzing the subgroups, zonulin levels between the control group and IBS-D patients at baseline did not differ significantly (p=0.091); whereas IBS-C patients showed significantly lower zonulin concentrations (p=0.006) compared to the control group. *(Figure 22)*

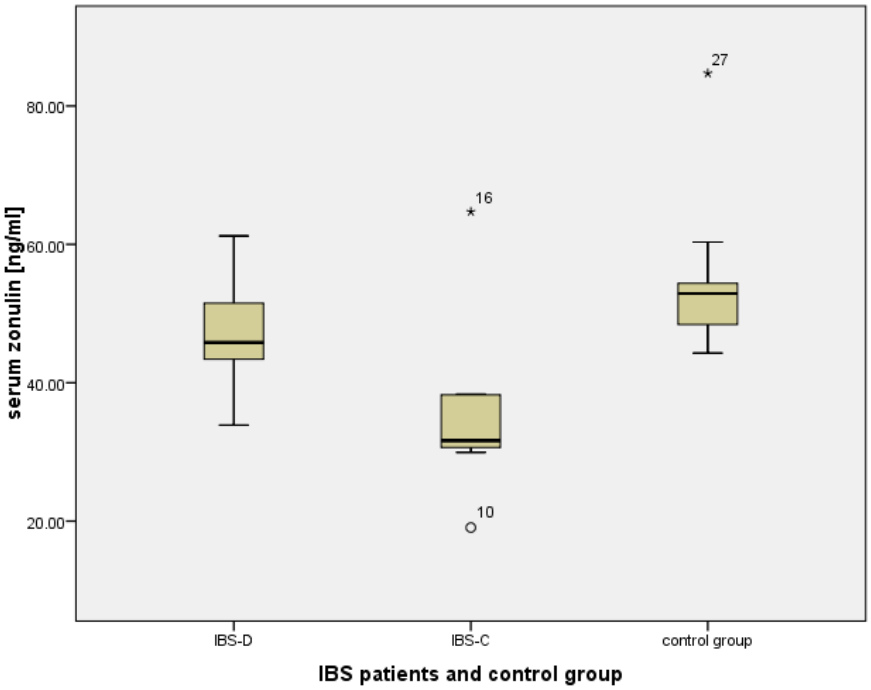

Figure 22: Comparison of serum zonulin levels in IBS subtypes and healthy controls. Serum zonulin concentrations in the control group (n=10) were significantly higher compared to IBS-C (n=8) patients (p=0.006). Serum zonulin levels did not differ significantly between healthy controls and IBS-D (n=11) patients (p=0.091).

3.5 Comparison of breath test devices

Five patients, who used the substitute breath test device at both time points or either at study visit 1 or 2, were analyzed separately. Two out of 5 were classified as IBS-D, three as IBS-C patients. Breath hydrogen values measured by the backup device were lower (not significantly) compared to the actual standard device. Table 19 summarizes breath hydrogen rises after 60, 90 and 120 minutes measured by the actual standard device (n=14) and the backup device (n=5). We did not perform further statistical analysis within the backup group due to the small sample size. *(Table 19)*

	60 min H2 rise		90 min H2 rise		120 min H2 rise	
	Actual n=14	***Backup n=5***	***Actual n=14***	***Backup n=5***	***Actual n=14***	***Backup n=5***
Mean	16	1	36	9	53	37
SD	±25	±5	±36	±12	±44	±46
Median	3	-1	22,5	6	42	19
Min	-12	-4	-20	-5	3	1
Max	61	8	90	23	143	114
Asymp. Sign (2-tailed)	p=0,126		p= 0,116		p= 0,431	

Table 19: Comparison of H2 increases in different breath test devices at time point 60, 90 and 120 min: H2 increases measured by the backup device were lower, yet not significantly, compared to the standard device at time point 60, 90 and 120 min. (Significant p-values are marked with *)

3.5.1 Analysis of patients measured with both devices

All statistical analysis which involved breath hydrogen were also performed in patients measured with both devices (n=19). When combining measured data from both devices, results did not show a major difference compared to results calculated by data only from the standard device. However, we observed a few differences, e.g. 90-minute hydrogen rises were normally distributed in the standard-group (n=14), but not in the standard + backup-group (n=19). As a matter of course, this had an impact on the choice of the statistical tests we used and with-it the results and the strength of significances we found. For example, 90-minute hydrogen rises of patients measured with the standard device (n=14) showed a significant (p=0.020) negative correlation of moderate strength (-0.611) with serum zonulin concentrations. In contrast, 90-minute hydrogen rises of patients measured by both devices (n=19) showed a less significant (p=0.046) negative correlation of low strength (-0.463)

4 Discussion

Although the irritable bowel syndrome is the most commonly diagnosed gastrointestinal disorder, the pathophysiology of the disease is still insufficiently explored. Therefore, therapeutic options still lack a causal background and target symptom improvement. Regarding the recently discovered associations between gut microbial alterations and IBS, the effect of therapeutic modulation of intestinal microbiota, either via antibiotic intervention or, more recently, via fecal microbiota transplantation, is currently under investigation. However, studies investigating the effect of the non-absorbable antibiotic rifaximin have shown a benefit only in a subset of patients. Therefore, it was postulated that only IBS patients with evidence of SIBO may benefit from antibiotic therapy. Hence, we tried to examine prevalence of SIBO in our IBS patients using LHBT and to test different parameters of LHBT regarding their predictive value for symptom improvement in response to rifaximin. Our second aim was to study the prevalence of elevated serum zonulin levels in our IBS patients and to correlate serum values with IBS subgroups, symptom severity, LHBT findings and treatment response.

4.1 IBS-SSS

Global IBS symptoms improved in 44% (8/18) of all IBS patients and in 70% (7/10) of IBS-D patients. This confirms the efficacy of rifaximin in IBS patients, especially those suffering from diarrhea-predominant subtype. Most studies published in this context report an adequate relief in global IBS symptoms in 42% to 52% of IBS-D patients treated with rifaximin (192). Although none of our patients went into remission, IBS-D patients showed a strong trend ($p=0.063$) for an improvement from clinical severity classification severe to non-severe (i.e. mild or moderate), which was not evident for IBS-C patients.

4.2 Lactulose breath test and prediction of treatment response

In most studies evaluating LHBT, presence of SIBO is defined as an increase of hydrogen excretion ≥ 20ppm within 90 minutes. In our study, patients with low hydrogen increases (< 20 ppm within 90 minutes) showed statistically significant symptom improvement ($p=0.028$), which was not seen in patients with high hydrogen increases ≥ 20 ppm in 90 minutes. Furthermore, also patients with 60-minute H2 increases < 20 ppm showed a trend for improvement ($p=0.068$). Some authors suggest elevated basal hydrogen levels ≥ 10

ppm to be indicative of SIBO in the proximal part of the small intestine (187,188). We could not confirm this SIBO criterion to be predictive for a positive response to antibiotics in our study cohort. In contrast, patients with basal hydrogen values < 10 ppm showed a significant improvement (p=0.049). Following our hypothesis, we would have expected an amelioration of symptoms in those patients with higher hydrogen rises, indicating presence of SIBO. However, our results are consistent with the findings of Kasir R et al in 2016, who conducted a retrospective study with 561 IBS patients to evaluate the predictive value of LHBT. In his study, patients with hydrogen + methane rises < 20 ppm throughout the duration of the whole LHBT improved in 94.7% of cases. All other study groups showed less symptom improvement, for example patients with a rise of > 50 ppm after 90 min. ameliorated only in 47.2% of cases (194). Kasir et al stated that their findings are in fact contradictive to previous pathophysiologic considerations, but very predictive for a positive response to antibiotics. This might indicate that improvement due to rifaximin does not result from its impact on SIBO in most cases, because SIBO would present with elevated breath hydrogen levels as previous reviews describe. (169,181) This is also emphasized by our finding that patients who symptomatically improved after rifaximin did not show any significant alterations in H2 values between before and after antibiotic treatment.

Furthermore, studies regarding the value of LHBT in predicting treatment results have shown contradictive results and therefore, question the relevance of lactulose in this setting: Long et al (188) evaluated in 2016 the predictive value of LHBT (15 g lactulose) in 100 IBS patients receiving antibiotic therapy, either rifaximin or amoxicillin/clavulanate. Patients were considered SIBO positive if baseline breath values were elevated, early hydrogen rise or second hydrogen peak were present. In total, 74% of all patients and 66% of SIBO negative patients improved. The authors concluded that a response to antibiotic therapy in IBS patients with suspected SIBO is not predictable by LHBT. A recent study from China also investigated the effectiveness of rifaximin (400mg twice per day for 4 weeks) in connection with SIBO and LHBT. Again, also SIBO negative IBS-D patients significantly improved (197). Regarding the selection of the test procedure in our study, the lactulose breath test was initially performed to assess changes of methane production before and after fecal microbiota transplantation, rather than detecting SIBO. As mentioned in chapter “Introduction”, several reviews and meta-analyses conclude that the glucose hydrogen breath test is more suitable to detect SIBO than LHBT, due to a higher

specificity (169,181,185,201). Furthermore, we have to mention, that patients in our study cohort did not use antiseptic mouth wash to eliminate lactulose fermentation by oropharyngeal bacteria prior to LHBT (202). Early hydrogen peaks in our patients might, at least in part, also be triggered by these oropharyngeal bacteria and therefore, interfere with our analyses.

4.3 Lactulose breath test and symptom severity

In 2013, Lasa JS et al described a significant correlation between the global H2 excretion (AUC) of 160 IBS patients (Rome III) and symptom severity assessed by IBS-SSS (189). A LHBT (10 g lactulose) was performed every 20 minutes for 180 minutes. Interestingly, no correlation was found between early or late H2 increase and IBS-SSS, which provides evidence against the SIBO as a cause. However, we could not confirm these findings in our study cohort, as we did not find a correlation between global H2 excretion and IBS-SSS. (Pearson: 0.157; p=0.593)

4.4 Methane

When analyzing methane levels in our study cohort, 50% (7/14) reached methane values ≥ 3 ppm at study visit 1. Patients with methane ≥ 3 ppm improved significantly (p=0.006) after administration of rifaximin. The group of patients with methane values ≥ 3 ppm mainly consisted of IBS-D patients (6/7), which are known to benefit from rifaximin. (192) After rifaximin at study visit 2, even 64.3% (9/14) had methane values ≥ 3 ppm, which argues against the hypothesis that improvement is dependent from eradication of methanogens via rifaximin. When applying the methane ≥ 3 ppm criterion, we could not confirm higher methane volumes in IBS-C compared to IBS-D patients, as described by previous studies (157–159).

4.4.1 SIBO criteria

A recent consensus (190) recommends 90-min H2 increases ≥ 20 ppm and methane values ≥ 10 ppm to be diagnostic of SIBO. When applying the early peak criterion (90 min H2 rise ≥ 20 ppm) on our study cohort, 50% (7/14) would have been diagnosed of SIBO at study visit 1 (44% of IBS-D patients and 60% of IBS-C patients). Ghoshal UC et al describe in a review, that the mean frequency of SIBO, detected by LHBT, among IBS

patients is 45% (169), which is very similar to our results. However, based on this diagnostic criterion, even 64.3% (9/14) of our study cohort would be diagnosed of SIBO on study visit 2 (56% of IBS-D patients and 80% of IBS-C patients). Ghoshal et al summarize, that SIBO is eradicated by rifaximin in 40.8% to 64.1% of cases (169), which makes a higher SIBO frequency after rifaximin intake in our study cohort highly unlikely.

Given the fact that the frequency of SIBO among IBS patients measured by jejunal aspiration (gold standard) is 23% (169), we critically question this early peak criterion, because it clearly leads to an overestimation of SIBO, as reviewed and demonstrated by numerous other studies. (169) This again points to the limitations of LHBT in diagnosing SIBO as already discussed above. The early peak criterion presumes oro-caecal transit times in all patients of at least 90 minutes. In fact, rises of ≥ 20 ppm in 90 min are more likely to result from colonic fermentation due to shortened oro-caecal transit times (especially in IBS-D patients), rather than SIBO. A study from 2011 with concurrent LHBT and oro-caecal scintigraphy in 40 IBS patients demonstrated that the oro-caecal transit time varies between 10 and 220min. Further, at the time of H2 increase, more than 5% of ingested lactulose has already arrived in the caecum in 88% of cases (203), which clearly shows the drawback of early peak criterion in LHBT.

Moreover, we used 25g lactulose instead of 10g, which is recommended by a recent consensus (190). Lactulose is known to be able to accelerate oro-caecal-transit time, which could be the case in our study and thus contribute to the high number of false positive results. The higher amount of ingested lactulose could also lead to higher breath hydrogen/methane values due to fermentation and thus also trigger false positive results.

Beside early hydrogen increases, elevated methane values ≥ 10 ppm regardless of the time point are suggested to be indicative of SIBO. Only two patients fulfilled this criterion in our study cohort, of whom one was classified as IBS-D and the other as IBS-C.

4.5 Serum zonulin

IBS-D patients showed significantly higher serum zonulin concentrations compared to IBS-C, both before ($p=0.009$) and after rifaximin intake ($p=0.029$). This confirms the findings of Barbara et al in 2015, who first described elevated serum zonulin levels in IBS patients with diarrhea-predominant subtype. However, the reason for elevated zonulin levels in IBS-D patients is still unclear and needs further investigation.

We did neither observe a significant correlation between serum zonulin and IBS-SSS nor between the changes of both variables. However, serum zonulin concentrations of patients, whose symptoms improved, changed significantly (p=0.046) on average by -1.495 (±1.75). Patients, who did not improve, showed a non-significant (p=0.621) change of serum zonulin of -0.645 (±3.98). This might indicate a connection between increased intestinal permeability and development of IBS-like symptoms. Antibiotic treatment with rifaximin could reduce the abundance of intestinal bacteria and thus decrease the release of zonulin, as bacterial exposure is one of the main triggers for mucosal release of zonulin according to Fasano.

4.6 Serum zonulin and hydrogen excretion

The relationship between serum zonulin concentrations and breath hydrogen values - either increases at certain time points or the overall excretion - has not yet been described in previous studies.

According to Fasano et al, one main trigger for zonulin release in the small intestine is the exposure to enteric microorganisms, which could be interpreted as a defense mechanism against bacterial overgrowth (110). Based on this assumption, we expected a positive correlation between zonulin levels and early hydrogen rises, both values suspected to reflect high numbers of bacteria in the small intestine. In fact, the opposite was the case in our study cohort: We found significant negative correlations between zonulin concentrations and hydrogen rises at 90, 120, 150 and 180 minutes. In other words, patients with low zonulin levels had high H2 levels. No correlations of serum zonulin levels with hydrogen rises at 30 and 60 minutes were found. However, we would expect bacterial overgrowth in the proximal small intestines to be reflected by early increases in LHBT. The significant negative correlations with later, colonic, hydrogen values might indicate an influence of colonic microbiota or the reaction of colonic epithelia to different colonic bacteria on zonulin levels. The missing correlation of zonulin values with early hydrogen increases stands in contrast to the hypothesis of Fasano. However, this might be, at least in part, explained by the above-mentioned limitations of the LHBT.

The significant negative correlation of high strength between hydrogen levels and serum zonulin levels was only present in IBS-C patients, which then in turn led to the significant negative correlation in all IBS-patients. This might be explained by the fact, that IBS-C

patients had significantly lower serum zonulin concentrations compared to IBS-D patients, which could lead to the negative correlation with H2 values.

A possible explanation for our findings could be the presence of a higher amount of biomass in the intestinal tract of IBS-C patients compared to IBS-D patients due to slow transit time. Increased fermentation of carbohydrates due to larger amounts of available intestinal biomass could results in increased SCFA's production. The SCFA butyrate is known to regulate tight junctions and improve intestinal barrier function (145,147,148). This could lead to low zonulin levels in IBS-C patients as an expression of the integrity of tight junctions and to higher expiratory H2 levels. The fact that SCFA are able to cause a reduction in motility of proximal intestine due to release of certain peptides in the ileum (204) matches this theory. In our study cohort, hydrogen increases of IBS-D patients were slightly lower, yet not significantly, compared to IBS-C patients at all time points. We observed a trend towards statistical significance at time point 120 and 150 min. This matches the above mentioned theory. On the other side, Ghoshal UC et al showed in 2014 that the stool form (Bristol stool scale) is influenced by the amount of bacteria in the small bowel, with higher numbers of bacteria leading to looser stools (175). Accordingly, SIBO is more common among IBS-D than IBS-C patients. Another study showed that IBS-C patients have the lowest number of SIBO positives among all IBS subtypes, detected by glucose breath test with a hydrogen or methane rise > 12 ppm indicative of SIBO (205). Therefore, we expected higher breath hydrogen levels among IBS-D patients, especially at early time points indicating small bowel fermentation. In fact, the opposite was the case in our study cohort which led to the above-described hypothesis.

However, because we did not measure SCFA's such as butyrate in our patients, we cannot proof our theory and it still needs further investigation.

4.7 Zonulin control group

As a marker for gut barrier integrity, we analyzed serum zonulin levels in all IBS patients and in a healthy control cohort. Our control group showed significantly higher zonulin levels compared to all IBS patients and to IBS-C patients. As described in chapter "Material and Methods", an ELISA was used for zonulin measurement. Due to the fact that patient and control samples were not measured on the same day, it is possible that this led to a bias in our results. Healthy controls were recruited in the year 2015 at the Division of Gastroenterology and Hepatology and their zonulin levels were measured in this period.

Samples of IBS patients were measured during this prospective study in 2017. Hence, different charges of ELISA kits were used for zonulin measurement in controls and IBS patients. Due to the high sensibility of this method, minor differences in production and execution of this test may have led to these diverging results. To assess the suspected bias, we calculated the coefficient of variation, which was 18.08%. This indicates variability in the measurements and explains the patch bias.

4.8 Summary

In summary, IBS-D patients significantly improved after antibiotic therapy with rifaximin. Patients with low (< 20ppm) hydrogen rises at 90 minutes and low basal hydrogen values (< 10ppm) showed significant improvement after treatment with rifaximin. However, the significance of low hydrogen increases for prediction of treatment response is questioned since classification of patients according to symptom improvement/non-improvement did not show different 90-minute or 60-minute breath hydrogen rises before treatment. The questionable relevance of the LHBT is supported by a lack of generally accepted diagnostic criteria for SIBO. The positive effect of rifaximin in IBS-D patients is suspected to result from its impact on altered microbiota composition. However, we did not observe a difference in H2 excretion between before and after rifaximin, maybe due to the limitations of the LHBT or the fact that rifaximin-associated gut microbiota changes are not reflected by hydrogen excretion.

We could confirm significantly higher zonulin levels in IBS-D patients compared to IBS-C patients, indicating decreased intestinal permeability in this subset of patients. Serum zonulin levels decreased significantly in patients who showed symptom improvement after rifaximin, which might indicate an association between serum zonulin levels and symptom development. Several of our calculations showed trends towards statistical significance but did not reach the threshold of $p<0.05$, possibly due to the small amount of patients included in this analysis.

In conclusion, further studies with larger study cohorts and in comparison to glucose hydrogen breath tests are needed to confirm our findings.

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6 Appendix

IBS-SSS

ANWEISUNGEN

Dieser Bogen dient uns zur Erfassung und Überwachung des Schweregrads Ihres Reizdarmsyndroms. Es ist zu erwarten, dass sich Ihre Symptome mit der Zeit ändern können. Versuchen Sie daher bitte, die Fragen anhand Ihres derzeitigen Befindens (also während der letzten 10 Tage) zu beantworten.

Bei Fragen, bei denen mehrere verschiedene Antworten möglich sind, kreisen Sie bitte die für Sie passende Möglichkeit ein.

1. Manche Fragen erfordern es, dass Sie eine passende Antwort eintragen.
2. Bei manchen Fragen müssen Sie eine Linie mit einem Kreuz markieren, so dass wir den Schweregrad eines bestimmten Problems beurteilen können.

Zum Beispiel:

Wie schwer waren Ihre Schmerzen?

Bitte markieren Sie die Linie an irgendeiner Stelle zwischen 0 und 100 % mit einem Kreuz (x) und geben so den Schweregrad Ihres Symptoms möglichst korrekt an.

Dieses Beispiel zeigt einen Schweregrad von ungefähr 90 %.

0% |_____X___| 100%

keine Schmerzen | nicht sehr schwer | ziemlich schwer | schwer | sehr schwer

TEIL 1: SCHWEREGRAD-PUNKTZAHL

	JA	NEIN	*Diese Spalte bitte für die Auswertung freilassen*
			Zutreffendes Kästchen einkreisen

1. a) *Leiden Sie derzeit unter Bauchschmerzen?*

b) *Falls ja, wie schwer sind Ihre Bauchschmerzen?*

0% I——————————————————————I 100%

keine Schmerzen | nicht sehr schwer | ziemlich schwer | schwer | sehr schwer

c) *Tragen Sie bitte die Anzahl der Tage ein, an denen Sie in den letzten 10 Tagen Schmerzen haben.*
Wenn Sie beispielsweise eine 4 eintragen, bedeutet dies, dass Sie an 4 von 10 Tagen Schmerzen haben. Falls Sie jeden Tag Schmerzen haben, dann tragen Sie eine 10 ein

Anzahl der Tage mit Schmerzen ☐ x 10

	JA	NEIN	
			Zutreffendes Kästchen einkreisen

2. a) *Leiden Sie derzeit unter Bauchblähung**
(Völlegefühl im Bauch, geschwollener oder gespannter Bauch)
*(*Frauen: Bitte ignorieren Sie Blähungen in Zusammenhang mit Ihrer Periode)*

b) *Falls ja, wie schwer ist Ihre Bauchblähung/-spannung?*

0% I——————————————————————I 100%

keine Blähung | nicht sehr schwer | ziemlich schwer | schwer | sehr schwer

3. *Wie zufrieden sind Sie mit Ihren Stuhlgewohnheiten?*

0% I——————————————————————I 100%

sehr zufrieden | ziemlich zufrieden | unzufrieden | sehr unzufrieden

4. *Bitte geben Sie auf der nachstehenden Linie mit einem Kreuz an, wie sehr Ihr Reizdarmsyndrom Ihr Leben im Allgemeinen beeinträchtigt oder sich störend darauf auswirkt*

0% I——————————————————————I 100%

überhaupt nicht | nicht sehr stark | ziemlich stark | völlig

Punktzahl ☐