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***In Vivo* evaluation of novel topical niacin nutraceuticals
for improving gut microbiota and metabolic health**

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Zusammenfassung

In der Vergangenheit konnte ein Zusammenhang zwischen dem Darmmikrobiom und der Adipositas sowie deren Komorbiditäten festgestellt werden, weshalb die Darmmikrobiota als neuer Ansatzpunkt in der Prävention und Therapie dieser Erkrankungen erforscht werden. In einem Maus Modell wurde Niacin als möglicher Kandidat zur Veränderung des Darmmikrobioms identifiziert, da die Gabe von Niacin zu einer günstigen Beeinflussung der Wirt-Mikrobiom Interaktion führte. Die Niacinmoleküle Nicotinsäure (NA) und Nicotinamid (NAM) dienen als Vorstufe des Nicotinamadenindinucleotids (NAD), welches in adipösen und Typ 2 diabetischen Mäusen dysreguliert ist.

Eine Kohorte mit 511 Probanden wurde deshalb hinsichtlich des Niacin Status und der Zusammensetzung der Darmmikrobiota untersucht. Die Analysen zeigten reduzierte Niacin-Serumspiegel in adipösen Typ 2-Diabetikern, was auf einen veränderten Niacin-Metabolismus schließen lässt. Weiter konnte ein positiver Zusammenhang der diätetischen Niacinzufuhr und dem Vorkommen von *Bacteroidetes* in Adipösen gezeigt werden, wobei die Adipösen weniger *Bacteroidetes* aufwiesen. Dennoch lässt eine einfache Erhöhung des Niacingehaltes in der Nahrung keine ausreichende Bereitstellung der Moleküle in den Dickdarm erwarten, wo jedoch die meisten Darmbakterien angesiedelt sind, denn oral aufgenommenes Niacin wird bereits im oberen Gastrointestinaltrakt resorbiert. Außerdem können unter hohen Dosen von schnell systemisch verfügbarem Niacin negative Effekte, wie zum Beispiel die *Flush*-Symptomatik, beobachtet werden. Deshalb wurden mit Hilfe lebensmitteltechnologischer Verfahren neuartige, lebensmittelbasierte NA- und NAM- Formulierungen entwickelt, die das Niacin unter minimaler systemischer Resorption in die Region des Ileo-Kolons liefern sollen.

Nach der *in vitro*-Evaluation, wurden die entwickelten NA- und NAM-Mikrokapseln in einem humanen Interventions-Pilotprojekt, welches aus einer Bioverfügbarkeitsstudie und einer *Proof-of-concept- and Safety-study* bestand, evaluiert. Raster-Elektronen-Mikroskop-Analysen zeigten die Freisetzung von NA und NAM aus den Mikrokapseln im humanen Gastrointestinaltrakt. Die Bestimmung der Niacin-Serumspiegel bestätigte die verzögerte Freisetzung von NA/NAM unter minimaler systemischer Resorption in der Region des Ileo-Kolons. Weiter zeigte sich unter Gabe der NA- und NAM-Mikrokapseln ein wünschenswertes Sicherheitsprofil. Die Intervention mit NA-Mikrokapseln führte zu einem signifikanten Anstieg der *Bacteroidetes*, analysiert mit 16S rRNA-Gen Sequenzierung, was auf eine vorteilhafte Änderung der Darmmikrobiota in Adipösen hindeutet. Darüber hinaus induzierte die Gabe von NA-Mikrokapseln signifikante Veränderungen von Biomarkern, die auf eine Verbesserung der Insulinsensitivität in Leber und Muskel sowie auf eine Verringerung der metabolischen Entzündung hindeuten. Die systemisch metabolischen Effekte waren jedoch nur unter Gabe von mikroverkapseltem NA, aber nicht unter freiem NA oder NAM

zu beobachten, was auf einen indirekten Mechanismus über die veränderte Zusammensetzung der Darmmikrobiota und nicht auf einen direkten systemischen Effekt von Niacin schließen lässt.

Abschließend lässt sich sagen, dass die vorliegende Arbeit darauf hindeutet, dass eine gezielte Mikrobiom-Intervention über Lebensmittel, die mit NA-Mikrokapseln angereichert sind, in der Prävention von Adipositas und Typ 2 Diabetes genutzt werden könnte.

Summary

The gut microbiota have been linked to obesity and its comorbidities and are viewed as a novel target in the prevention and treatment of those diseases. In a mouse model it has been shown, that administration of niacin beneficially effects the host-microbiome interaction, indicating niacin to be a promising candidate for a gut-targeted nutritional intervention. In addition the niacin molecules nicotinic acid (NA) and nicotinamide (NAM) serve as precursors for nicotinamide adenine dinucleotide (NAD), whose metabolome is dysregulated in obesity and type 2 diabetes in mice.

Niacin status and gut microbial composition were therefore examined in a cohort of 511 human subjects. Analyses revealed reduced niacin serum concentrations in obese type 2 diabetic subjects suggesting an altered niacin metabolism. Further dietary niacin intake was positively correlated with *Bacteroidetes* abundance in obese, which showed a reduced *Bacteroidetes* abundance. However, to perform a gut targeted intervention simply increasing niacin in human food was not expected to deliver sufficient amounts into the colon where most of the gut microbiota are located, since orally administered niacin will be rapidly resorbed in the upper gastrointestinal tract. Further, negative side effects such as facial flush can occur under high and immediate systemic available doses of niacin. Thus, novel food-based delayed-release NA and NAM formulations were developed using food technology in order to deliver NA and NAM under minimal systemic resorption into the ileo-colonic region.

After *in vitro* evaluation, the developed NA and NAM microcapsules were evaluated in a first human intervention project consisting of a bioavailability and a proof-of-concept and safety study. Scanning electron microscope analyses of niacin microcapsules revealed the release of NA and NAM from microcapsules in the human gastrointestinal tract. Determination of niacin serum levels proofed the delayed-release of NA/NAM under minimal systemic resorption in the ileo-colonic region. Further NA and NAM microcapsules showed a preferable safety profile with no flush occurring. The intervention with delayed-release NA produced a significant increase of *Bacteroidetes* abundance, analysed by 16S rRNA gene sequencing, indicating a favourable microbiome change in obese. In addition, delayed-release NA induced significant biomarker changes indicating improved insulin sensitivity of liver and skeletal muscle and reduced adipose tissue inflammation. Of importance, delayed-release NA, but not native NA or NAM resulted in systemic metabolic effects, suggesting an indirect mechanism via the microbiome rather than a direct effect of the nutrient itself.

In conclusion, the present thesis presents evidence of beneficial use of a targeted microbiome intervention by food enriched with delayed-release NA in the prevention of obesity and type 2 diabetes.

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List of abbreviations

AADAT	kynurenine aminotransferase II
ACMSD	amino-carboxymuconic-semialdehyde-decarboxylase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
BfR	German federal Institute for risk assessment
BMBF	German Federal Ministry of Education and Research
BMI	body mass index
CCBL1	kynurenine aminotransferase I
CCBL2	kynurenine aminotransferase III
CRP	C-reactive protein
DGE	German Nutrition Society
DNA	deoxyribonucleic acid
eGFR	glomerular filtration rate
ELISA	Enzyme-linked Immunosorbent Assay
enc.	encapsulated (microencapsulated)
EPIC	European Prospective Investigation into Cancer and Nutrition (EPIC)
FFQ	food frequency questionnaire
FoCus	Food Chain Plus
HAAO	3-hydroxyanthranilate 3,4-dioxygenase
HDL	high density lipoprotein
HOMA-IR	Homeostasis Model Assessment Insulin Resistance index
IDO1	indoleamine 2,3 dioxygenase I
IDO2	indoleamine 2,3 dioxygenase II
IKMB	Institute of Clinical Molecular Biology
IL-6	interleukin-6
IQ	interquartile range
KMO	kynurenine 3-monooxygenase
LDL	low density lipoprotein
Lp (A)	lipoprotein (a)
LPS	lipopolysaccharide
MLHB	Medical laboratory Bremen
mmHg	mm of mercury column

n.s.	not significant
NA	nicotinic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADS	nicotinamide adenine dinucleotide synthetase
NAM	nicotinamide
NAMDA	nicotinamide deamidase
NAMNAT	nicotinic acid mononucleotide adenylyltransferase
NAMPRTase	nicotinamide phosphoribosyltransferase
NAPRTase	nicotinic acid phosphoribosyltransferase
NE	niacin equivalents
NMNAT	nicotinamide mononucleotide adenylyltransferase
NR	nicotinamide riboside
OTU	operational taxonomical unit
PARP	poly(ADP-ribose)-polymerase
PCR	polymerase chain reaction
PPi	pyrophosphate
PRPP	phosphoribosyl-pyrophosphat
QPRTase	quinolate phosphoribosyltransferase
rho	spearman rank correlation coefficient
RNA	ribonucleic acid
ROS	reactive oxygen species
SCF	Scientific Committee on Food
SD	standard deviation
SEM	scanning electron microscope
SIRT1	sirtuin-1
T2D	type 2 diabetes
TDO2	tryptophan 2,3-dioxygenase
TRP	tryptophan
VLCD	very low calorie diet
γ -GT	gamma-glutamyl transpeptidase

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1 Introduction

Rather than being viewed as simple commensals, the gut microbiome is now seen as playing an active role in the control of energy homeostasis and in mediation of the adverse consequences of obesity. The first landmark studies in this field showed that gut microbial transplantation from conventionally raised animals to germ-free mice results in increased body fat content and insulin resistance despite reduced food intake [1]. Furthermore, colonization of germ free mice with microbiota from genetically obese mice (ob/ob) resulted in a greater increase in body fat than colonization with microbiota of lean donors [2]. The data suggested that an obesogenic microbiome increases the energy harvest from the diet thereby increasing body weight and inducing obesity associated co-morbidities [1, 2]. More recently, it has been shown that the two major phyla of mouse and human gut microbiota whose abundance differs between normal weight and obese hosts are the *Bacteroidetes* and the *Firmicutes* [3, 4]. In rodents, obesity is associated with an increase in the relative abundance of *Firmicutes* and a decrease in the relative abundance of the *Bacteroidetes* [4]. Also in humans, analogous differences in the *Firmicutes/Bacteroidetes* ratio of lean versus obese individuals have been observed in most [3, 5-7], but not all studies [8, 9]. In addition, obesity is associated with an overall reduction in gut microbial gene richness [6, 10, 11]. Thus, the gut microbiome is now viewed as a novel target in the prevention and treatment of obesity and its comorbidities.

Of physiological relevance, the composition of the gut microbiota can be altered by diet, as weight loss diets have been reported to influence the abundance of *Bacteroidetes* and *Firmicutes* [3, 8] and the overall microbial diversity [10, 12]. As reported in our co-authored publication in 2016, we also conducted a study with 18 obese subjects, performing a multimodal obesity program, to examine if a very low calorie diet (VLCD) can alter gut microbial composition [13]. Dietary intervention of the program (Optifast⁵²) included a three months VLCD, which was formula based, fully balanced according to micronutrients and consisted of approximately 800 kcal/day. Subsequently a weight maintenance phase of three months was performed, where formula meals were replaced by conventional meals step by step. A lean and an obese control group were included, which didn't undergo an intervention. The microbiome was characterized by performing high-throughput dual-indexed 16S rDNA amplicon sequencing. Gut microbiome analyses revealed a significant difference in *Firmicutes/Bacteroidetes* ratio between lean and obese individuals. Further, three months VLCD resulted in significant alterations of gut microbial diversity and bacterial metabolism. Of importance, the observed changes in gut microbial composition diminished during weight maintenance phase, despite sustained reductions in body weight and sustained improvement of insulin sensitivity. It was concluded that gut microbiota alterations were mainly induced by diet and not by weight change, and thus, did not sustain during weight maintenance phase. But

maintenance of beneficially alterations in gut microbiota may play an important role especially in long-term weight management, which seems to be still challenging. Therefore, new factors should be identified that target gut microbiota in obesity, also on long term.

Nicotinamide adenine dinucleotide (NAD⁺) is the central co-factor of metabolism, mediating fuel oxidation, adenosine triphosphate (ATP) generation, reactive oxygen species (ROS) detoxification, biosynthetic processes, DNA repair and nutritionally sensitive gene regulation [14]. In vertebrates, NAD⁺ is synthesized *de novo* from tryptophan (Trp) and from three vitamin precursors [15]. Nicotinamide (NAM) and nicotinic acid (NA), here termed niacin, are the classical NAD⁺ precursor vitamins. Nicotinamide riboside (NR) was discovered to be a NAD⁺ precursor vitamin much more recently [16]. The NAD⁺ metabolome has been shown to be dysregulated in obesity and type 2 diabetes in mice [17]. Moreover, NR repletion has been shown to blunt weight gain on high fat diet [18] and to oppose fatty liver on high fat high sucrose diet [19], largely by increasing the activity of SIRT1, an NAD⁺-dependent protein lysine deacetylase.

The effect of NAD⁺ precursors as modulators of the microbial contribution to host metabolism has not been thoroughly investigated. However, administration of Trp and niacin has been shown to beneficially effect the host-microbiome interaction in a mouse model especially when administered in controlled released formulation targeting the ileo-colonic region [20, 21]. These effects are thought to be mediated by an improvement of the host-microbiome interface involving signaling pathways in intestinal epithelial cells that result in beneficially altered production of antimicrobial peptides and thereby affecting the intestinal microbiome [20]. Thus, Trp, NA and NAM might be promising candidates for a gut-targeted microbiome intervention.

Because the upper gastrointestinal tract efficiently resorbs amino acids and soluble vitamins, simply increasing the Trp, NA and/or NAM content in the food in humans would not be expected to deliver these molecules into the colon, where most of the microorganisms are located [22]. Furthermore, negative side effects such as facial flush or gastrointestinal symptoms can occur under high and immediately systemic available dosages of NA and/or NAM [23]. Thus, novel food-based delayed-release NA and NAM formulations were developed, using food technology (Department of Food Technology, Christian-Albrechts-University of Kiel, Germany), to deliver NA and NAM under minimal systemic resorption into the ileo-colonic region to target the gut microbiome. The developed NA and NAM microcapsules were evaluated *in vitro* and showed a promising release profile. Hence, the novel formulations were used to produce food-grade NA and NAM microcapsules for human interventions studies, in order to also evaluate the niacin microcapsules *in vivo*.

The aim of the present thesis was therefore (I) to examine Trp, NA and NAM in humans in relation to obesity, T2D and the gut phylogenome in a large human cohort of >500 well characterised individuals with different BMIs and metabolic abnormalities, (II) to evaluate bioavailability, systemic resorption and (III) the safety profile of the developed NA and NAM microcapsules under dose escalation in a pilot *in vivo* project and finally (IV) evaluate effects of NA and NAM microcapsules on gut microbial composition and human systemic metabolism.

2 Theoretical background

2.1 Niacin – an overview

Niacin is a term used to describe compounds that have the biological activity associated with NAM (3-Pyridincarboxamide) and NA (3-Pyridincarboxylic acid) (**Figure 1**) [24, 25]. In the past niacin was identified as a pellagra-preventive factor (vitamin PP) by Joseph Goldberg [24], but nowadays the term vitamin B3 is commonly used for niacin. NA and NAM are white crystalline solids which are stable in air and at room temperature, whereby NA is moderately soluble in water and NAM is highly soluble in water [24]. Both, NA and NAM, can be converted to the biologically active NAD and NADP coenzyme forms. NAD belongs to the group of dinucleotides and consists of NAM and adenine which are connected by ribose and phosphoric acid. In analogy NADP shows a similar structure, but the 2'-hydroxyl group is esterified with phosphoric acid (**Figure 1**) [25].

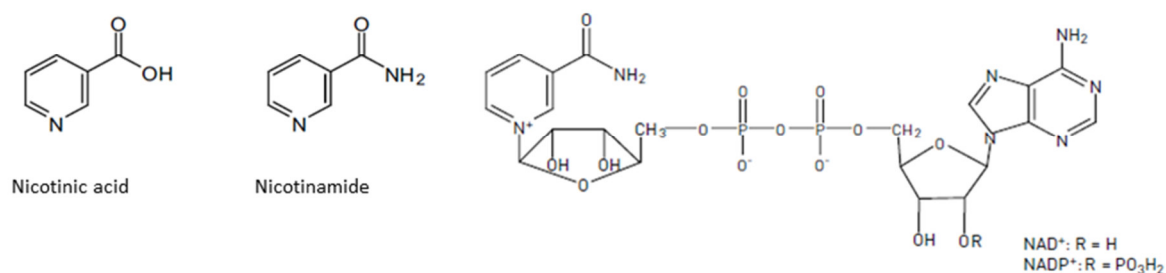


Figure 1: Structural formula of nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (based on Stahl & Hesecker [25])

Abbreviations: NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

2.1.1 Food content, dietary requirements and niacin status

Niacin is present in plant and animal foods and is widely distributed in human food [26]. Good sources are yeast, meat (including offal), cereals, legumes and seeds, fish, coffee and tea [26-28]. In animal products niacin is mainly present as NAM or its coenzymes NAD and NADP [24, 29, 30]. Coenzymes tend to release NAM during aging or cooking [24] or they are metabolised to NAM in the upper small intestine and the released NAM can be absorbed [24, 27]. Thus niacin

originating from animal food shows good bioavailability [27]. In contrast, plant food mainly contains NA, but much of it occurs in bound forms [24]. These bound forms (sometimes referred to as niacytin), for example in cereals, are heterogeneous mixtures of polysaccharides and glycopeptides to which NA is esterified. Therefore NA from plant products is less bioavailable [24, 26, 27, 30]. The bioavailability can be increased by alkali treatment, which frees the NA for absorption, as it is commonly performed in Central America with maize prior to the preparation of tortillas [24, 26, 27]. Further, roasting of coffee beans releases NA from its bound form [26, 27, 30].

Stability of NA and NAM in food products is good, since both are stable in the presence of oxygen, acids, light and heat. Thus, in normal food preparation it can hardly be destroyed. It can be washed out by water (e.g. boiling or blanching) due to its water solubility, but loss can be minimized when cooking water is not discarded [26].

Because of the metabolic formation of niacin from Trp, intake recommendations not only include the levels of niacin, but also that of the essential amino acid Trp. Thus intake recommendations are given as niacin equivalents (NE) [28], which are commonly calculated as niacin intake (mg) plus $1/60$ Trp intake (mg) [31, 32]. Since niacin (NAD) plays a central role in energy homeostasis reference values depend on energy intake. WHO recommended intake of 6.6 mg NE per 1000 kcal per day in 2000 [26]. Reference values of the German Nutrition Society (DGE) are stratified by gender and age due to dependency on energy intake and are shown in **Table 1**. Only reference value for infants younger than 4 months depends on content of NE in breast milk and is set at 2 mg/day [28].

Due to its wide distribution in human food also highest reference values for NE intake can be reached with a complete nutrition [28]. Niacin intake within the German population was evaluated by Max-Rubner-Institute and results were reported in 2008. The median niacin intake (NE) of men was 36 mg/day and median niacin intake of women was 27 mg/day. Thus, niacin intake was even higher than recommendations for niacin intake, and only 1 % of the men and 2 % of the women did not reach intake recommendations. Niacin intake decreased with age in men older than 19–24 years. In contrast, age-dependent fluctuations in niacin intake of women were negligible. Main sources of niacin intake were meats and sausages, non-alcoholic beverages, bread, milk and dairy products including cheese [33].

Table 1: Recommendations for niacin intake (DGE, 2015)

age	niacin equivalents ^a mg/day	
	male	female
infants		
0 to < 4 months ^b	2	
4 to < 12 months ^c	5	
children and adolescent^c		
1 to < 4 years	8	
4 to < 7 years	9	
7 to < 10 years	11	10
10 to < 13 years	13	11
13 to < 15 years	15	13
15 to < 19 years	17	13
adults^c		
19 to < 25 years	16	13
25 to < 51 years	15	12
51 to < 65 years	15	11
65 years and older	14	11
pregnant women^d		
2. Trimester	14	
3. Trimester	16	
breastfeeding women^e		
	16	

^amg niacin equivalents= 1 mg niacin= 60 mg Tryptophan; ^bestimated value, which refers to preformed niacin; ^cbased on age- and gender-specific reference values for energy intake; ^dbased on reference values for women between 19 and 25 years (PAL-value 1.4) plus 250 kcal/day in the second and 500 kcal/day in the third trimester; ^ebased on reference values for women between 19 and 25 years (PAL-value 1.4) plus 500 kcal/day for exclusively breastfeeding mothers during the first 4–6 months

2.1.2 Absorption and metabolism of niacin

Both NA and NAM can be absorbed in their free forms in the stomach and upper small intestine. NAD and NADP are first metabolized to NAM by mucosal enzymes in the upper small intestine, which can be absorbed afterwards [24, 29]. At low concentrations NA and NAM are absorbed via sodium-dependent facilitated diffusion [29], or via high affinity and a specific carrier mediated mechanism [34] making use of proton cotransporters and anion antiporters [35]. Higher concentrations can be absorbed by passive diffusion [24].

NAM that was absorbed from gut lumen into enterocytes can either be converted to NAD via Dietrich pathway (s. pathway below, **Figure 2**) to store niacin within the cells or it can be released into the portal circulation. Also NA can move into the blood in its native form, but the bulk will be converted to NAD via Preiss-Handler pathway (s. below, **Figure 2**). Through NAD glycohydrolases NAM can be released from NAD (s. pathway below) into the plasma as the principal circulating form of niacin, if required [24].

After entering the portal circulation NA and NAM are either internalized by erythrocytes, which take up niacin compounds by facilitated diffusion and convert them to nucleotides to maintain concentration gradient, or NA and NAM are transported to the liver. Both NA and NAM can be metabolized in the liver to NAD (s. pathway below) or to yield compounds for urinary excretion, depending on niacin status. Moreover, NR (nicotinamide riboside) from diet has been discovered to serve as a precursor for NAD synthesis [16]. Built NAD can be stored in the liver. The storage of NAD can serve to supply other tissues with NAM by releasing it from NAD into the circulation mediated through glycohydrolases (s. pathway below). For urinary excretion a variety of methylation and hydroxylation products of the niacin compounds are formed in the liver [24, 29]. The spectrum of urinary products includes NA, NAM, N¹-methyl-nicotinamide, N-oxide, N¹-methyl-4-pyridon-5-carboxamid, N¹-methyl-2-pyridon-5-carboxamid, nicotinic acid, 6-hydroxynicotinic acid and 6-hydroxynicotinamide [26, 29]. Under lower doses of niacin the 2-pyridon form and under higher doses N-methyl nicotinamide and nicotinic uric acid are built primary [26, 29]. The N-oxide has been reported to be a minor excretion product [29]. Further NA and NAM itself are excreted under high doses of niacin [26], whereas NA and nicotinic uric acid were absent under NAM administration [29]. The ratio of N-methyl-nicotinamide and Methyl-2-pyridon-5-carboxamid has been reported as a parameter to assess niacin status [24, 26, 36].

Besides the oral supply with niacin compounds, NAD can be built *de novo* from the essential amino acid Trp via the kynurenine pathway. As described above, not all dietary Trp will be converted to NAD, because Trp is also used for other pathways such as protein synthesis or methoxyindole pathway, which leads to the formation of serotonin. Within the kynurenine pathway, dietary Trp is metabolized through kynurenine and quinolinic acid to NA mononucleotide, which is then converted to NAD within Preiss-Handler pathway. Conversion of Trp, NA, NAM and NR to NAD through Kynurenine-, Dietrich- and Preiss-Handler pathway is summarised in **Figure 2**.

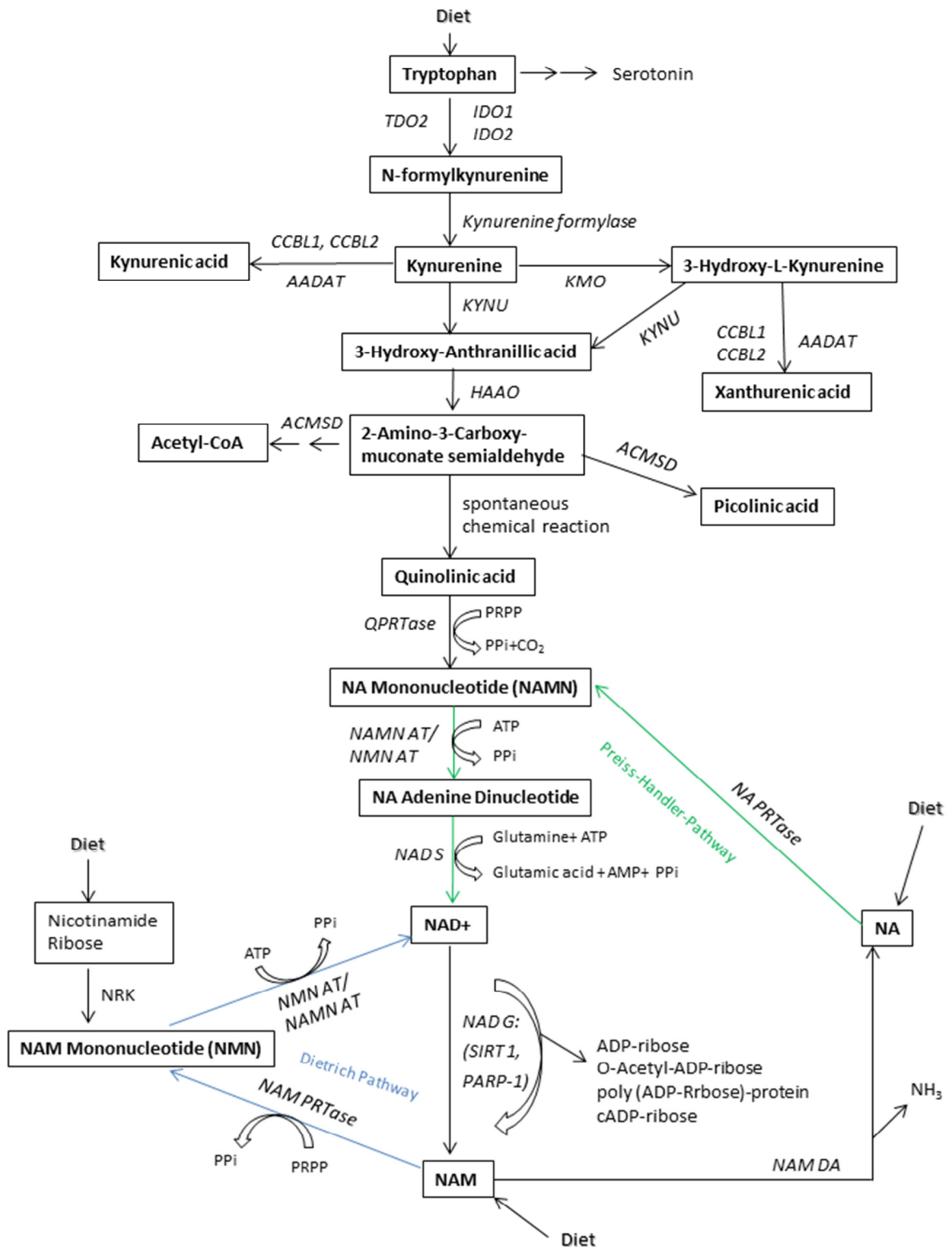


Figure 2: Pathways of nicotinamide adenine dinucleotide (NAD) synthesis (based on [24, 37-39])

Abbreviations: NA, nicotinic acid; NAM, nicotinamide; NAD, nicotinamide adenine dinucleotide; TDO2, tryptophan 2,3-dioxygenase; IDO1, indoleamine 2,3-dioxygenase I; IDO2, indoleamine 2,3-dioxygenase II; CCBL1, kynurenine aminotransferase I, CCBL2, kynurenine aminotransferase III; AADAT, kynurenine aminotransferase II; KMO, kynurenine 3-monooxygenase; KYNU, kynureninase; HAAO, 3-

hydroxyanthranilate 2,3-dioxygenase; ACMSD, amino-carboxymuconic-semialdehyde-decarboxylase; QPRTase, quinolinate phosphoribosyltransferase; PRPP, phosphoribosylpyrophosphat; PPi, pyrophosphate; NAMNAT, nicotinic acid mononucleotide adenyltransferase; NADS, nicotinamide adenine dinucleotide synthetase; ATP, adenosinotriphosphat; SIRT1, sirtuin-1; PARP, poly(ADP-ribose)-polymerase; NAMPRase, nicotinamide phosphoribosyltransferase; NRK, nicotinamide riboside kinase; NMNAT, nicotinamidmononucleotide adenyltransferase; NAMDA, nicotinamide-deamidase; NAPRTase, nicotinic acid phosphoribosyltransferase.

2.1.3 Function of niacin

Niacin has universal biochemical functions in human metabolism. The coenzyme forms NAD and NADH act as cofactors in redox reactions. NAD⁺ is reduced to NADH during oxidation of energy sources (e.g. glycolytic reactions, oxidation of acetate during citric acid, oxidation of alcohol, β -oxidation of fatty acids). The resulting NADH is used as an electron donator in electron transport chain (respiratory chain) to generate ATP. In contrast to NAD(H), which plays a central role in energy expenditure, NADP(H) is used for biosynthetic reactions. NADP is produced by phosphorylation of NAD via NAD kinase [24, 27]. Through reduction of NADP⁺ during the pentose-phosphate pathway NADPH is produced, which acts as a reducing agent for biosynthetic pathways (e.g. fatty acid production, cholesterol synthesis).

Further, niacin functions as a substrate for reactions that affect DNA-repair. For example, NAD is consumed by ADP-ribose transferase and poly(ADP-ribose) polymerase to create an ADP-ribosyl modification or synthesize an ADP-ribose polymer. Poly (ADP) ribosylation plays a diverse role in molecular and cellular processes such as DNA-damage response and epigenetic modification [14, 24].

NA in high doses also plays a pharmacological role, due to its lipid-lowering effects. These effects are mediated through the G-protein receptor GPR109A resulting in an inhibition of adenylate cyclase and thus in reduction of cAMP levels. This leads to a decreased mobilization of fatty acids through lipolysis. Thus formation of triglycerides and VLDL (very low density lipoprotein) is reduced in the liver causing a subsequent drop of LDL (low density lipoprotein) levels. Additionally to other lipid-lowering agents, NA also increases circulating levels of the beneficial HDL (high density lipoprotein) [24, 27, 40]. However, pharmacological doses of niacin can lead to toxic and severe side effects, as it will be discussed below.

2.1.4 Niacin deficiency

Early symptoms of niacin deficiency are unspecific during the first phases of deficiency. For example, insomnia, anorexia, weight loss, lassitude, burning sensation on the tongue, diarrhea, abdominal pain, headache, dizziness, lack of concentration, confusion and forgetfulness have been reported. Manifestation of clinical niacin deficiency leads to the pellagra, showing changes in the skin (dermatitis), gastrointestinal (diarrhea) and nervous system (dementia). Parts of the skin that are exposed to direct sunlight show symmetrically distributed erythema, which are hyperpigmented and accompanied by burning and itching. In the course of pellagra the affected skin party will be hardened and scaling and exfoliation can be observed. In the gastrointestinal tract glossitis, stomatitis and diarrhea can be observed. Further, patients complain of nausea or vomiting. Symptoms affecting nervous system can be anxiety, depression, fatigue, tremors or irritability, but also mental aberration can be observed which can lead to dementia [26, 27].

Niacin deficiencies due to insufficient alimentary intakes are not common, since there is adequate supply within the German population (s. above). Nevertheless other causes can lead to deficiency symptoms: interaction with drugs, disorders in absorption, chronic diarrhea, chronic dialysis or deficiency in other vitamins involved in the NAD synthesis from Trp (e.g. Vitamin B6) [27].

2.1.5 Niacin toxicity

No adverse reactions are known under doses of physiological amounts of niacin appearing in human food [27], but additional intake of high niacin doses can lead to health impairment [23]. Vasodilatation is commonly seen under dosage with NA, which is associated with burning or stinging sensations in the face, hands and arms and is commonly called flush/flushing [23, 24, 27, 30]. Flush symptomatic does not occur under NAM application [30]. Further, short-term side effects under niacin administration can include gastro-intestinal symptoms such as nausea, vomiting or diarrhea [23, 24, 30]. Moreover, hepatotoxicity can be observed under high niacin dosage [23, 27, 30], but occurrence of hepatotoxic effects might depend on niacin formulation [23]. NA has also been reported to induce impaired glucose tolerance [23, 24, 30]. Comparing NA and NAM, NAM shows fewer adverse side effects and observed side effects mainly occur under higher doses of NAM than under NA [23].

2.2 Human gut microbiome – an overview

Humans are associated with a diverse community of microorganism. This microbial community is mainly composed of bacteria but also includes archaea, viruses, fungi and protozoa. The sum of all microorganisms is called microbiota and they reside in and/or on the host or a specified part of the host. The microorganisms mainly cover mucosal surfaces of the host. Since the gut has a huge surface area that can function as the habitat for bacteria and it is also rich in molecules that can be used as nutrients by bacteria, most bacteria reside within the gastrointestinal tract and thus are called gut microbiota [22, 41].

2.2.1 Composition of the human gut microbiome

The ‘normal’ gut microbiota is dominated by anaerobic bacteria and approximately consists of 500–1000 species in total. In the adult human gut $\sim 10^{14}$ bacterial cells are located. That is a tenfold of the number of human body cells [22, 41]. But the density of gut bacteria cells varies between the different sections of the human gut. It increases from 10^1 – 10^3 bacteria per gram of content in the stomach and duodenum to 10^4 – 10^7 bacteria cells per gram in the Jejunum and Ileum. But most of the gastrointestinal bacteria are located within the colon with up to 10^{12} bacteria cells per gram of content [22].

Cell culture of the complex community was difficult, due to unknown growth requirements, stress imposed by the cultivation procedure and difficulties on simulating the interactions of bacteria with other microbes and host cells. Thus a culture-independent sequencing was introduced as a new approach, which made detection of microbial genes and disease associated patterns in our gut microbiota possible. Using this new approach it was possible to identify the main phyla in the human gut [42, 43], which have been identified to be the *Bacteroidetes* and the *Firmicutes* [3, 44, 45], whereas other phyla such as *Proteobacteria* and *Actinobacteria* are present in minor proportions [44].

Colonization of the human gut begins during birth, when babies are exposed to microbes from different environments. Depending on delivery mode (vaginal vs. cesarean section) infants show different microbial composition [22, 42]. During the first year of life the gut harbors very low diversity of bacteria [22, 46], but the composition changes and diversity increases as the infant grows and by the end of the first year of life gut microbial composition resembles that of a young adult [47]. The initial colonization of the human gut is presumed to shape microbial composition through adulthood [22]. Besides that, host genome has been reviewed to impact the microbial composition [22, 48]. For instance, the *Firmicutes/Bacteroidetes* ratio is altered in genetically obese mice compared to their lean littermates [4].

Though the major bacterial groups dominating the human intestine are conserved, the proportion of the groups can vary. The gut microbial composition shows high inter-individual variability, but was reported to be stable during adult life within an individual. The constancy of microbial composition considers that numerous variables are held constant, since microbial composition can be influenced by many factors such as diet, disease or antibiotic treatment [22, 42, 48].

As already mentioned, sequencing technologies are used to determine the composition of the gut microbiota nowadays. In specific, the targeted amplicon sequencing of the 16S ribosomal RNA (16S rRNA) gene is currently the most used strategy [49]. The sequenced gene regions ('reads') of 16S rRNA are mapped to known 16S rRNA gene sequences of different microbial taxa, such as genera or phyla. Further, sequences can be clustered into operational taxonomical units (OTUs), according to their similarity and the allowed threshold (e.g. 97 % similarity on species level) [49, 50]. In addition to the abundance of specific genera or phyla, indices of diversity, such as alpha-diversity, can also be determined to evaluate the human gut microbiota. The alpha-diversity is a measure of the compositional complexity of a community within a site [49]. It thus represents a within sample taxonomic diversity (intra-individual) [50]. There is no consensus definition of alpha-diversity and therefore several measures for alpha-diversity have been established. These include richness indices that estimate the number of different species, for example by simple counting the number of different taxonomical or OTU levels (species) within a sample. Since some rare species can be lost during analyses, an underestimation of species richness is possible. Therefore richness estimators (e.g. Chao1 index) were developed in order to predict the true richness of a sample by correcting the observed richness for the number of lost species, considering the distribution of the rarest species. Besides richness indices also evenness indices, which consider the species relative abundances without focusing on their total number, and diversity indices, which account for both species abundance and total number of species (e.g. Shannon index), are established [49]. Further the breadth or depth of phylogenetic branches spanned by a microbiome can be considered by phylogenetic measures [50].

2.2.2 Metabolic function and host-microbiome interactions

The collective genomes of the bacteria are known as the microbiome, which includes ~ 100 times as many genes as the human host's genome [45, 51]. This large number of genes enriches the host's genetic potential of biochemical and metabolic activities. Thus, the intestinal microbiota can perform metabolic activities that humans cannot and therefore have been referred to as an additional organ [41, 42, 52]. For example, microbiota can ferment otherwise indigestible polysaccharides and thus increase the energy harvest for the host [1-3], and they play a role in supplying humans with essential vitamins [53, 54]. Further gut microbiota are required for the

development and differentiation of host's intestinal epithelium and immune system, and they protect the host from incoming pathogens [22, 41, 42, 48].

Beyond the involvement in 'normal' host physiology, gut microbiota can be contributing factors in the development of several diseases [22]. The increased energy harvest through the gut microbiome indicates that gut microbiota may play a role in the development of obesity. Besides an increased energy harvest, several other mechanisms are involved such as an improvement of the absorption of nutrients and a microbial regulation of host genes that promote deposition of lipids in host's adipocytes [1, 55]. Although caloric intake is a main cause of human obesity, manipulation of host's genes and metabolism through gut microbial composition, may predispose to obesity [55]. In addition to energy homeostasis, gut microbiota also impact on other levels of host metabolism. For instance, an altered composition of the gut microbiota has been linked to insulin resistance and can contribute to the development of diabetes. Further the obese gut microbiome has been reported to promote activation of inflammatory pathways [22, 42].

3 Material and methods

3.1 Study population of the FoCus cohort and study design

Within the network of competence *Food Chain Plus* (FoCus), which was funded by the German Ministry of Education and Research (BMBF), a cohort of n=2000 human individuals was established from 2011-2015 in Kiel (Germany). The FoCus cohort was built-up to perform nutritional intervention studies, examine genetic and environmental factors in the pathogenesis of nutrition associated diseases, develop functional food products with anti-inflammatory properties and evaluate these novel food products in human intervention studies (<http://www.focus.uni-kiel.de/>). 500 subjects of the FoCus cohort were recruited from obesity outpatient clinic of the Department of Internal Medicine I of the University Hospital of Kiel, and 1500 subjects were recruited from the regional registration office as cross-sectional controls. Participants of the FoCus cohort were phenotyped and genotyped extensively, including: anthropometric measurements, collection of blood samples to determine several metabolic and inflammatory parameters as well as microbiome 16S rRNA gene sequencing of collected stool samples. Subjects were advised to come fasted to the study center for examinations and blood sample collection. Furthermore subjects completed a 12 months retrospective food frequency questionnaire (EPIC), which served to evaluate nutritional intake according to EPICsoft database [56, 57]. Study was proofed by local ethic committee and written confirmed consent was obtained from each subject.

As a subset of the described FoCus cohort, n=511 human subjects were included in the present investigation in order to examine the NA, NAM and Trp serum levels and the gut microbial composition of subjects with different metabolic phenotypes. The subjects were subdivided into four groups of different metabolic phenotypes: (I) underweight (BMI <20 kg/m², n=66), (II) lean (BMI 20-25 kg/m², n=149), (III) obese without type 2 diabetes (T2D) (BMI>30 kg/m², n=148) and (IV) obese with T2D (BMI>30 kg/m², n=148). Obese and lean groups were matched by age and sex. Baseline characteristics of this FoCus subset are shown in **Table 2**. In addition to routine laboratory parameters, NA, NAM and Trp serum levels were measured in samples of the sub-cohort subjects. The food frequency questionnaire was completed by n=481 subjects. Three variables of niacin and Trp intake were calculated from food frequency questionnaires (EPIC): niacin intake (including NA+NAM intake), Trp intake and intake of NE.

3.2 Production of NA and NAM microcapsules

Within a joined project, funded by the Cluster of Excellence *Inflammation at Interfaces*, niacin microcapsules were developed and produced at the Department of Food Technology of the Christian-Albrechts-University of Kiel. The desired formulation was one that would deliver niacin molecules in a pH-dependent manner and under minimal systemic resorption into the colon, where most of the gut microorganisms are located [22], and thus, avoid major side effects of NA and NAM. To be able to dissect potential differences of NA versus NAM, two separate microcapsules were developed, the first carrying only NA the second only NAM. In Brief:

At first a core of NA or NAM (0.3 – 0.5 μm) was prepared. Due to the higher daily doses of NAM, the NAM cores were prepared by a continuous granulation process using a ProCell fluidized bed granulator with Vario 3 from an external company (Glatt Ingenieurtechnik, Weimar, Germany). For the production of the NA microcapsules, a NA/HPMC (hydroxyl-propyl-methyl-cellulose) solution was applied to indigestible cellulose-cellets via fluidized bed spray coating. NA and NAM cores were subsequently microencapsulated using a double shellac coating separated by an intermediate pH-modulating layer consisting of sodium bicarbonate and citric acid for NA and NAM cores, respectively. Both the double shellac coating and the pH-modulating layer were applied via fluidized bed spray coating and aimed at modulating the pH-depending degradation or dissolution of the shellac coating.

To verify the homogenous distribution of the coating material, SEM pictures were taken after the coating process. Further the dissolution profile was evaluated *in vitro* by subjecting NA and NAM microcapsules to simulated gastrointestinal (GIT) fluids according to modified pharmaceutical standards [58]. The *in vitro* release profiles of the developed NA and NAM microcapsules proofed resistance against the gastric fluid simulating fasten or fed status, and indicated a sufficient release of NA and NAM during the exposure to pH-values which can be found in the human ileum and colon. For the *in vivo* studies NA and NAM microcapsules (**Figure 3**) and NA and NAM powder (free niacin) were filled into standard size 0 gelatin capsules using a manual capsule filler for 60 capsules for a better handling. Differential dosing was achieved by administration of different amounts of full or partially filled capsules (for detailed description: Eva Theismann, Department of Food Technology, Christian-Albrechts-University, Kiel; Germany).

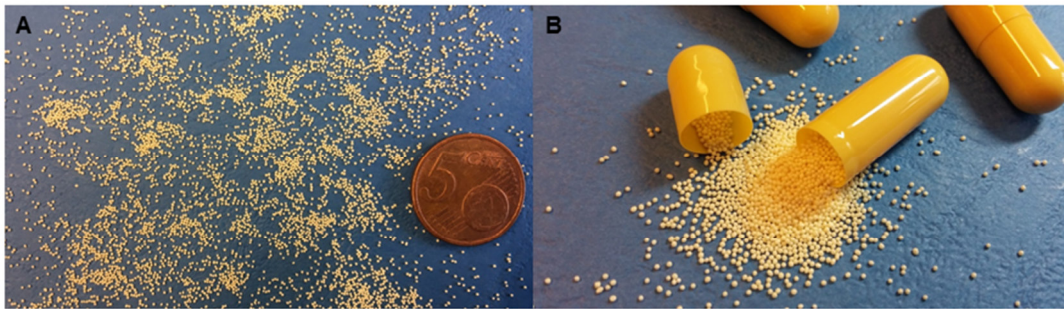


Figure 3: Photography of niacin microcapsules (supplied by Eva Theismann, Department of Food Technology, Christian-Albrechts-University; Kiel)

Niacin microcapsules (A) were filled into standard size 0 gelatine capsules (B) for *in vivo* studies.

3.3 Study cohort and study design of niacin interventions

After an *in vitro* evaluation of the developed food-grade delayed-release niacin microcapsules, two human intervention studies were performed to evaluate the niacin microcapsules *in vivo*: (I) a bioavailability study (including pilot and main bioavailability study) and (II) a proof-of-concept and safety study. Subjects for niacin interventions were recruited and studies were performed at the Department of Internal Medicine 1 of the University Hospital Schleswig-Holstein, Campus Kiel (Kiel Germany). Niacin interventions were proofed by local ethic committee and written confirmed consent was obtained from each subject.

3.3.1 Study cohort and study design of pilot bioavailability study

A small self-experiment was performed in order to get a first impression of the release profile of niacin from microcapsules, and thus, to further design main bioavailability study. Three healthy volunteers (investigators of the project) ingested either 900 mg of unformulated NAM (first week) or 900 mg of microencapsulated NAM (second week) packaged in gelatin capsules. Before self-administration of the dose, subjects fasted for twelve hours with water only. Blood samples were taken at baseline (before ingestion, hour 0), every hour until eight hours after ingestion as well as after ten and twelve hours. Venous catheters were used to reduce the number of venous punctures. During time of blood sampling subjects remained fasted with water only, except for two light meals with minimal vitamin B3 content. One such “low niacin meal” consisted of 150 g apple, 150 g red pepper and 100 g cucumber and approx. 3.2 mg NE (calculated with EBISpro nutritional software, <http://www.ebispro.de/>). NA and NAM serum levels were measured in all blood samples and volunteers stated their height and weight. Baseline Characteristics of the volunteers are shown in **Table 3**.

3.3.2 Study cohort and study design of main bioavailability study

To determine systemic exposure profile of different doses of NA and NAM microcapsules, main bioavailability study was performed including 20 healthy volunteers. Subjects were allocated into two groups: one group received NA capsules (n=10; “NA-group”) and the other group received NAM capsules (n=10; “NAM-group”). Exclusion criteria were: kidney- or liver-disorders, pregnancy, pre-existing rheumatic diseases or infections, smoking, medication intake (except birth control and thyroxine) and intake of nutritional supplements (protein- or vitamin-preparations). Baseline characteristics of the study populations are shown in **Table 4**.

Bioavailability study consisted of four study days, each separated by one week of washout and regeneration. At each study day, subjects came to the study center after an overnight fast (with water only) and fasted blood samples were collected. After administering the respective dose of NA or NAM (with a cup of water), blood samples were collected in hourly intervals for eight hours. The two last blood samples were collected ten and twelve hours after administration of niacin dose. Venous catheters were used to reduce the number of venous punctures. One subject of NA-group dropped out after two study days in bioavailability study due to persistent difficulties in blood sample collection. NA and NAM serum levels were determined in each blood sample (s. Biochemical analyses). In addition, safety parameters were analysed at baseline of each study day: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (γ -GT) were measured to monitor liver function, and creatinine and estimated glomerular filtration rate (eGFR) were measured to monitor kidney function. In addition uric acid was determined, since niacin can induce high uric acid levels [23].

To avoid an influence of niacin rich foods on niacin serum levels, subjects remained fasted (with water only) during blood sample collection, except for two light meals with minimal vitamin B3 content. Due to the personal experiences of the investigators of self-experiment (pilot bioavailability), composition of low niacin meal was changed and consisted of three rice wafers, one apple and one carrot and was supplied by the study center. Vitamin B3 content was calculated with the nutritional software EBISpro (<http://www.ebispro.de/>) and consisted of approx. 2.3 mg NE. These meals were consumed after blood samples were taken at hour three and eight. At the baseline study day, weight was measured and subjects stated their height. Daily routine of the study days are shown in **Figure 4**. On the calibration day of the study a reference dose of free NA (30 mg) or free NAM (900 mg) was administered, thus, each subject was its own control. Reference dose was chosen due to recommended daily allowance in the case of NAM (900 mg) and due to vasodilatory effects that can occur under doses >30 mg in the case of NA [23, 30]. Different doses of microencapsulated NA or NAM were administered on the other three study days: 30 mg NA or 900 mg NAM, 150 mg NA or 1500 mg NAM, and 300 mg NA or 3000 mg NAM.

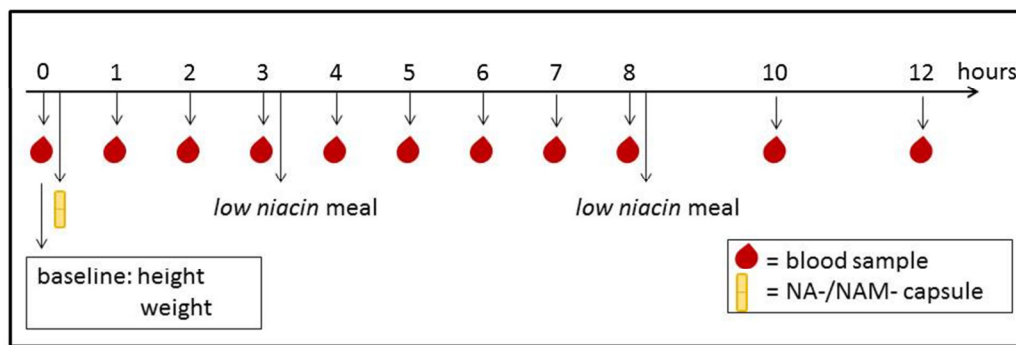


Figure 4: Study design of the niacin bioavailability study

Abbreviations: NAM, nicotinamide; NA, nicotinic acid

3.3.3 Study cohort and study design of the proof-of-concept and safety study

The safety profile of niacin microcapsules was examined during a proof-of-concept and safety study for a total of six weeks. Study was performed with 10 healthy volunteers allocated into NA and NAM group with 5 subjects each. Exclusion criteria were: kidney- or liver-disorders, pregnancy, pre-existing rheumatic diseases or infections, smoking, medication intake (except birth control and thyroxine) and intake of nutritional supplements (protein- or vitamin-preparations). Baseline characteristics of the study population are shown in **Table 5**. In the NAM-Group one drop-out occurred in week two due to an accident not associated to the intervention and one subject of the NA-group had to be excluded in week five due to elevated aspartate transaminase (AST) levels.

Within the proof-of-concept and safety study subjects ingested the test items in the morning from Monday to Thursday of every week, followed by a three day washout period from Friday to Sunday (**Figure 5**). Subjects ingested the capsules while they were fasted (with water only) and remained fasted until 30 min after administration or until the second blood sampling (on Mondays) in order to (I) not influence the stomach-pH through pH-increasing or pH-decreasing foods, because niacin is released pH-dependent and (II) not influence niacin serum levels due to niacin rich foods. During week one reference dose of free NA or free NAM, respectively, was applied. From week two until week six NA or NAM microcapsules were administered with weekly dose increase of 30 mg NA or 300 mg NAM, beginning with an initial dose analogous to free reference dose. Same reference doses were used as in bioavailability study. Due to low systemic exposure in week two till four, doses were increased by 105 mg NA or 750 mg NAM in week five and week six, reaching final dose of 300 mg NA and 3000 mg NAM. Blood samples were taken before ingestion of the capsules (Mondays, hour 0) and after last ingestion of each dose (Thursday, hour

72) to determine systemic NA and NAM levels and safety parameters. Differential blood count, NA+, K+, creatinine, AST, ALT, γ -GT, bilirubin and c-reactive protein (CRP) were determined as safety parameters to monitor human metabolism, liver and kidney function in specific. Further, glucose, insulin, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides and lipoprotein (a) (Lp(a)) levels were monitored, since niacin can affect glucose and lipid metabolism [23]. An additional blood sample was taken at hour two, in order to identify immediate release of niacin from the capsules by determination of NA and NAM serum levels. Stool samples were collected at the first study day (baseline) and the end of each dosage week. Subjects stated their height at the beginning of the study. Body weight and blood pressure were determined at the beginning of every week. Additionally, blood pressure was measured again on Thursday. Subjects were advised not to change nutritional habits and they kept a nutritional diary during dosage with niacin capsules to monitor nutritional intake. EBISpro software was used to analyse nutritional intake.

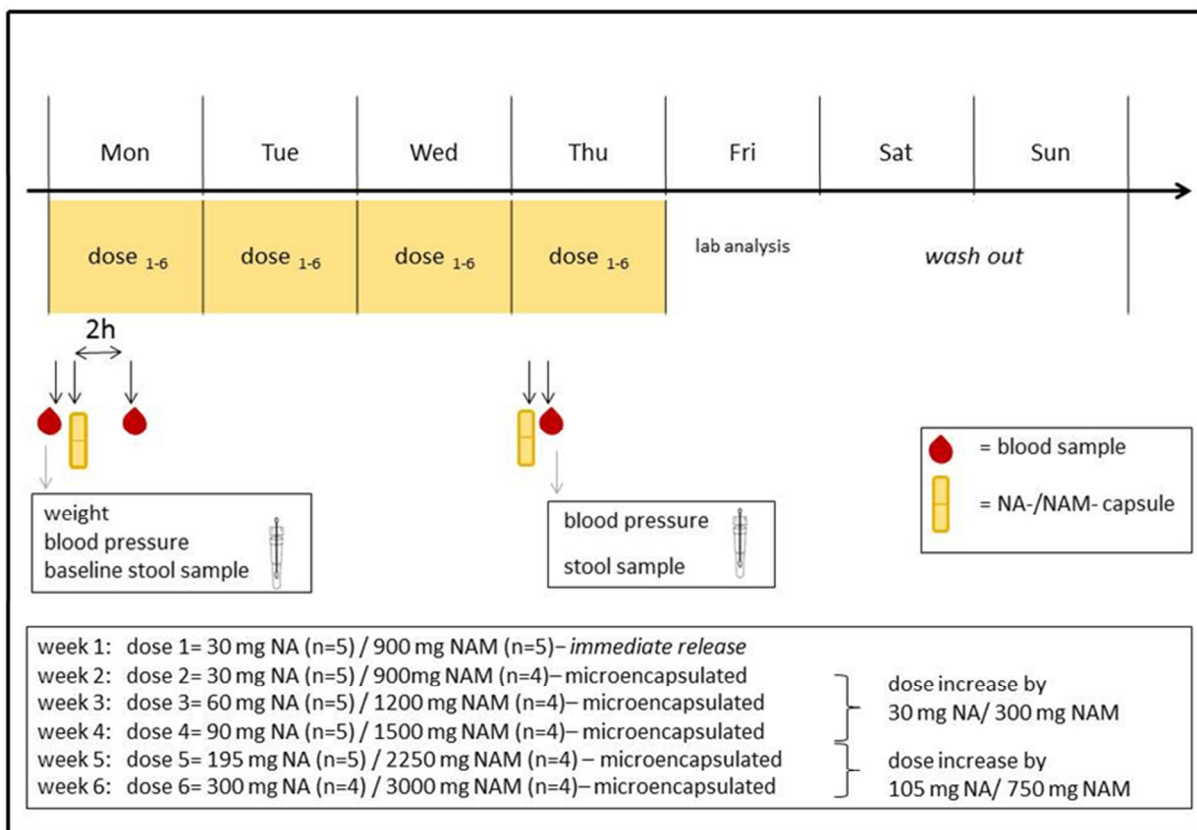


Figure 5: Study design of the niacin proof-of-concept and safety study

Abbreviations: NAM, nicotinamide; NA, nicotinic acid.

3.4 Anthropometrics

Body weight was measured with Tanita Scale (Body Composition Analyzer; Type BC- 418 MA; Tanita Corporation) and subjects either stated their height (niacin intervention) or it was measured with a stadiometer (FoCus cohort). Anthropometric values were used to calculate BMI (weight (kg)/(height (m))²).

3.5 Biochemical analyses

Serum was stored immediately at -80°C and blood samples were subjected to routine laboratory analyses at the central laboratory of the University Hospital Schleswig-Holstein in Kiel, Germany (<http://www.zentrallabor.uni-kiel.de/>). Uric acid, triglycerides, HDL, LDL and γ -GT were determined by enzymatic colorimetric assay (Hitachi Modular, Roche) and CRP via Immunoturbidimetric assay (Hitachi Modular Roche). Enzymatic colorimetric assay according to IFCC (Hitachi Modular, Roche) was used to measure AST, ALT and γ -GT. Also, creatinine was determined with an enzymatic assay and photometric measurement. Lp (a) was measured with an Immunoephelometric assay (Dade Behring). After determination of fasting insulin by electrochemiluminescence immunoassay (ECLIA; Elecsys system; Roche) and fasting glucose by glucose-hexokinase-UV-test (Hitachi Modular; Roche) the Homeostasis Model Assessment (HOMA) index was calculated as glucose(mg/dl)*insulin (μ U/ ml)/405. NA and NAM serum levels were measured by liquid chromatography and mass spectrometry (LC-MS/MS; Agilent 1100 HPLC/CTC-PAL Autosampler/Sciex API 4000 Triple Quadrupole) in an external specialized laboratory (Medizinisches Labor Bremen, www.mlhb.de). Trp serum levels were determined in the same specialized laboratory by LC-MS/MS (Agilent 1100 HPLC/CTC-PAL Autosampler/Sciex API 4000 QTRAP).

ELISA-Enzyme linked Immunosorbent Assay

Systemic metabolic parameters in the serum samples were measured by Enzyme-linked Immunosorbent Assay (ELISA) using myostatin test kit (SEB653Hu, Cloud-Clone Corp.; sensitivity: < 0.244 ng/ml), fetuin-A test kit (SEA178Hu, Cloud-Clone Corp.; sensitivity: < 0.37 ng/ml), adiponectin test kit (EH2593, Fine Test; sensitivity: < 0.938 ng/ml), FGF21 test kit (SEC918Hu, Cloud-Clone Corp.; sensitivity: < 12.1 pg/ml) and osteopontin test kit (SEA899Hu, Cloud-Clone Corp.; sensitivity: < 0.263 ng/ml), following the manufacturer's instructions. According to the manufacturers ELISA had excellent specificity for detection of the measured biomarkers and no significant cross-reactivity or interference with analogues was observed.

Photometric measurement was performed using Mithras LB940 photometer (Berthold Technologies) and MicroWin2000 software (Berthold Technologies).

3.6 Microbiome Analyses

Stool samples of FoCus cohort and niacin proof-of-concept study cohort were immediately stored at -80°C after collection from the subjects and subsequently subjected to the Institute of Clinical Molecular Biology (IKMB) in Kiel (Germany) for microbiome sequencing.

3.6.1 Microbiome Analyses within the FoCus cohort

Samples were examined as described in the following procedure, which has been already reported in co-authored publication on FoCus cohort samples by the IKMB and our working group:

DNA extraction according to Heinsen et al. (2016): “DNA was extracted using the QIAamp DNA stool mini kit automated on the QIAcube (Qiagen). Approximately 200 mg stool was transferred to 0.70 mm Garnet Bead tubes (Dianova) filled with 1.1 ml ASL lysis buffer. Bead beating was performed using the a SpeedMill PLUS (Analytik Jena) for 45 sec at 50 Hz. Samples were then heated to 95°C for 5 min with subsequent continuation of the manufacturer’s protocol” [13].

Bacterial 16S rRNA gene sequencing and quality control according to Heinsen et al. (2016): “Variable regions V1 and V2 of the 16S rRNA gene were amplified using the primer pair 27F-338R in a dual-barcoding approach according to Caporaso et al. (2012) [59]. PCR products were normalized using the SequelPrep Normalization Plate Kit (Life Technologies), pooled equimolar and sequenced on the Illumina MiSeq. Demultiplexing after sequencing was based on zero mismatches in the barcode sequences. Forward and reverse reads were merged using the FLASH software allowing an overlap of the reads between 250 und 300 bp [60]. To eliminate low-quality sequences a filtering step was applied to the data, removing sequences with a sequence quality of less than 30 in less than 95% of the nucleotides. Chimeras were removed using UCHIME [61]” [13].

Microbiome Bioinformatic Analysis according to Heinsen et al. (2016): “Taxonomical classification was carried out using the RDP classifier [62], where classifications with low confidence at genus level (<0.8) are organized in an arbitrary taxon of "unclassified_[family]". For each sample, 10.000 sequences were randomly chosen to construct a taxon-by-sample abundance table”[13].

3.6.2 Microbiome analyses within the niacin proof-of-concept study

Since the niacin intervention was performed within the frame of a joined project of the Cluster *Inflammation at Interfaces*, stool samples were subjected to the Institute of Clinical Molecular Biology (IKMB) in Kiel (Germany) and examined as described by research group of Philip Rosenstiel:

“From stool samples total genomic DNA was extracted using DNeasy Powersoil Kit (Qiagen, Germany) according to the manufacturer’s instructions. Aliquots of extracted DNA were used to amplify the V3-V4 variable region of 16S rRNA using composite primers (319F and 806R), as described by Fadrosch et al. [63]. Amplification was performed by Phusion® hot start flex 2X master mix (New England Biolabs, Frankfurt/M., Germany) in a GeneAmp PCR system 9700 (Life Technologies/Applied Biosystems, Darmstadt, Germany) using the following cycling conditions: an initial denaturation of 3 min at 98°C followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, and a final extension step at 72°C for 10 min. PCR performance was assessed by agarose gel electrophoresis for quality (expected amplicon size) and quantity (band intensity). Quantitative normalization was performed using the SequalPrep™ kit (Invitrogen, Germany) to pool equal amounts of amplicons per sample. Sequencing reads were primarily processed for quality control using the software mothur [64]. Forward and reverse reads (fastq) were assembled to contig sequences and discarded if they had more than 475 bases, any ambiguous base or more than 8 homopolymers. Sequences were aligned against the mothur-curated silva alignment database and screened to have alignment in the amplified specified V3-V4 region only. Chimeric sequences were detected with the Uchime algorithm [61] and were also removed from analysis. Sequences were classified using mothur-formatted greengenes reference taxonomy (release August 2013) and eliminated if classified as unknown, archaea, eukaryote, chloroplast or mitochondria. Sequences were clustered in phylotypes according to their phylogenetic affiliation at phylum level.” (Ateequr Rehman, IKMB).

3.7 Scanning electron microscopy of niacin microcapsules

Microcapsules from stool samples of the proof-of-concept and safety study were isolated from stool samples, washed in demineralized water and dried overnight. Afterwards microcapsules were prepared on a holder with Leit-C conductive carbon cement. Before examination in a Hitachi S-4800 scanning electron microscope (SEM) at an accelerating voltage of 3kV, microcapsules were sputter-coated with a layer of 8-10 nm gold-palladium using a Leica EM SCD 500 high-vacuum sputter coater. SEM pictures of the capsules isolated from stool samples were compared with SEM pictures of undigested microcapsules. The undigested capsules were mounted and analysed in the same way as microcapsules from stool samples.

3.8 Statistical Analyses

Statistical analyses were carried out employing SPSS version 22.0 for windows (SPSS, Chicago, IL; USA) and graphic data analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data were checked for normality by using Shapiro-Wilk-test and presented as means \pm SDs (normal distribution) or median and interquartile range (not normally distributed). Independent samples t-test and Mann-Whitney-U-test were used to determine between group differences of the study groups for continuous variables, and χ^2 -test was used for categorical variables. To compare more than two groups Kruskal-Wallis-test was used for non-parametric data for overall comparison and Mann-Whitney-U-test for pairwise comparison. Welch-ANOVA with Games-Howell-Post-Hoc-test was used for parametric data with heterogeneity of variances. According to distribution Spearman correlation analysis was performed. Bonferroni adjustment was used for multiple testing. P-values are given as nominally significant (pnom), if they are above the multiple testing significant limit. In order to evaluate the total uptake of niacin, area under the curves (AUCs) were calculated with GraphPad Prism. In case of serum levels after 12 hours not matching baseline serum levels, the NAM-serum level curves were extrapolated. Extrapolation was performed by means of trend lines in Excel version 2010 (Excel, Microsoft Cooperation, Redmond, WA, USA). According to normal distribution paired sample t-test was used to determine differences between AUCs of unformulated niacin and AUCs calculated under the application of microencapsulated niacin. Changes in systemic metabolic parameters (measured by ELISA) during the study weeks were determined by Friedman-test for overall comparison of all three time points and by Wilcoxon test for comparison of two time points. To determine significant changes in the microbial composition under niacin intervention GraphPad was used to perform over time repeated ANOVA and paired t-test. Statistical significance was set at $p < 0.05$.

Remarks

Within FoCus study cohort, insulin and HOMA values were missing for one lean subject and lipoprotein (a) values were missing for 152 subject. Values below detection limit of lipoprotein (a) ($<95\text{mg/l}$ / $<93.1\text{mg/l}$) were set at 95mg/l or 93.1mg/l ($n=114$), respectively. IL-6 values below detection limit of 1.5pg/ml were regarded as equivalent to 1.5pg/ml (91 samples). Same was applied to CRP values below 0.9mg/l (150 samples).

Within niacin safety study two subjects showed lipoprotein (a) values below detection limit of 7nmol/l , these values were set at 7nmol/l . Same was applied for one γ -GT value ($>7\text{U/l}$) within bioavailability study.

Further, three extreme nicotinamide serum level values $>100 \mu\text{g/l}$ (1276 $\mu\text{g/l}$, 206 $\mu\text{g/l}$, 183 $\mu\text{g/l}$) of the Focus cohort are not shown in the figures within results section (see figure legends) in order to use a proper scaling for the figures, but values were included into analyses.

4 Results

4.1 Characterisation of the study cohorts

FoCus study cohort and study populations of niacin interventions were characterised by several anthropometric, metabolic and inflammatory parameters and the study groups were compared according to these parameters.

4.1.1 Characterisation of the FoCus subset

Subjects of the FoCus cohort were subdivided into four groups: underweight (BMI < 20 kg/m²), lean (BMI 20-25 kg/m²), obese without T2D (BMI > 30 kg/m²) and obese with T2D (BMI > 30 kg/m²). Their baseline characteristics are shown in **Table 2**. Lean and obese groups did not significantly differ in gender distribution and age, but underweight group was significantly younger and percentage of female subjects was higher in this group. According to their classification by BMI and metabolic phenotypes, subjects of the four groups significantly differed in metabolic and inflammatory parameters, whereby obese subjects with T2D were showing the highest levels, followed by obese without T2D and lean subjects. Underweight were showing lowest metabolic and inflammatory parameters. However, underweight and lean subjects did not significantly differ according to glucose, insulin, Il-6 and CRP. Similarly there was no difference in CRP-values of obese groups.

Table 2: Characteristics of the FoCUS subset study cohort

	BMI < 20 kg/m ² n=66	BMI 20-25kg/m ² n=149	BMI > 30 kg/m ² without T2D n=148	BMI > 30 kg/m ² with T2D n=148	P _{total}	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆
age (years)	45.53 ± 15.58 ^a	52.90 ± 10.82	52.82 ± 10.86	52.93 ± 10.83	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.	n.s.
gender (% female)	86.4 %	67.1 %	66.9 %	66.2 %	<0.05	<0.01	<0.01	<0.01	n.s.	n.s.	n.s.
height (m)	1.70 ^b (1.64; 1.75)	1.72 (1.68; 1.79)	1.70 (1.64; 1.78)	1.70 (1.64; 1.80)	n.s.	-	-	-	-	-	-
weight (kg)	55.45 (50.38; 58.43)	66.80 (61.35; 74.40)	111.30 (95.73; 132.20)	123.25 (102.50; 148.08)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
BMI (kg/m ²)	19.09 (18.18; 19.73)	22.81 (21.45; 23.98)	37.08 (32.40; 45.13)	42.80 (36.75; 47.94)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
mmHg (sys)	120.00 (110.00; 130.00)	120.00 (115.00; 130.00)	130.00 (130.00; 140.00)	140.00 (130.00; 140.00)	<0.001	0.004	<0.001	<0.001	<0.001	<0.001	n.s.
mmHg (dia)	80.00 (70.00; 80.00)	80.00 (70.00; 80.00)	80.00 (80.00; 90.00)	80.00 (80.00; 90.00)	<0.001	0.005	<0.001	<0.001	<0.001	<0.001	<0.001
glucose (mg/dl)	88.00 (85.00; 95.25)	93.00 (87.00; 99.00)	100.00 (91.00; 108.00)	123.00 (104.25; 162.00)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	<0.001
insulin (mIU/l)	5.25 (3.90; 8.30)	6.50 (5.10; 9.28)	17.20 (11.00; 24.23)	25.50 (15.53; 43.88)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	<0.001
HOMA-IR index	1.14 (0.83; 1.89)	1.49 (1.08; 2.26)	4.15 (2.57; 5.71)	7.87 (4.18; 16.94)	<0.001	0.005	<0.001	<0.001	<0.001	<0.001	<0.001
triglycerides (mg/dl)	68.00 (54.75; 92.50)	87.00 (64.00; 112.50)	121.50 (91.25; 179.25)	166.00 (122.00; 237.75)	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lp(a) (mg/l)	117.00 (95.00; 361.00)	121.00 (95.00; 320.00)	148.00 (95.00; 378.50)	108.00 (95.00; 324.25)	n.s.	-	-	-	-	-	-

Continued in part 2 below

Table 2: Characteristics of the FoCus subset study cohort (part 2)

IL-6 (pg/ml)	2.15 (1.50; 3.73)	2.50 (1.50; 4.20)	4.00 (2.90; 5.35)	5.20 (3.33; 7.08)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
CRP (mg/l)	0.90 (0.90; 1.13)	0.90 (0.90; 1.90)	4.35 (1.75; 8.65)	5.85 (2.90; 10.88)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	<0.001	n.s.

^amean ± sd (all such values); ^bmedian; 25th, 75th percentiles in parentheses (all such values); normally distributed data were analysed with ANOVA and Post Hoc-Test. Overall comparison for non-parametric data was performed with Kruskal-Wallis-test and pairwise comparison with Mann-Whitney-U-test. Significance level was set at p<0.05 for overall comparison and for pairwise comparison it was set at p<0.0083 due to Bonferroni-adjustment. For categorical variables χ^2 -test was used. p_{total} = p-value for overall comparison, p_{1-6} =pairwise comparison: p_1 =BMI < 20 kg/m² vs. BMI 20-25 kg/m²; p_2 =BMI < 20 kg/m² vs. BMI > 30 kg/m² without T2D; p_3 = BMI < 20 kg/m² with T2D; p_4 = BMI 20-25 kg/m² vs. BMI > 30 kg/m² without T2D; p_5 = BMI 20-25 kg/m² vs. BMI > 30 kg/m² with T2D; p_6 =BMI > 30 kg/m² without T2D vs. BMI > 30 kg/m² with T2D. Abbreviations: BMI, body mass index; mmHg, mm of mercury column; HOMA-IR, Homeostasis Model Assessment insulin resistance index; Lp(a), lipoprotein (a); IL-6, interleukin-6; CRP, C-reactive protein; n.s, not significant.

4.1.2 Characterisation of the subjects of pilot bioavailability study

The three investigators of the research group, who ingested the NAM capsules in a self-experiment, were healthy, normal-weight volunteers of different age. Baseline Characteristics are shown in **Table 3**.

Table 3: Baseline characteristics of the pilot bioavailability study cohort

	subject 1	subject 2	subject 3
gender	male	female	female
age (years)	43	52	26
height (m)	1.78	1.70	1.68
weight (kg)	78	67	62
BMI (kg/m ²)	24.62	23.18	21.97
NA (µg/L)	<12	<12	<12
NAM (µg/L)	45.8	16.6	17.9

Abbreviations: BMI, body mass index; NA, nicotinic acid serum levels; NAM, nicotinamide serum levels.

4.1.3 Characterisation of the main bioavailability study population

To monitor function of liver and kidney, safety parameters were measured in blood samples of the bioavailability subjects in the course of the study. Baseline values of these parameters are shown in combination with age, anthropometric values and NAM serum levels in **Table 4**, demonstrating no abnormalities according to the liver- and kidney-safety parameters in the healthy subjects. Further, gender distribution of NA and NAM group did not differ and subjects of both groups showed median BMI within normal range. NAM serum levels differed between the groups, but serum levels of both groups were within normal range (8.0–52.0 µg/l; reported by MLHB, Bremen).

Table 4: Baseline characteristics of the main niacin bioavailability study cohort

	NA group n=10	NAM group n=10	all subjects n=20
age (years)	26.20 ± 5.07 ^a	27.50 ± 4.81	26.85 ± 4.86
gender	5 male, 5 female	5 male, 5 female	10 male, 10 female
height (m)	1.78 ± 0.79	1.75 ± 0.08	1.76 ± 0.08
weight (m)	75.16 ± 19.22	73.70 ± 13.66	74.43 ± 16.25
BMI (kg/m ²)	22.91 (20.40; 24.90) ^b	22.78 (21.66; 26.80)	22.78 (21.25; 25.57)
NAM (µg/l) ^c	18.53 ± 7.52*	12.67 ± 2.76*	15.60 ± 6.28
uric acid (µmol/l)	296.20 ± 63.60	284.80 ± 81.79	290.50 ± 71.55
creatinine (µmol/l)	86.70 ± 9.56	80.80 ± 13.11	83.75 ± 11.57
eGFR (ml/min/1.73)	94.20 ± 13.26	102.80 ± 12.18	98.50 ± 13.16
AST (U/l)	20.65 (19.82; 29.88)	20.15 (17.85; 26.70)	20.65 (19.03; 26.35)
ALT (U/l)	20.55 (11.80; 26.33)	18.85 (9.15; 31.55)	20.55 (11.43; 30.75)
γ-GT (U/l) ^d	16.50 (12.00; 26.75)	17.00 (9.00; 28.28)	17.00 (12.00; 26.50)

^amean ± sd (all such values); ^bmedian; 25th, 75th percentiles in parentheses (all such values); ^cbaseline NA serum levels of all subjects were below the detection limit of 12 µg/l and are therefore not included; ^d for γ-GT one value was slightly below the detection limit of 7 U/l and set tot 7.0 for the calculations. Independent samples t-tests and Mann-Whitney-U-tests were used to determine between group differences at baseline. *significant differences between NA- and NAM-group; unpaired t-test (p<0.05). Abbreviations: BMI, body mass index; NAM, nicotinamide; GFR, glomerular filtration rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, gamma-glutamyl transpeptidase

4.1.4 Characterisation of the proof-of-concept and safety study population

Study cohort of proof-of-concept and safety study consisted of more female than male subjects. Male subjects were only included in NA group, however there was no significant difference between the groups according to gender distribution. Further, NA and NAM groups did not differ in age. Both groups showed a median BMI slightly higher than cut-off for normal weight BMI. Still, subjects were not obese according to median BMI and of importance there was no significant difference between the groups. Mean/median of metabolic and inflammatory parameters was within normal range for both groups, apart from LDL, which occurred to be slightly above normal range within NA group. There was no significant difference according to metabolic and inflammatory parameters and NAM serum levels between the groups (Table 5).

Table 5: Baseline characteristics of the proof-of-concept and safety study cohort

	NA group n=5	NAM group n=5	all subjects n=10
age (years)	50.20 ± 7.50 ^a	39.40 ± 12.10	44.80 ± 11.06
gender	2 male, 3 female	5female	2 male, 8 female
height (m)	1.75 ± 0.06	1.70 ± 0.08	1.72 ± 0.73
weight (m)	85.64 ± 15.50	73.52 ± 10.23	79.58 ± 13.93
BMI (kg/m ²)	27.97 ± 4.33	25.55 ± 2.86	26.76 ± 3.68
mmHg (sys)	119.00 ± 11.40	121.00 ± 7.42	120.00 ± 9.13
mmHg (dia)	75.00 ± 11.18	72.00 ± 4.47	73.50 ± 8.18
NAM (µg/l) ^b	10.90 (9.6, 34.75) ^c	24.90 (18.65, 27.35)	19.80 (10.70, 26.42)
fasting glucose (mg/dl)	96.80 ± 7.63	91.40 ± 5.50	94.10 ± 6.89
fasting insulin (mIU/l)	8.56 ± 3.24	7.10 ± 5.77	7.83 ± 4.48
HOMA-IR index	2.06 ± 0.81	1.56 ± 1.19	1.81 ± 1.00
uric acid (µmol/l)	304.20 ± 37.38	274.60 ± 45.23	289.40 ± 42.11
LDL (mmol/l)	3.50 ± 1.08	2.88 ± 1.02	3.19 ± 1.04
HDL (mmol/l)	2.07 ± 0.52	1.75 ± 0.32	1.91 ± 0.44
Lp(a) (nmol/l) ^d	24.50 (7.35, 127.70)	17.10 (7.60, 193.95)	20.80 (7.53, 160.45)
triglycerides (mmol/l)	1.06 ± 0.17	1.04 ± 0.52	1.05 ± 0.37
CRP (mg/l)	1.52 (0.89, 7.71)	2.07 (0.99, 4.13)	1.82 (0.97, 3.35)

^amean ± sd (all such values); ^bbaseline NA serum levels of all subjects were below the detection limit of 12 µg/l and are therefore not included in this table; ^cmedian; 25th, 75th percentiles in parentheses (all such values); ^dfor Lp(a), values for two subjects were slightly below the detection limit of 7 nmol/l and set to 7.0 for the calculations. Independent samples t-tests and Mann-Whitney-U-tests were used to determine between group differences at baseline for continuous variables, and χ^2 -test was used for categorical variables. Statistical significance was set at p<0.05. No significant differences between the two intervention groups. Abbreviations: BMI, body mass index; mmHg, mm of mercury column; NAM, nicotinamide; HOMA-IR, Homeostasis Model Assessment insulin resistance index; LDL, low density lipoprotein; HDL, high density lipoprotein; Lp(a), lipoprotein (a); CRP, C-reactive protein.

4.2 Tryptophan and niacin nutritional intake and serum concentrations in relation to obesity and type 2 diabetes

To gain insights into the physiology of Trp, NA and NAM in human obesity and T2D, their nutritional intake and serum levels were examined in subjects with different metabolic phenotypes originating from the FoCus cohort. In that analysis no significant differences in the nutritional intake of Trp, niacin (including NA and NAM intake) or NE could be observed between lean, obese and the obese with T2D groups. Underweight individuals, however, were found to have a significant lower intake of Trp, niacin and NE (**Figure 6A–C**). These results are consistent with measured Trp serum levels, showing lowest levels in the underweight group (**Figure 6D**). In contrast to Trp, major differences between the groups could be observed for serum NAM levels: underweight subjects showed the highest levels, followed by lean subjects and obese subjects without T2D. Lowest NAM serum levels could be observed in the obese group with T2D (**Figure 6E**). With respect to NA, serum levels of all subjects were below the detection limit (12 µg/l), apart from two subjects: one subject of the lean group showed a NA serum level of 19.8 µg/l and the serum level of another obese subject was 20.7 µg/l.

Taken together, the analysis shows that obese subjects especially with T2D exhibit lower serum levels of NAM compared to healthy controls, despite comparable nutritional intake. These results in terms of nutritional intake and serum levels could also be reflected by correlation analysis within the whole cohort, whereby Trp, niacin and NE intake levels were weakly positive correlated with Trp serum levels (**Figure 7A–C**) but not with NAM serum levels (**Figure 7D–F**).

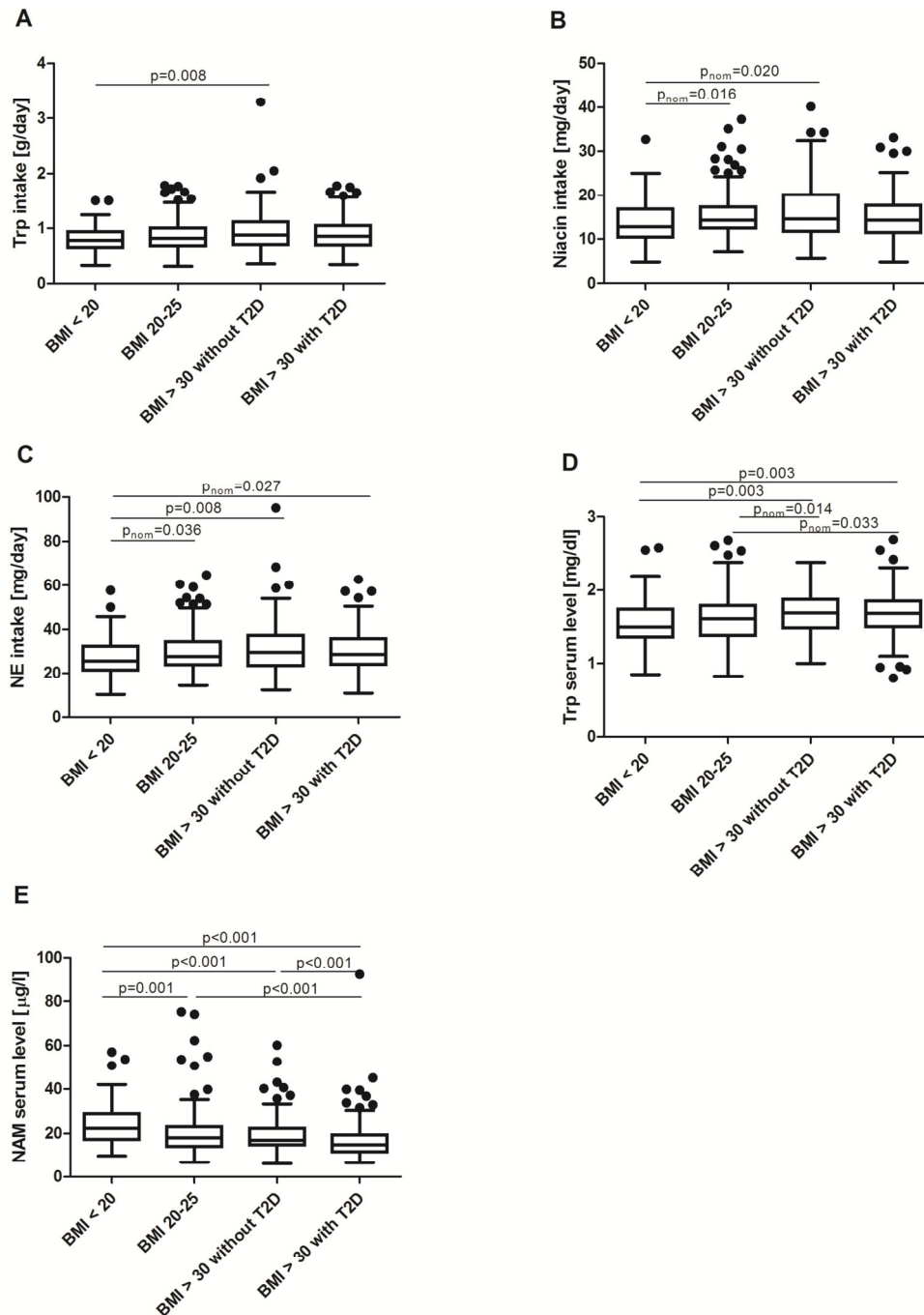


Figure 6: Tryptophan, NA and NAM nutritional intake and serum levels in humans with different metabolic phenotypes

Differences in tryptophan (A), niacin (B) and niacin equivalent (C) intake, determined by food frequency questionnaire, and measured tryptophan (D) and nicotinamide (E) serum levels (three extreme nicotinamide values ($>100 \mu\text{g/l}$) are not shown in the figure, but were included in analyses) between the groups of different metabolic phenotypes. Statistical significance was tested using Kruskal-Wallis-test for overall comparison ($p < 0.05$) and Mann-Whitney-U-test for pairwise comparison with $p < 0.0083$ due to Bonferroni-adjustment. Abbreviations: Trp, tryptophan; NE, niacin equivalents; NAM, nicotinamide; BMI, Body Mass Index; T2D, type 2 diabetes.

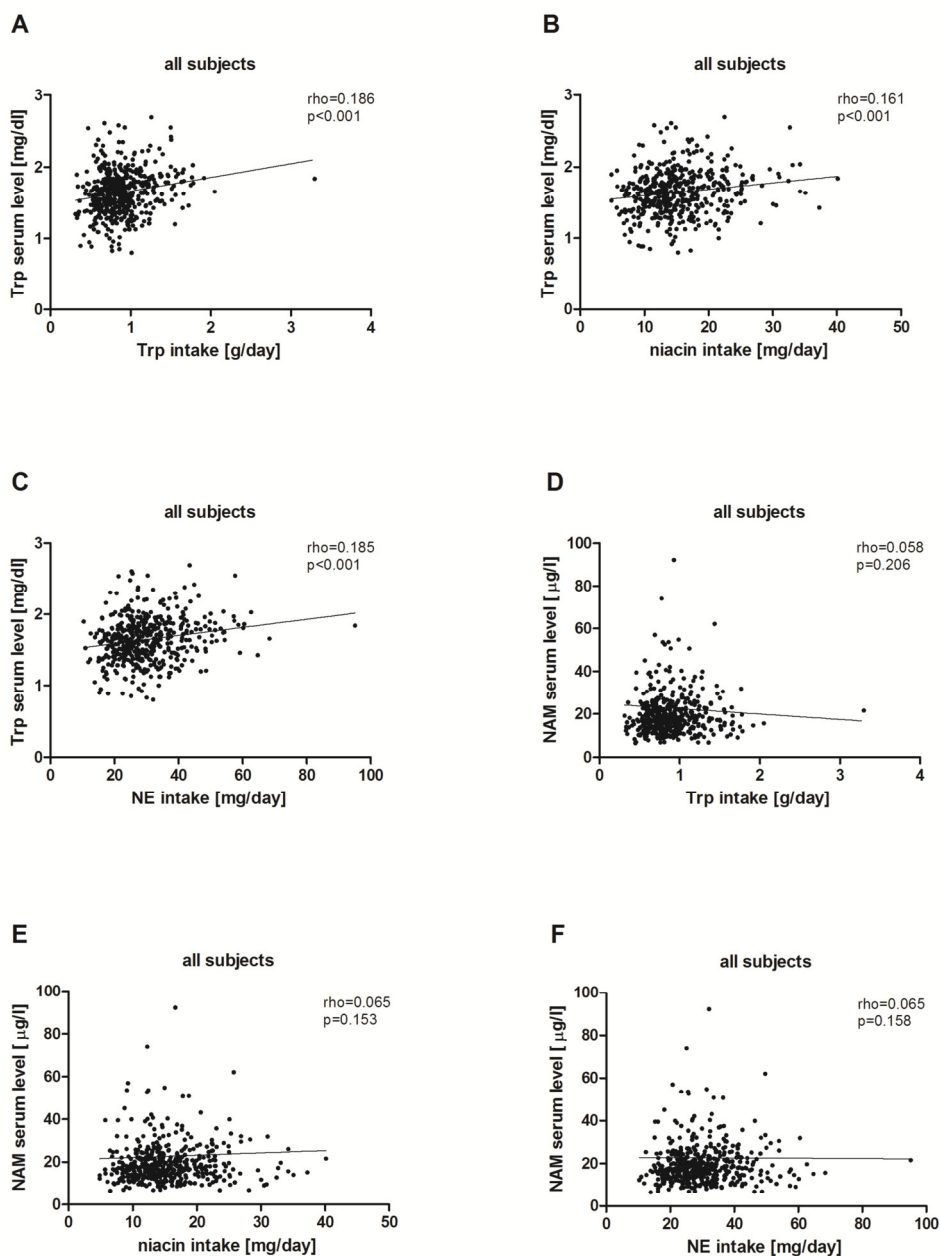


Figure 7: Association of tryptophan and niacin nutritional intake with tryptophan and nicotinamide serum levels

Scatterplots and spearman correlation analyses of tryptophan, niacin and niacin equivalent nutritional intake levels with tryptophan serum levels (A-C) and with nicotinamide serum levels (three extreme nicotinamide values ($>100 \mu\text{g/l}$) are not shown in the figures, but were included in analyses) (D-F) within the whole cohort. Significance level was set at $p<0.0167$ due to Bonferroni-adjustment for correlation analyses with Trp or NAM serum level, respectively. Abbreviations: Trp, tryptophan; NE, niacin equivalents; NAM, nicotinamide; rho, Spearman correlation coefficient.

4.3 Tryptophan and niacin in relation to the human gut microbiome

Gut microbial composition was characterized using 16S rRNA gene sequencing and bioinformatic analysis of fecal samples of the FoCUS study subjects. Seven measures of alpha diversity (Genera_Number, Chao1_Genera, Shannon_Genera, OTU_Number, Chao1_OTU, Shannon_OTU, Phylodiversity) and five measures on phylum level (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Firmicutes/Bacteroidetes* ratio) were determined and analysed. In this analysis, obese subjects showed significantly lower alpha diversity measures of genera, OTU (operational taxonomical unit) and phylodiversity compared to underweight and lean subjects (**Figure 8A–E**). Furthermore, obese subjects showed significantly lower abundance of *Bacteroidetes* on phylum level compared to lean subjects (**Figure 8F**).

Association of NAM serum levels and niacin nutritional intake with the gut microbial composition was further analysed. NAM serum levels (**Figure 9A–D**) and niacin intake (**Figure 9E–F**) were significantly positive correlated with alpha-diversity within the cohort, but correlations were weak. With further stratification by metabolic phenotypes, correlation of niacin intake and microbial composition was exclusively observed within obese subjects, showing higher correlation coefficients than analyses within the whole cohort (**Figure 10A–C**). Moreover a significant positive correlation of *Bacteroidetes* abundance with niacin and NE nutritional intake could be observed in relation to human obesity (**Figure 10D&E**).

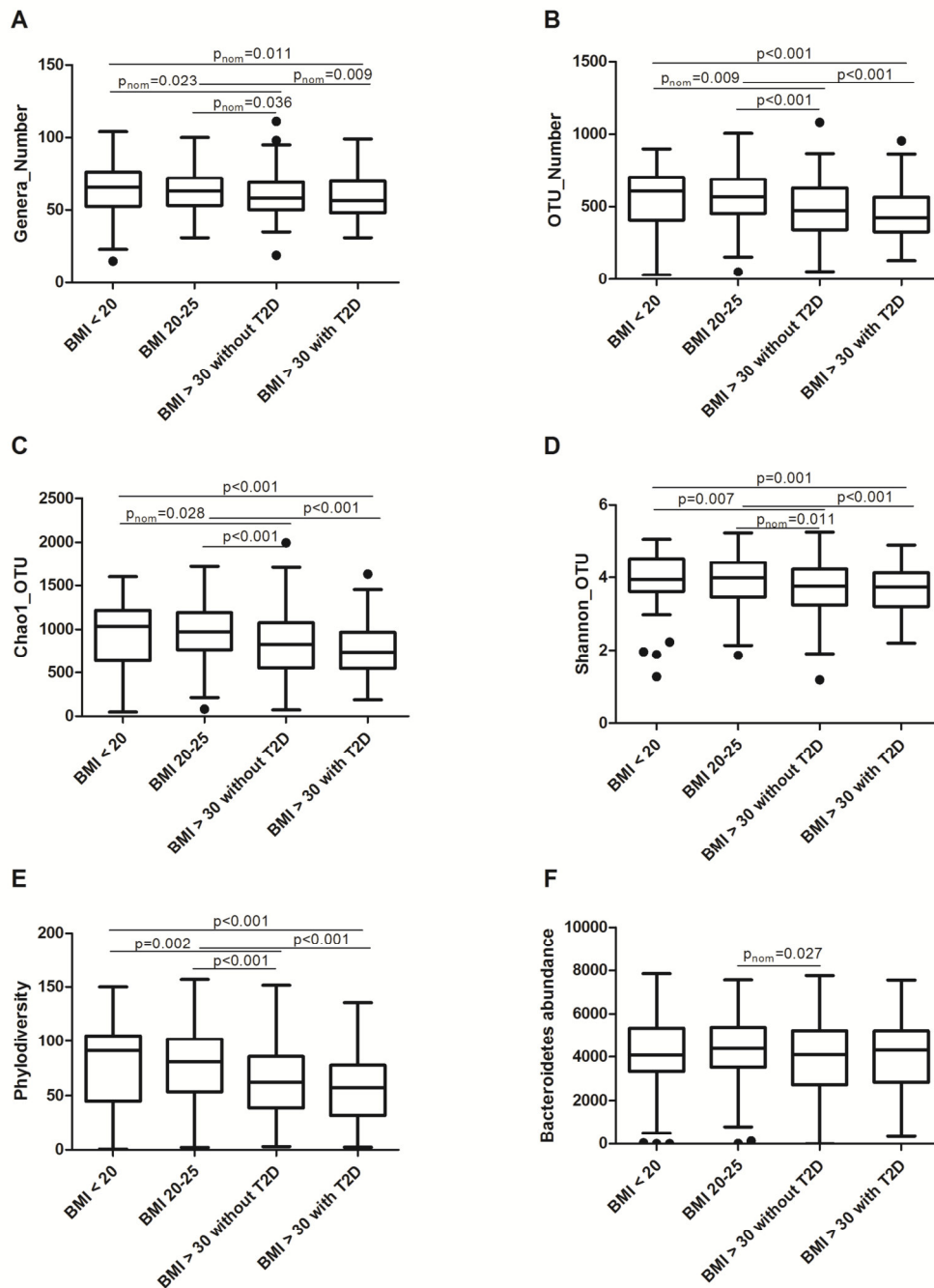


Figure 8: Microbial composition in humans with different metabolic phenotypes

Differences in the alpha-diversity measures Genera_Number (A), OTU_Number (B), Chao1_OTU (C), Shannon_OTU (D) and Phylodiversity (E) and in the *Bacteroidetes* abundance (F) between humans with different metabolic phenotypes. Statistical significance was tested using Kruskal-Wallis-test for overall comparison ($p<0.05$) and Mann-Whitney-U-test for pairwise comparison with $p<0.0083$ due to Bonferroni-adjustment. Abbreviations: BMI, Body Mass Index; T2D, type 2 diabetes; OTU, operational taxonomical unit.

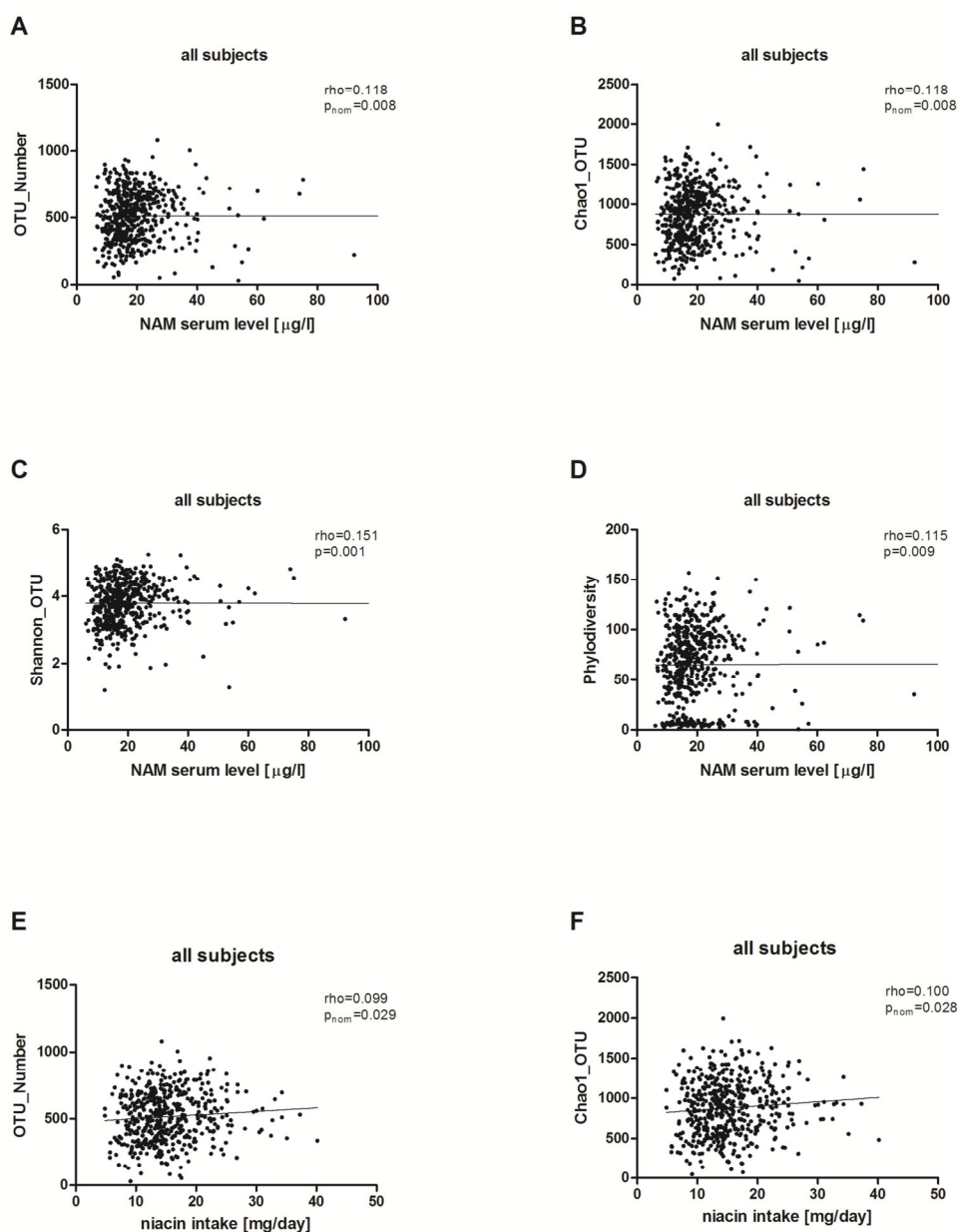


Figure 9: Association of nicotinamide serum levels and niacin intake with alpha-diversity measures within the whole cohort

Scatterplots and spearman correlation analyses of nicotinamide serum levels (three extreme nicotinamide values ($>100 \mu\text{g/l}$) are not shown in the figure, but were included in analyses) and alpha diversity measures within the whole cohort (A–D) and alpha diversity measures and niacin nutritional intake within the whole cohort (E&F). Significance level was set at $p < 0.0071$ due to Bonferroni adjustment. Abbreviations: NAM, nicotinamide; OTU, operational taxonomical unit; rho, Spearman correlation coefficient.

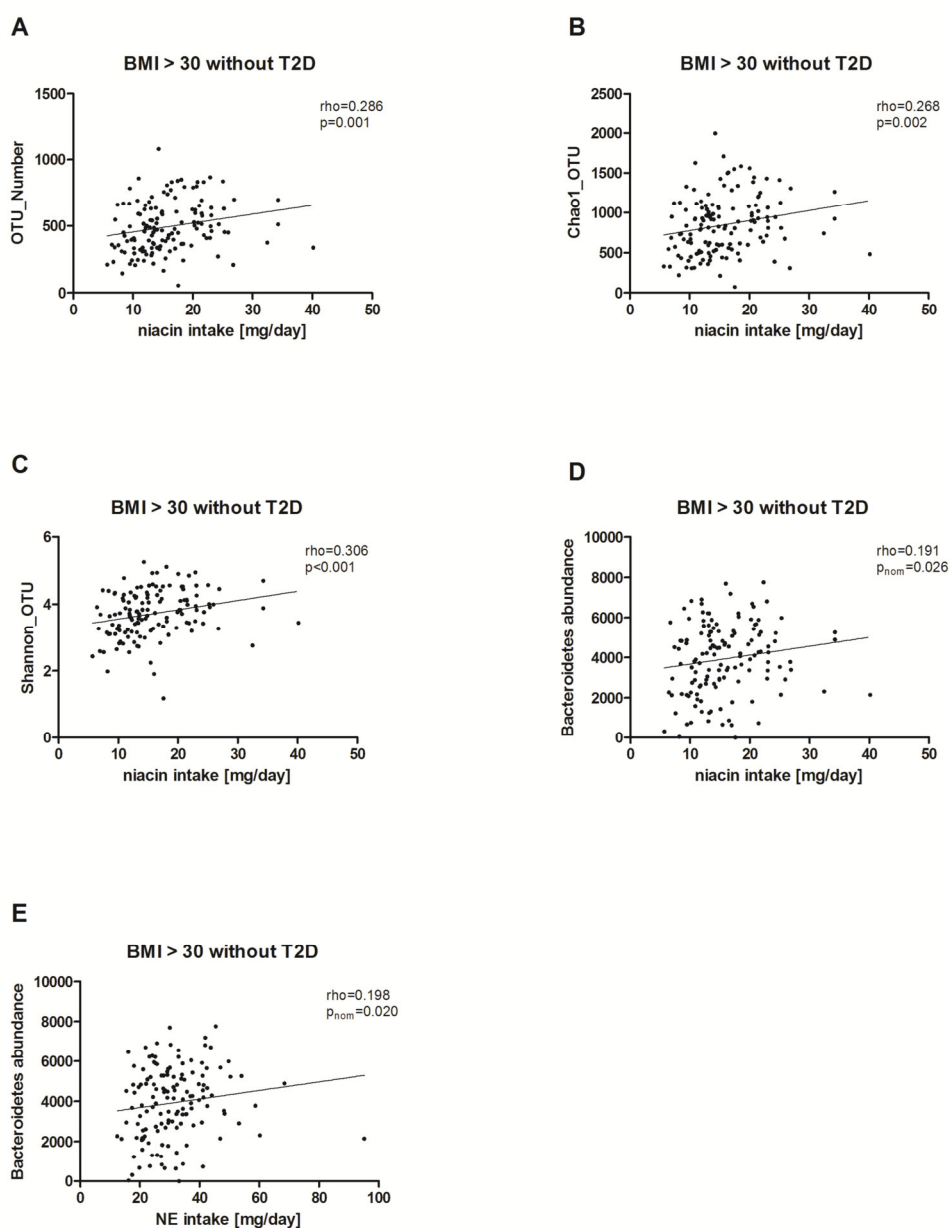


Figure 10: Association of niacin intake with alpha diversity measures and *Bacteroidetes* abundance within obese subjects

Scatterplots and spearman correlation analyses of niacin nutritional intake and alpha-diversity measures (A–C) and niacin nutritional intake and *Bacteroidetes* abundance (D&E) of obese subjects. Significance level was set at $p<0.071$ for analyses of alpha-diversity measures and $p<0.01$ for analyses of phylum level due to Bonferroni-adjustment. Abbreviations: T2D, type 2 diabetes; BMI, body mass index; OTU, operational taxonomical unit; NE, niacin equivalents.

4.4 Bioavailability of delayed-release niacin from microcapsules in healthy human subjects

Having shown significant reductions in NAM serum concentrations associated with obesity and T2D and an association of the niacin status with the composition of the gut microbiome, it was aimed to develop a safe and effective niacin supplementation strategy for obese and T2D subjects. Furthermore, in a mouse model it has been shown, that Trp and its downstream metabolites (e.g. niacin) can alter gut microbial composition [20]. Thus, the desired formulation was one that would deliver NA and NAM molecules under minimal systemic resorption into the colon where most of the gut microorganisms are located [22]. The delayed-release NA and NAM formulations were developed using food-grade microencapsulation procedure and evaluated *in vitro* (Department of Food Technology, Christian-Albrechts-University of Kiel). Subsequently, developed NA and NAM microcapsules were evaluated *in vivo* in a first-in-man project, that consisted of two human intervention studies (I) a bioavailability study including 20 healthy volunteers and (II) a proof-of-concept and safety study including 10 healthy volunteers. In those human interventions, NA- and NAM-effects observed under different dosage of microencapsulated NA (=”NA group”) or microencapsulated NAM (=”NAM group”) were compared with effects after ingestion of a reference dose of free NA (30 mg) or free NAM (900 mg).

4.4.1 Scanning electron microscopy-examination of digested niacin microcapsules to determine bioavailability of niacin

To determine the release of niacin from microcapsules, composition of the NA and NAM microcapsules was analysed after gastrointestinal passage in the human volunteer by applying SEM. Also undigested NA and NAM microcapsules were analysed by SEM and served as controls. As shown in **Figure 11A**, digested capsules collected from stool samples of the NAM group showed open end empty coating shells. These coating shells exhibited a spongy structure in cross section (**Figure 11B**) and a porous and cracky surface could be observed (**Figure 11C**). In contrast, the undigested coating of NAM microcapsules had a smooth surface structure with no cracks (**Figure 11D**) and cross-section of undigested NAM capsules proofed the tight coating around the NAM core before digestion (**Figure 11F**). Crystalline surface structure of NAM cores (**Figure 11E**) was not found in stool samples.

Also the digested NA capsules appeared with opened coating shells (**Figure 12A**). In analogy to samples of NAM group, coating shells exhibited a similar spongy structure in cross section (**Figure 12B**) and a similar porous surface (**Figure 12C**). Undigested NA microcapsules served as controls, showing a smooth surface structure instead (**Figure 12D**). In contrast to NAM capsules a core appeared within the opened coating shell (**Figure 12A**). Higher magnification of the core proofed

the same fibrous structure (**Figure 12E**) as indigestible cellulose cores of NA microcapsules (**Figure 12F**) and not as NA-cores (cellulose-cellets with NA-coating) (**Figure 12G**), which showed crystalline surface structure instead.

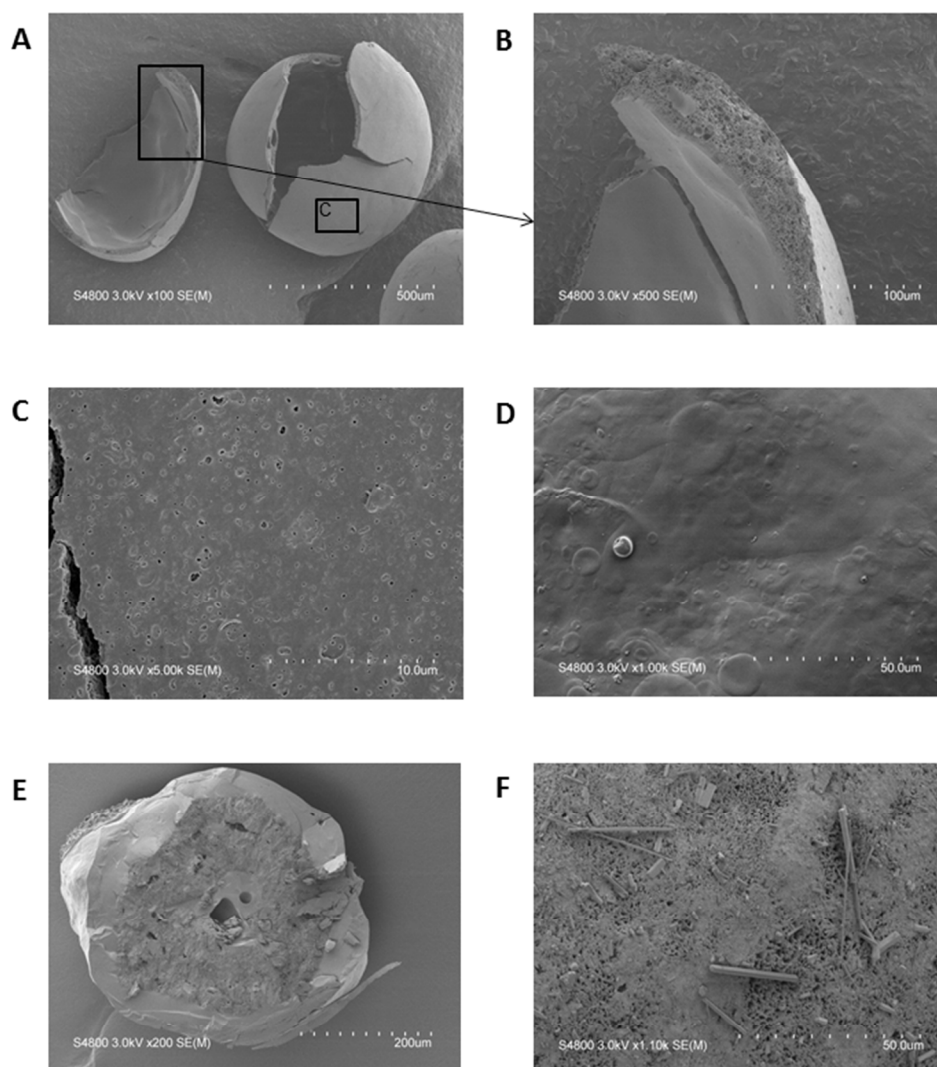


Figure 11: Appearance of undigested and digested nicotinamide microcapsules

Scanning electron microscope pictures of opened coating shells of digested nicotinamide microcapsules (A), higher magnified digested coating shells (B), higher magnified surface of digested coating shells showing porous surface (C), smooth surface of undigested coatings (D), cross section of an undigested nicotinamide microcapsule showing tight coating with nicotinamide core inside (E) and uncoated nicotinamide cores (undigested) with crystalline surface structure (F).

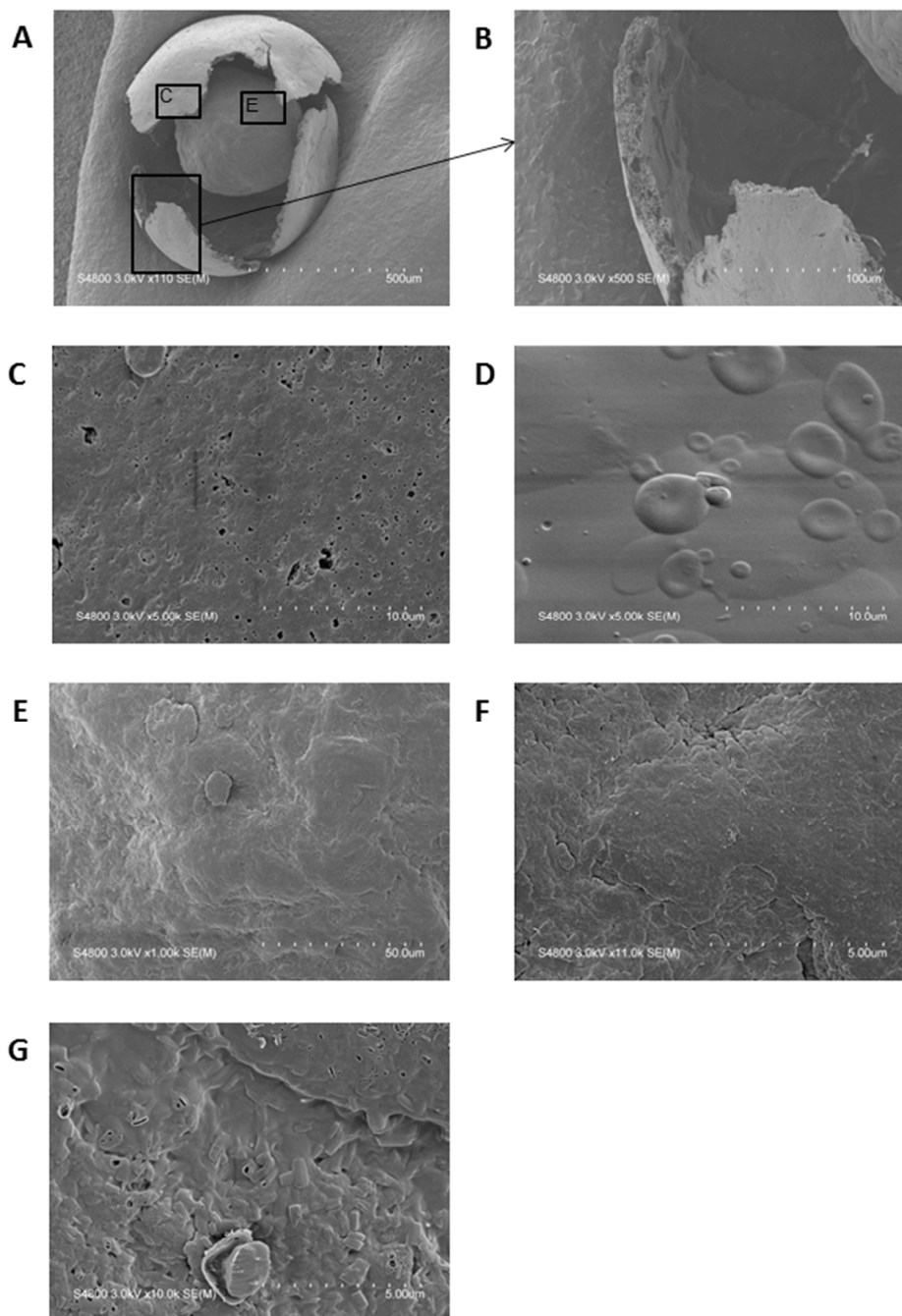


Figure 12: Appearance of undigested and digested nicotinic acid microcapsules

Scanning electron microscope pictures of digested nicotinic acid microcapsules showing opened coating shells with indigestible core inside (A), higher magnified digested coating shells (B), higher magnified surface of digested coating shells showing porous surface (C), smooth surface of undigested coatings (D), higher magnified surface of indigestible cores found in stool samples (E), uncoated indigestible cellulose cores (F) and an undigested cellulose-cellet with nicotinic acid coating (G).

Since the niacin microcapsules were evaluated *in vitro* and *in vivo* within a joined project with the Department of Food Technology (s. above), SEM analyses were performed in cooperation and may also be part of dissertation of Eva-Maria Theismann.

4.4.2 Systemic exposure profile of nicotinamide from microcapsules during pilot bioavailability study

Within the pilot bioavailability study the systemic exposure profile was examined by measuring NA and NAM serum concentrations after ingestion of microencapsulated NAM in comparison to the systemic exposure under reference dose of free NAM in three volunteers, in order to get a first impression of the release of niacin from microcapsules. Therefore blood samples were collected at baseline and for twelve hours after microcapsules ingestion in specific intervals (s. 3.3.1 Study cohort and study design of pilot bioavailability study). As shown in **Figure 13**, ingestion of free NAM led to higher NAM serum levels than ingestion of microencapsulated NAM. Further, NAM serum peak levels consistently occurred one hour after ingestion of free NAM, whereas serum peaks under microencapsulated NAM varied between three and six hours (**Figure 13**, **Table 6**). Twelve hours after ingestion baseline NAM serum levels were nearly reached again (**Figure 13**). In addition to NAM serum levels, NA serum levels were measured (**Table 7**). Apart from the time points corresponding to peak serum levels after ingestion of free NAM, NA serum levels were below the detection limit (12 µg/l).

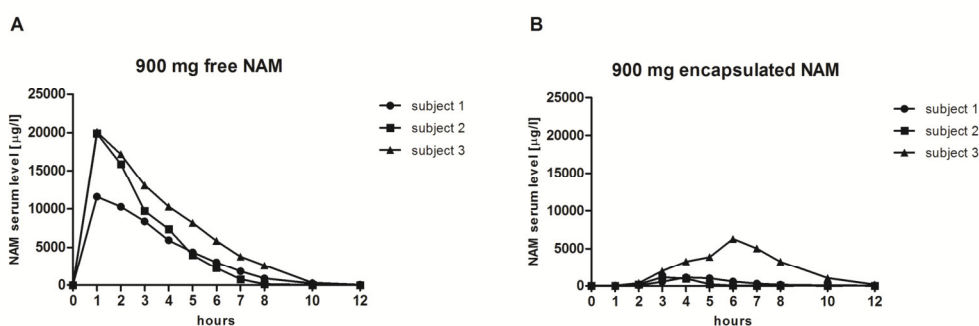


Figure 13: Nicotinamide serum levels in the course of pilot bioavailability study

Nicotinamide (NAM) serum level curves of pilot bioavailability study subjects under dosage of 900 mg free nicotinamide (A) and 900 mg microencapsulated nicotinamide (B).

Table 6: Nicotinamide serum levels with marked serum peak levels during pilot bioavailability study

NAM µg/l	subject 1		subject 2		subject 3	
	free NAM	enc. NAM	free NAM	enc. NAM	free NAM	enc. NAM
hour 0	45.8	26.8	16.6	16.3	17.9	15.7
hour 1	11,600	41.5	19,900	19.9	20,100	26.4
hour 2	10,285	63.9	15,900	203.5	17,200	386
hour 3	8,375	551	9,740	1,155	13,100	1,975
hour 4	5,920	1,145	7,390	997	10,300	3,290
hour 5	4,355	1,040	3,980	224.5	8,190	3,890
hour 6	3,030	589	2,270	41.75	5,840	6,310
hour 7	1,815	297	781	25.9	3,790	5,030
hour 8	892	160	135	19.3	2,610	3,270
hour 10	233	82.3	21.9	26.9	318	1,055
hour 12	45.9	49.7	15.2	18.5	61.9	170

bold: serum peaks. Abbreviations: NAM, nicotinamide; enc., encapsulated (microencapsulated NAM)

Table 7: Nicotinic acid serum levels of pilot bioavailability study subjects

NA µg/l	subject 1		subject 2		subject 3	
	free NAM	enc. NAM	free NAM	enc. NAM	free NAM	enc. NAM
hour 0	< 12	< 12	< 12	< 12	< 12	< 12
hour 1	< 12	< 12	17.6	< 12	15.9	< 12
hour 2	13.2	< 12	12.9	< 12	12.9	< 12
hour 3	< 12	< 12	< 12	< 12	13.0	< 12
hour 4	< 12	< 12	< 12	< 12	< 12	< 12
hour 5	< 12	< 12	< 12	< 12	< 12	< 12
hour 6	< 12	< 12	< 12	< 12	< 12	< 12
hour 7	< 12	< 12	< 12	< 12	< 12	< 12
hour 8	< 12	< 12	< 12	< 12	< 12	< 12
hour 10	< 12	< 12	< 12	< 12	< 12	< 12
hour 12	< 12	< 12	< 12	< 12	< 12	< 12

bold figures represent measurements above the detection limit of 12 µg/l. Abbreviations: NA, nicotinic acid; NAM, nicotinamide; enc., encapsulated (microencapsulated NAM).

4.4.3 Systemic exposure profile of nicotinamide from microcapsules in healthy human subjects of main bioavailability study

The pilot bioavailability study already indicated the delayed release of niacin from microcapsules and NAM serum levels to reach baseline levels after approx. twelve hours. Therefore the main bioavailability study was performed mainly following study design of the pilot bioavailability study (**Figure 4**), with only few adaptations being included (e.g. modification of low niacin meal, bigger sample size). In addition to pilot bioavailability study, different doses of microencapsulated NAM were administered and systemic exposure profile was determined by measuring NA and NAM serum concentrations in specific time intervals after dosage (**Figure 4**).

Within the NAM group the serum concentration curves clearly showed delayed release of microencapsulated NAM, since the serum peak levels of free NAM (**Figure 14A**) were measured 60 min after ingestion and the serum peak levels of the same dose of microencapsulated NAM were mainly observed 4 h after ingestion (**Figure 14B, Table 8**), indicating delayed release of NAM. With increasing dose of microencapsulated NAM (1500 and 3000 mg) serum peak levels were measured at later time points (4–8 h) (**Figure 14 C&D**). Individual serum peak levels of the subjects are shown in **Table 8**. In addition, for the 1500 mg and 3000 mg doses the inter-individual variance in NAM serum concentrations increased (**Figure 14**).

Also NA serum levels were measured within NAM group. Under dosage with free NAM, most NA serum levels were below the limit of detection, apart from five subjects (**Table 9**). They showed NA serum levels slightly above the detection limit 1–3 h after ingestion. In contrast, under highest dosage of microencapsulated NAM, NA serum levels above the detection limit occurred at later time points and in more subjects. During dosage with 900 mg and 1500 mg all NA serum levels were below the detection limit of 12 µg/l.

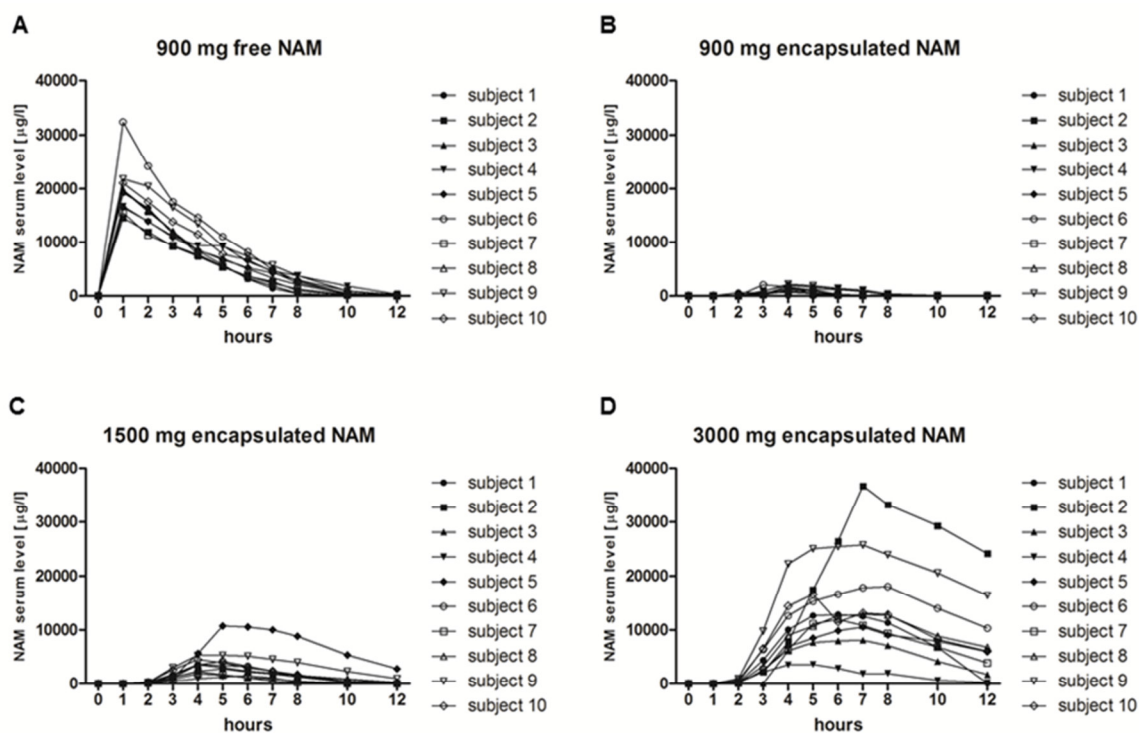


Figure 14: Nicotinamide serum levels of NAM group in the course of main bioavailability study days under different doses of nicotinamide

Nicotinamide (NAM) serum level curves of NAM group under dosage of 900 mg free nicotinamide (A), 900 mg microencapsulated nicotinamide (B), 1500 mg microencapsulated nicotinamide (C) and 3000 mg microencapsulated nicotinamide (D). Nicotinamide serum level curves were designed by measured nicotinamide serum level at baseline (fasted state) and measured serum levels for the following twelve hours.

Table 8: Nicotinamide serum levels with marked serum peak levels of NAM group within main bioavailability study

NAM µg/l	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
900 mg free NAM										
hour 0	14.1	15.3	4.9	23.0	16.3	26.8	14.1	13.0	30.6	38.4
hour 1	19,200	14,500	19,600	16,700	16,500	32,400	15,500	19,700	21,800	21,000
hour 2	16,400	11,900	15,700	13,800	13,900	24,200	11,300	16,000	20,400	17,500
hour 3	11,500	9,190	12,000	11,100	10,900	17,500	9,310	12,000	16,500	13,800
hour 4	8,330	7,350	7,660	9,300	8,470	14,600	7,610	8,410	13,400	11,400
hour 5	5,740	5,300	5,470	9,270	6,730	11,000	5,380	6,930	9,190	7,730
hour 6	3,160	3,640	3,280	6,370	5,200	8,150	3,660	5,010	7,330	6,670
hour 7	1,400	2,670	1,910	4,600	4,260	4,820	2,380	3,360	5,670	4,450
hour 8	437	994	532	3,760	2,980	2,830	1,280	2,110	3,790	2,400
hour 10	57.2	189	79.9	1,860	945	459	245	355	920	565
hour 12	25.6	75.9	48	366	250	40.7	63.0	47.7	130	117
900 mg microencapsulated NAM										
hour 0	15.3	11.3	13.3	9.8	14.0	7.4	13.4	10.9	15.9	15.4
hour 1	34.8	21.5	20.8	36.5	45.2	5.2	17.9	17.7	27.9	22.8
hour 2	615	50.1	87.6	93.6	84.0	67.6	26.9	63.0	52.6	36.4
hour 3	626	348	510	919	540	2,090	210	348	881	199
hour 4	927	830	671	2,030	1,370	1,670	1,150	1,780	2,180	72.3
hour 5	617	587	468	1,610	1,170	1,080	984	321	1,930	17.1
hour 6	343	304	230	1,370	1,220	366	202	165	1,400	42.3
hour 7	79.6	135	152	1,110	817	144	69.7	84.9	1,060	35.0
hour 8	45.0	219	161	401	360	25.5	51.5	63.4	380	24.5
hour 10	31.4	18.3	46.2	80.0	84.4	18.0	29.5	30.0	116	20.8
hour 12	28.0	18.8	35.9	46.9	53.5	18.7	18.8	32.0	73.3	45.5
1500 mg microencapsulated NAM										
hour 0	21.5	24.6	30.2	23.6	27.0	25.3	21.1	18.7	49	45.1
hour 1	30.7	33.3	31.9	35.9	35.5	34.8	14.1	30.2	38.2	37.4
hour 2	149	180	146	116	209	165	73.9	311	296	326
hour 3	1,220	1,280	1,240	585	1,190	1,610	882	1,780	3,010	2,640
hour 4	3,600	2,350	2,270	936	5,640	3,540	1,880	3,610	5,310	4,500
hour 5	3,000	2,820	1,710	1,310	10,700	4,220	1,650	3,420	5,300	3,920
hour 6	2,390	2,230	1,170	1,540	10,500	3,290	1,190	3,230	5,140	3,010
hour 7	–	1,880	951	1,140	9,980	2,360	513	2,420	4,560	2,390
hour 8	–	1,320	543	378	8,810	1,490	278	1,630	4,000	1,710
hour 10	–	290	96.0	214	5,360	228	48.5	929	2,330	443
hour 12	38.5	40.3	39.0	87.5	2,820	79.9	26.1	269	975	131
3000 mg microencapsulated NAM										
hour 0	18.8	19.7	21.1	30.6	26.2	27.3	16.8	23.4	21.5	40.4
hour 1	20.8	51.9	26.8	48.0	38.0	36.8	13.9	22.1	42.3	37.1
hour 2	331	240	147	429	211	738	125	239	974	385
hour 3	4,400	2,260	57.4	2,220	2,300	6,370	2,590	3284	9,760	6,550
hour 4	10,000	7,700	6,160	3,560	6,910	12,600	6,330	9,000	22,200	14,400
hour 5	12,600	17,400	7,660	3,630	8,450	15,300	11,100	10,680	25,000	16,600
hour 6	12,800	26,400	7,990	2,880	9,840	16,600	11,900	12,600	25,400	11,440
hour 7	12,500	36,700	8,070	1,870	10,500	17,800	10,800	12,800	25,700	13,100
hour 8	11,300	33,200	7,130	1,960	9,040	18,000	9,360	12,700	23,900	12,800
hour 10	6,780	29,300	4,180	673	7,920	14,000	6,830	8,840	20,500	8,240
hour 12	101	24,100	1,740	251	5,980	10,300	3,870	6,770	16,400	6,060

bold: serum peaks. Abbreviations: S 1–S 10: subject 1–subject 10; NAM, nicotinamide; –blood sampling was not possible.

Table 9: Nicotinic acid serum levels of NAM group within main bioavailability study

NA µg/l	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
900 mg free NAM										
hour 0	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 1	<12	<12	<12	<12	14.4	20.3	<12	<12	17.5	15.2
hour 2	<12	<12	<12	13.5	<12	13.5	<12	<12	13.8	<12
hour 3	<12	<12	<12	<12	<12	15.0	<12	<12	12.1	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 5	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 6	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 7	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 8	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 10	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
900 mg microencapsulated NAM										
hour 0	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 5	<12	<12	<12	<12	<12	<12	<12	<12	<12	-
hour 6	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 7	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 8	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 10	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
1500 mg microencapsulated NAM										
hour 0	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 5	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 6	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 7	-	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 8	-	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 10	-	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
3000 mg microencapsulated NAM										
hour 0	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 4	<12	<12	<12	<12	12.4	13.1	<12	<12	22.5	18.6
hour 5	19.0	16.7	12.9	<12	15.9	12.2	12.6	<12	26.9	18.0
hour 6	<12	22.9	<12	<12	15.8	21.6	<12	14.2	27.3	18.9
hour 7	13.0	33.3	14.7	<12	16.7	16.9	<12	<12	27.0	23.6
hour 8	13.7	28.9	12.2	<12	14.7	18.8	13.2	<12	23.1	17.9
hour 10	<12	20.3	<12	<12	<12	15.4	<12	<12	21.0	17.6
hour 12	12.3	22.1	<12	<12	<12	<12	<12	<12	17.5	14.6

bold figures represent measurements above the detection limit of 12 µg/l. Abbreviations: S 1 –S 10: subject 1–subject 10; NA; nicotinic acid; NAM, nicotinamide; –blood sampling was not possible

As shown above (**Figure 14**), systemic NAM levels are lower under 900 mg and 1500 mg microencapsulated NAM than under dosage of free NAM. Most subjects also showed lower NAM serum levels under 3000 mg dose. Still, total systemic niacin resorption under 3000 mg could be higher than exposure under reference dose. Furthermore NAM serum levels after twelve hours were above baseline serum levels in most cases under 3000 mg dose. Thus, areas under the curves (AUCs) of extrapolated NAM serum level curves were calculated in order to determine total systemic resorption of niacin (**Table 10**). Though NA serum levels under dosage of 3000 mg microencapsulated NAM were low (shown above, **Table 9**), they were included in calculation of total systemic niacin resorption. However, the AUCs under 3000 mg did not significantly differ from AUCs observed under reference dose of 900 mg free NAM. In fact for the 900 mg and 1500 mg microcapsules significantly lower AUCs could be demonstrated.

Table 10: Areas under the curves of NAM group of main bioavailability study

Dose NAM	900 mg free	900 mg enc.	1500 mg enc.	3000 mg enc. (non-extrapol.) ^a	3000 mg enc. (extrapol.) ^b
subject 1	66533	3408	16941	83272	83338
subject 2	56503	2665	13386	223262	371351
subject 3	66628	2516	8580	50917	52070
subject 4	80878	7982	6757	19189	19460
subject 5	72578	6016	65023	73642	99657
subject 6	117887	5519	18003	134758	167627
subject 7	57620	2822	6754	74437	79227
subject 8	75339	2972	19383	92137	107436
subject 9	101960	8415	35314	202337	259718
subject 10	87416	556.5	20428	104272	117598
MW	78334.20	4287.15	21011.90	105822.30	135748.20
± SD	19538.11	2571.91	17616.94	64276.47	105826.64

^anon-extrapol., non extrapolated: area under the curves calculated with raw data; ^bextrapol., extrapolated: if serum levels after twelve hours were above baseline, curves were extrapolated (as described in Statistical analyses) and total niacin uptake (NA serum levels + NAM serum levels) was considered for calculation of area under the curves. According to normal distribution, paired t-test was used to compare areas under the curves of encapsulated NAM with area under the curves under dosage of free NAM. No statistically significant differences could be observed. Abbreviations: NAM, nicotinamide; enc., microencapsulated.

4.4.4 Systemic exposure profile of nicotinic acid from microcapsules in healthy human subjects of main bioavailability study

In analogy to NAM group systemic exposure was also determined under dosage with free and microencapsulated NA.

In the NA group, all NA serum levels were below the limit of detection of 12 µg/l (**Table 11**). In addition to NA serum levels in the NA group also NAM concentrations were measured. In that analysis only minimal fluctuations of NAM serum levels on a high background level could be observed, but no consistent and dose-dependent increase, despite 10-fold dose increase (**Figure 15A–D**).

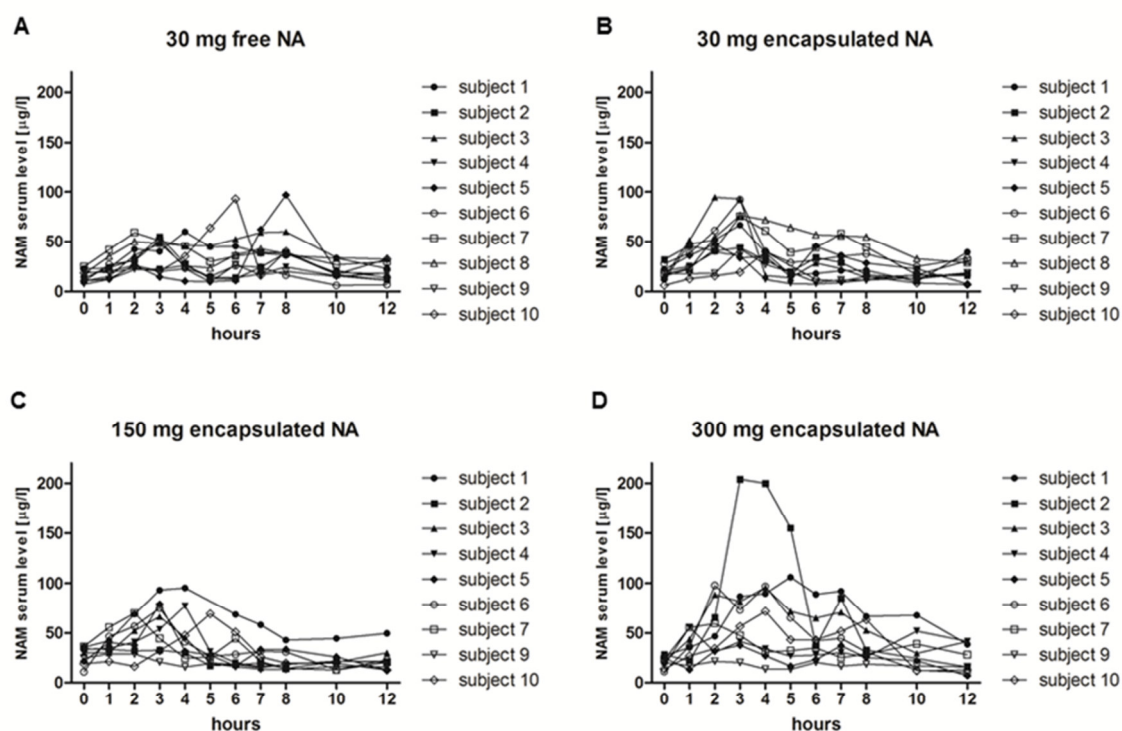


Figure 15: Nicotinamide serum levels of NA group in the course of main bioavailability study days under different doses of nicotinic acid

Nicotinamide (NAM) serum level curves of NA group under dosage of 30 mg free nicotinic acid (NA) (A), 30 mg microencapsulated nicotinic acid (B), 150 mg microencapsulated nicotinic acid (C) and 300 mg microencapsulated nicotinic acid (D). Nicotinamide serum level curves were designed by measured nicotinamide serum level at baseline (fasted state) and by measured serum levels for the following twelve hours.

Table 11: Nicotinic acid serum levels within NA group of main bioavailability study

NA µg/l	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
30 mg free NA										
hour 0	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 5	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 6	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 7	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 8	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 10	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
30 mg microencapsulated NA										
hour 0	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 5	<12	<12	<12	<12	<12	<12	<12	<12	<12	-
hour 6	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 7	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 8	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 10	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
150 mg microencapsulated NA										
hour 0	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 5	-	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 6	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 7	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 8	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 10	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
300 mg microencapsulated NA										
hour 0	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 1	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 2	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 3	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 4	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 5	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 6	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 7	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 8	<12	<12	<12	<12	<12	<12	<12	-	<12	<12
hour 10	<12	-	<12	<12	<12	<12	<12	-	<12	<12
hour 12	<12	<12	<12	<12	<12	<12	<12	-	<12	<12

Abbreviations: S 1–S 10: subject 1–subject 10; NA, nicotinic acid; –blood sampling was not possible; -drop-out of subject 8

4.4.5 Safety parameters under dosage with delayed-release niacin microcapsules during main bioavailability study

For safety reasons, routine safety parameters were assessed during main bioavailability study and are summarized in **Table 12** (NAM group) and **Table 13** (NA group). No safety signals were observed.

Table 12: Safety parameters of NAM group during main bioavailability study

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
uric acid (µmol/L)										
day 1	181	365	225	339	348	241	398	243	338	170
day 2	175	427	244	334	355	218	387	207	361	168
day 3	137	453	239	344	434	228	366	244	373	146
day 4	137	392	275	343	445	198	404	219	388	158
creatinine (µmol/L)										
day 1	73	82	83	91	98	73	101	80	61	66
day 2	67	77	83	87	99	71	97	79	61	67
day 3	67	77	83	88	104	75	102	83	66	65
day 4	66	82	89	91	116	68	100	85	66	64
estimated glomerular filtration rate (eGFR) (mL/min/1.73)										
day 1	97	119	112	101	90	95	92	90	123	109
day 2	107	125	112	100	89	98	–	–	123	107
day 3	107	125	112	105	84	92	91	86	113	112
day 4	109	118	103	101	73	103	93	83	113	114
aspartate aminotransferase (AST) (U/L)										
day 1	24	19.1	47.9	25.7	29.7	13.3	19.0	15.6	18.6	21.2
day 2	25.9	27.8	32.9	24.3	24.2	10.6	19.5	14.6	22.1	21.1
day 3	19.7	20.4	35.0	25.0	31.0	17.7	23.8	18.2	21.0	26.1
day 4	24.3	38.2	37.6	26.2	39.9	17.5	24.2	16.9	18.9	21.8
alanine aminotransferase (ALT) (U/L)										
day 1	10.6	29.7	39.5	31.1	32.9	7.9	24.3	8.4	9.4	13.4
day 2	12.9	22.6	35.1	29.4	29.9	6.3	23.3	8.4	13.3	12.6
day 3	15.3	20.4	31.8	28.0	37.5	10.8	23.9	13.4	11.9	16.7
day 4	11.0	34.4	38.5	29.8	41.2	13.6	24.6	13.2	11.7	14.5
gamma-glutamyl transpeptidase (γ-GT) (U/L)										
day 1	12	19	27	18	32	9	32	<7	9	16
day 2	11	18	24	16	30	10	31	<7	11	15
day 3	10	19	25	13	28	8	29	10	10	18
day 4	9	20	25	15	32	<7	32	<7	10	16

S1–S10: subject 1–10; –drop-out or missing value

Table 13: Safety parameters of NA group during main bioavailability study

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
uric acid (µmol/L)										
day 1	389	411	298	266	317	194	257	277	268	285
day 2	396	370	264	249	283	248	220	254	270	269
day 3	367	391	266	327	327	253	187	-	261	257
day 4	332	351	243	268	328	194	160	-	244	238
creatinine (µmol/L)										
day 1	90	101	85	82	100	87	70	92	82	78
day 2	98	99	82	87	103	92	76	88	81	83
day 3	97	107	88	84	98	93	73	-	92	86
day 4	98	105	79	78	102	87	68	-	84	68
estimated glomerular filtration rate (eGFR) (mL/min/1.73)										
day 1	95	89	116	113	93	79	103	76	85	93
day 2	86	92	121	105	90	74	94	80	86	87
day 3	87	83	111	109	70	73	98	-	74	83
day 4	86	85	126	118	91	79	107	-	82	110
aspartate aminotransferase (AST) (U/L)										
day 1	40.0	25.9	18.4	26.5	43.0	20.6	20.7	19.3	20.2	20.0
day 2	30.9	14.2	21.2	22.7	32.4	24.5	19.3	45.4	16.0	20.9
day 3	33.8	17.9	23.1	34.1	32.1	21.1	18.1	-	15.3	18.6
day 4	38.5	17.7	25.1	24.1	346	20.5	19.2	-	16.3	21.7
alanine aminotransferase (ALT) (U/L)										
day 1	81.4	33.9	11.4	16.5	23.8	11.9	22.4	11.5	20.3	20.8
day 2	63.9	19.0	14.3	14.4	21.7	15.1	17.8	22.0	14.1	25.3
day 3	63.0	21.8	16.8	17.5	18.8	14.1	21.2	-	15.7	21.2
day 4	62.7	18.7	16.0	14.4	17.7	14.5	19.6	-	11.3	18.6
gamma-glutamyl transpeptidase (γ-GT) (U/L)										
day 1	33	32	14	12	12	25	10	12	19	19
day 2	26	27	13	11	13	27	10	14	16	20
day 3	28	25	15	13	12	28	14	-	15	20
day 4	25	25	13	10	12	27	12	-	12	18

S1–S10: subject 1–10; -drop-out of subject 8

4.5 Safety profile under dosage with delayed-release niacin microcapsules during proof-of-concept and safety study

The safety profile was examined during a proof-of-concept and safety study for a total of six weeks (Figure 5). Healthy human subjects were provided with NA or NAM microcapsules with weekly dose increase. In that setting, four days of treatment (Monday to Thursdays) were followed by a three-days wash-out period (Friday to Sunday). In the first week, the reference dose of free NA (30 mg) or free NAM (900 mg) was administered. Blood samples were taken at the beginning of each week and after 2 and 72 h to determine NA and NAM serum levels and routine laboratory safety parameters.

Safety parameters were measured in baseline blood samples and at the end of each dosage week. One subject of the NA group experienced a significant elevation of aspartate aminotransferase (AST)-levels at week five of the intervention and was therefore withdrawn from the study. In the subsequent observation period, AST-levels returned to normal range within four days. Apart from

that single subject, no safety signals occurred in laboratory parameters. Significantly, no flush phenomenon or other adverse events were observed or reported by the subjects. All safety-relevant parameters are summarized in **Table 14**, showing baseline safety parameters, safety parameters after application of free NA/NAM and after application of highest dose of microencapsulated NA/NAM (end of week six).

Table 14: Safety parameters of the proof-of-concept and safety study population

<i>Part 1</i>	NA group					NAM group				
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
age (y)										
w1h0	48	56	40	48	59	29	45	51	48	24
height (m)										
w1h0	1.76	1.80	1.68	1.70	1.80	1.78	1.72	1.58	1.76	1.64
weight (kg)										
w1h0	91.2	109.2	83.0	69.4	75.4	83.7	83.6	61.6	65.1	73.6
w6h0	91.3	109.0*	80.2	70.5	73.3	–	84.7	61.4	64.5	73.5
body mass index (BMI) (kg/m²)										
w1h0	29.40	33.70	29.41	24.01	23.27	26.42	28.26	24.68	21.02	27.36
w6h0	29.47	33.64*	28.42	24.39	22.62	–	28.63	24.60	20.82	27.33
blood pressure (mm Hg; systolic /diastolic)										
w1h0	125/75	130/90	120/70	100/60	120/80	120/70	110/70	120/80	125/70	130/70
w1h72	120/80	130/85	115/70	105/70	130/80	120/80	105/75	120/80	120/80	120/75
w6h72	110/70	130/80*	120/80	100/70	120/80	–	110/75	–	110/75	105/70
glucose (mg/dL)										
w1h0	91	97	101	88	107	86	94	95	97	85
w1h72	77	92	89	85	94	80	92	84	83	87
w6h72	85	99 (101)*	96	90	97	–	94	90	92	82
insulin (mIU/L)										
w1h0	10.2	8.8	12.8	4.4	6.6	17.2	3.8	5.6	3.0	5.9
w1h72	9.6	15.9	8.7	3.3	4.2	11.0	5.4	4.8	1.7	8.5
w6h72	13.4	12.2 (20.1)*	7.8	7.2	4.3	–	5.9	5.7	5.0	6.2
homeostasis model assessment insulin resistance (HOMA-IR) index										
w1h0	2.29	2.11	3.19	0.96	1.74	3.65	0.88	1.31	0.72	1.24
w1h72	1.83	3.61	1.91	0.69	0.97	2.17	1.23	1.00	0.35	1.83
w6h72	2.81	2.98 (5.01)*	1.85	1.60	1.03	–	1.37	1.27	1.14	1.26
uric acid (µmol/L)										
w1h0	261	347	325	319	269	260	313	262	325	213
w1h72	320	323	294	265	258	260	336	312	325	235
w6h72	300	351 (376)*	285	262	295	–	312	230	350	251
low density lipoprotein (LDL) (mmol/L)										
w1h0	1.68	3.54	4.49	4.05	3.75	2.05	2.38	2.79	4.63	2.53
w1h72	1.81	3.79	4.47	3.46	4.36	2.23	2.40	2.32	4.36	2.54
w6h72	1.74	4.06 (4.30)*	4.05	4.14	4.35	–	2.79	2.45	3.56	3.22

Continued in part 2 below.

Table 14: Safety parameters of the proof-of-concept and safety study population

Part 2	NA group					NAM group				
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
high density lipoprotein HDL (mmol/L)										
w1h0	2.21	1.20	2.31	2.56	2.08	2.03	1.94	1.94	1.52	1.30
w1h72	2.32	1.25	2.48	2.15	2.23	2.50	1.42	1.91	1.32	1.32
w6h72	2.08	1.35 (1.27)*	2.51	2.09	2.52	–	2.10	2.34	1.83	1.54
lipoprotein (a) [Lp(a)] (nmol/L)										
w1h0	<7	96.8	158.6	24.5	7.7	8.2	17.1	221.9	166.0	<7
w1h72	<7	98.8	168.9	21.3	16.9	7.4	11.0	189.5	173.9	<7
w6h72	<7	103.7 (109.8)*	171.7	23.3	12.2	–	15.7	225.8	200.5	<7
triglycerides (mmol/L)										
w1h0	1.2	1.2	1.1	0.8	1.0	1.6	0.7	0.5	1.6	0.8
w1h72	0.9	0.7	1.4	1.0	1.0	1.0	1.2	0.4	1.3	0.7
w6h72	1.3	0.9 (1.8)*	0.9	1.0	0.8	–	0.9	0.6	0.9	0.8
c-reactive protein (CRP) (mg/L)										
w1h0	1.05	2.41	13.00	1.52	0.72	6.17	2.07	0.41	1.57	2.08
w1h72	0.96	2.48	11.80	1.05	0.63	3.55	2.26	0.35	0.93	2.87
w6h72	1.30	3.34 (3.01)*	8.93	0.95	0.72	–	2.76	0.43	1.78	3.45
aspartate aminotransferase (AST) (U/L)										
w1h0	15.9	24.8	14.3	30.2	24.8	21.6	16.6	22.0	21.9	23.8
w1h72	15.0	22.7	16.5	25.2	28.0	19.8	21.8	22.4	22.4	21.1
w6h72	15.1	116.0 (32.4)*	16.7	30.3	22.6	–	23.9	22.0	25.0	21.9
alanine aminotransferase (ALT) (U/L)										
w1h0	16.4	14.3	19.2	30.1	19.1	27.5	9.7	10.8	21.5	20.3
w1h72	19.6	17.7	30.6	23.4	24.3	25.6	15.7	0.9	18.5	21.6
w6h72	15.9	48.8 (31.1)*	18.7	24.8	17.9	–	16.3	10.1	20.0	20.4
gamma-glutamyl transpeptidase (γ-GT) (U/L)										
w1h0	20	24	36	23	18	29	13	28	28	9
w1h72	19	26	32	22	21	29	16	24	27	10
w6h72	16	24 (29)*	27	20	16	–	17	28	35	10
bilirubin (μmol/L)										
w1h0	8.5	9.1	<2.0	7.6	6.3	5.2	6.7	4.7	5.3	6.1
w1h72	11.1	9.7	3.7	–	8.0	5.9	4.3	6.9	6.2	8.0
w6h72	10.3	8.2 (8.0)*	4.5	8.3	6.2	–	10.8	9.4	6.0	9.3
creatinine (μmol/L)										
w1h0	77	98	80	86	84	57	74	63	71	61
w1h72	87	99	76	73	–	61	91	81	62	61
w6h72	81	98 (94)*	77	80	91	–	80	62	73	67

S1–S10: subject 1–10; w1h0, week 1 hour 0 (baseline); w1h72, week 1 hour 72 (after dosage with free NA/NAM); w6h72, week 6 hour 72 (after dosage with highest dose of microencapsulated NA/NAM)

*measurements for subject 2 at week 5 (drop-out due to AST elevation), control values were measured 4 days after the end of the intervention (in parentheses); –missing values

In addition to safety parameters, NA and NAM serum levels were measured in blood samples of proof-of-concept and safety study. Interestingly, NA serum levels above detection limit mainly occurred under dosage with free NAM (900 mg) or higher doses of microencapsulated NAM (**Table 15**), similar to results of bioavailability study. However, NA serum levels were below the detection limit in most cases. Therefore only NAM levels are shown in the figures for both groups. Despite dose escalation with microencapsulated NA, no consistent or dose-dependent increase in NAM serum levels could be observed compared to serum levels obtained under dosage with free NA (**Figure 16**). Within NAM group, serum levels under microencapsulated NAM were far below those after ingestion of 900 mg free NAM (**Figure 17**). Comparing NAM serum levels at 72 h and baseline, in neither group a consistent accumulation during the weeks could be observed (**Figure 16, Figure 17**).

Table 15: Nicotinic acid serum levels of the proof-of-concept and safety study population

NA ($\mu\text{g/L}$)		NA group					NAM group				
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
free NA/NAM 30/900 mg	w1h0	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12
	w1h2	< 12	< 12	< 12	< 12	< 12	< 12	12.7	16.1	< 12	< 12
	w1h72	< 12	< 12	< 12	< 12	< 12	< 12	12.8	21.8	20.3	< 12
enc. NA/NAM 30/900 mg	w2h0	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12
	w2h2	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12	< 12
	w2h72	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
enc. NA/NAM 60/1200 mg	w3h0	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w3h2	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w3h72	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
enc. NA/NAM 90/1500 mg	w4h0	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w4h2	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w4h72	< 12	< 12	< 12	< 12	< 12	–	21.3	< 12	< 12	< 12
enc. NA/NAM 195/2250 mg	w5h0	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w5h2	< 12	< 12	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w5h72	15.2	< 12	< 12	< 12	< 12	–	< 12	13	< 12	< 12
enc. NA/NAM 300/3000 mg	w6h0	< 12	–	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w6h2	< 12	–	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12
	w6h72	< 12	–	< 12	< 12	< 12	–	< 12	< 12	< 12	< 12

bold figures represent measurements above the detection limit of 12 $\mu\text{g/l}$. Abbreviations: S1 – S10: subject 1 – 10; w1h0 – w6h72: week 1 hour 0 – week 6 hour 72.

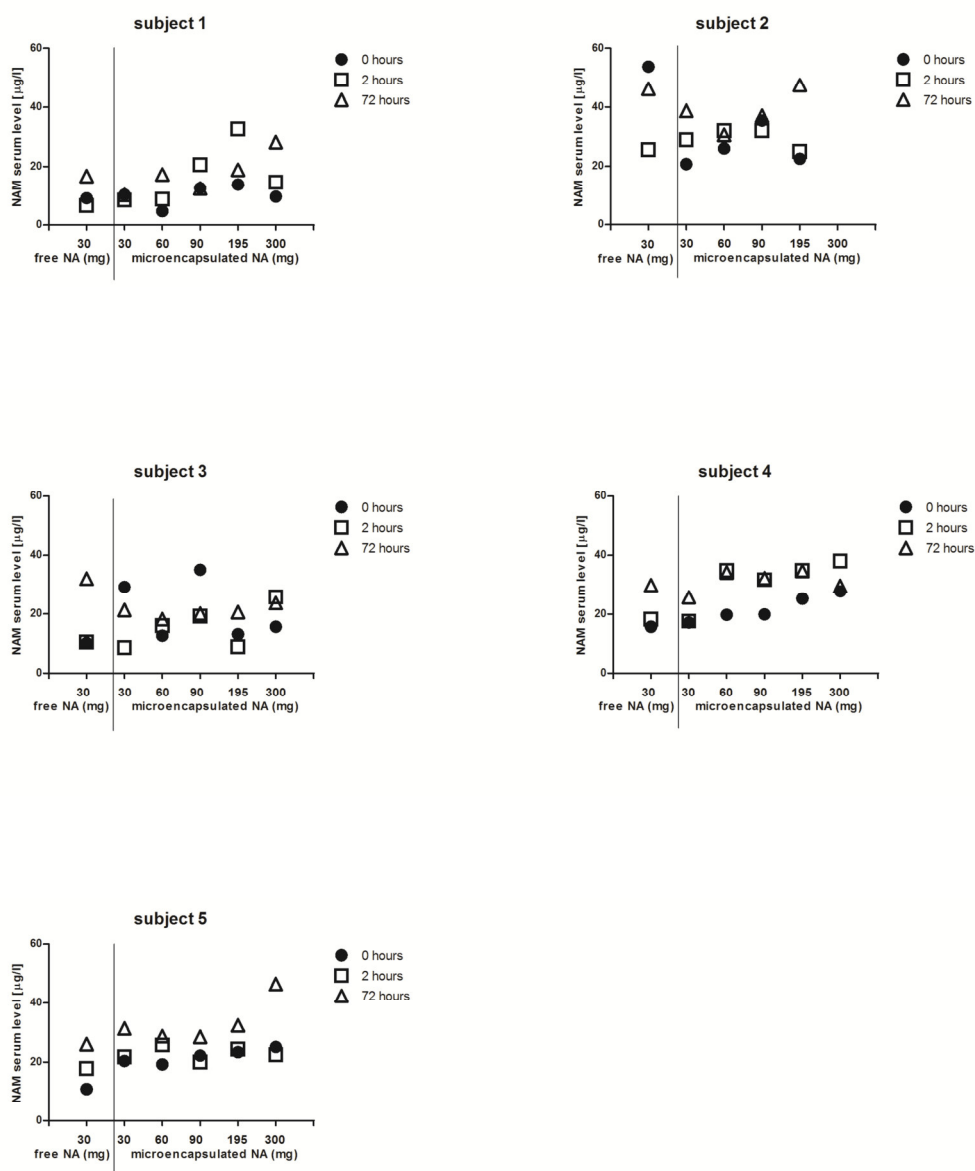


Figure 16: Nicotinamide serum levels of NA group in the course of proof-of-concept and safety study
Nicotinamide serum levels measured at baseline (fasted; 0 hours), two hours (2 hours) after first ingestion of nicotinic acid and after 72 hours (fourth day of dosage). Doses are shown on axis of abscissas. Abbreviations: NA, nicotinic acid; NAM, nicotinamide.

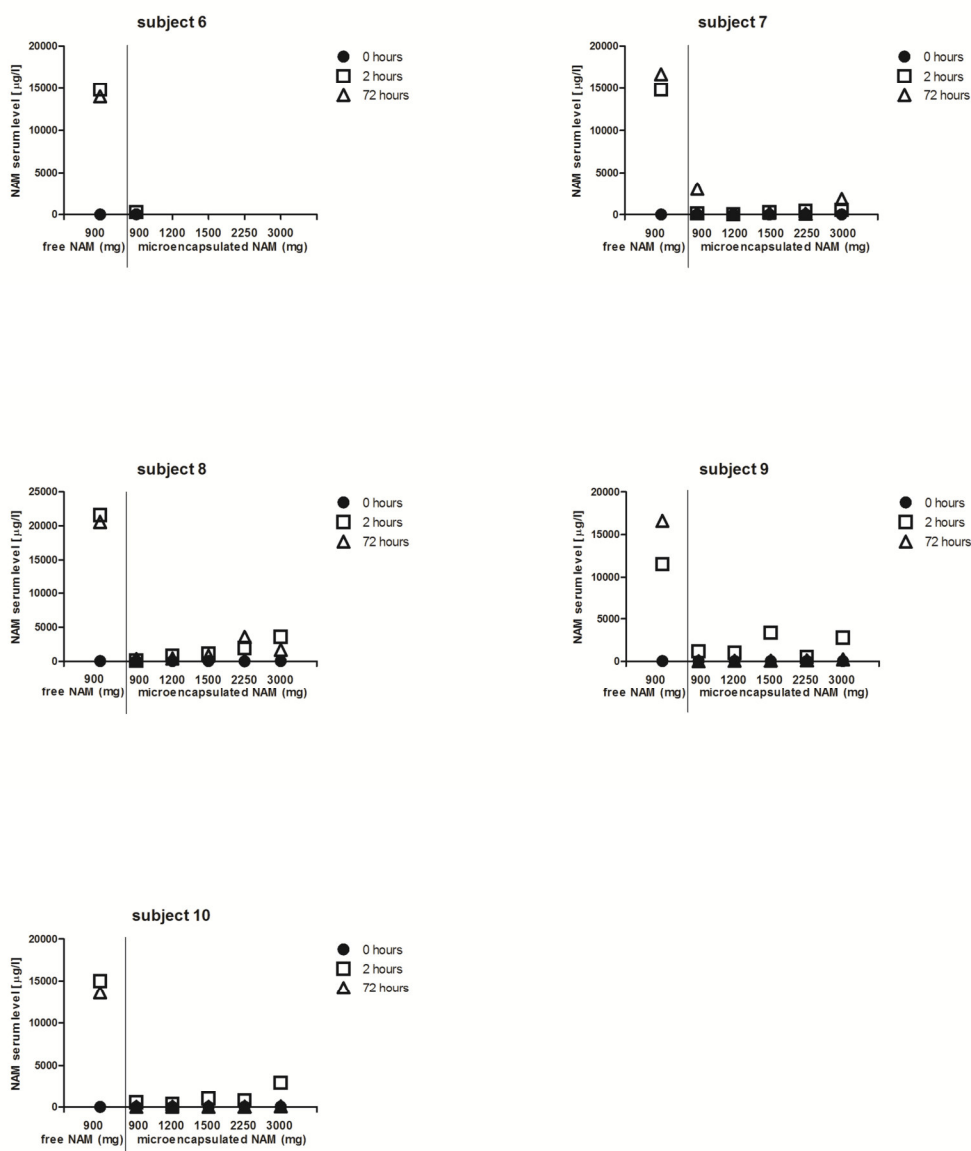


Figure 17: Nicotinamide serum levels of NAM group in the course of proof-of-concept and safety study Nicotinamide serum levels measured at baseline (fasted; 0 hours), two hours (2 hours) after first ingestion of nicotinamide and after 72 hours (fourth day of dosage). Doses are shown on axis of abscissas. Abbreviations: NA, nicotinic acid; NAM, nicotinamide.

4.6 Effects of delayed-release niacin microcapsules on gut microbial composition

Having shown that the microencapsulation procedure is able to induce delayed release of NA and NAM under minimal systemic resorption, it was next aimed to analyse the human gut microbiome before and after administration of delayed release NA and NAM microcapsule formulation (performed at IKMB, Kiel). Applying modern high-throughput sequencing technologies, a total of 976,351 16S rRNA gene sequences were obtained, of which 966,038 passed quality control. The majority of sequences were classified to the *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* phylum. The healthy volunteers of NA group showed a significant increase in *Bacteroidetes* over the time period of six weeks in the proof-of-concept study (**Figure 18B**). No significant changes occurred according to the abundance of the other phyla within NA group (**Figure 18**). However, a trend of decrease was recognisable in *Firmicutes* abundance (**Figure 18A**). In contrast to NA group, no significant change occurred in the *Bacteroidetes* abundance, and neither in the abundance of the other phyla within NAM group (**Supplemental Information, Figure S1**).

Further, diversity measures of gut microbiome were examined in the course of the proof-of-concept study. No consistent change could be observed according to the determined alpha-diversity measures within NA or NAM group (**Supplemental Information, Figure S2**).

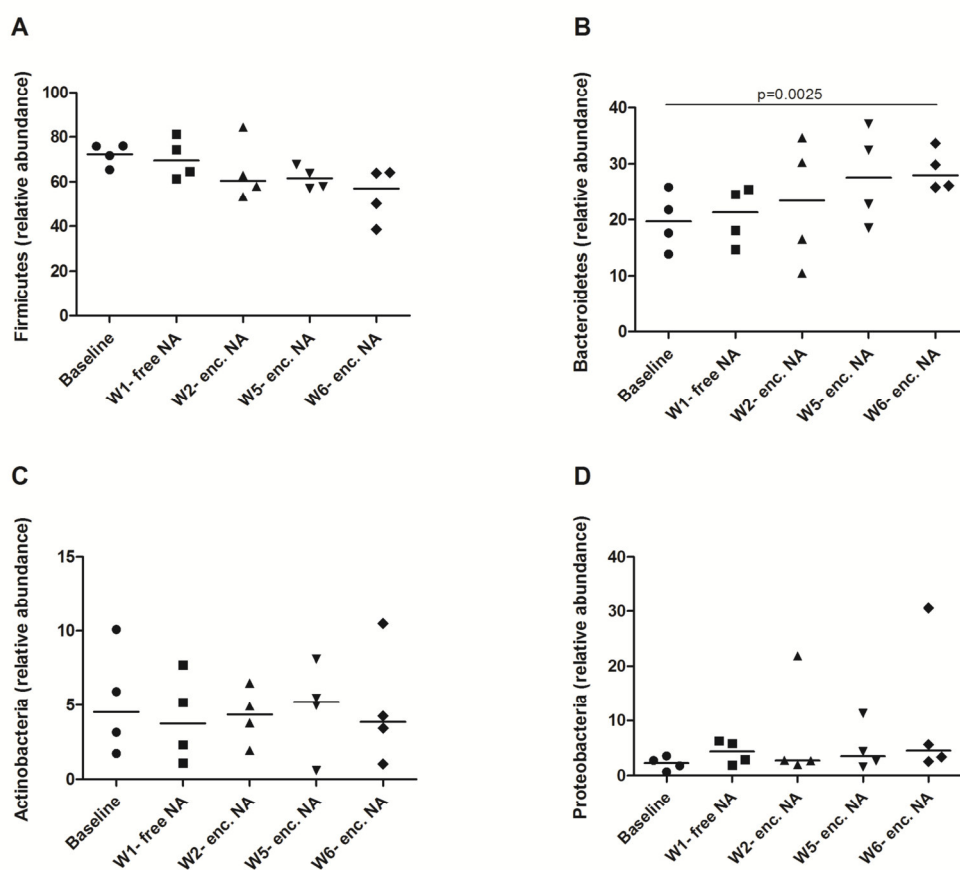


Figure 18: Relative abundance of the gut microbial phyla of NA group in the course of proof-of-concept and safety study

Relative abundance of *Firmicutes* (A), *Bacteroidetes* (B), *Actinobacteria* (C) and *Proteobacteria* (D) of NA group in the course of proof-of-concept and safety study. To determine significant changes in the microbial composition under niacin intervention over time repeated ANOVA and paired t-test were used. Statistical significance was set at $p < 0.05$ for ANOVA and at $p < 0.0125$ for paired t-test due to Bonferroni adjustment. Abbreviations: NA, Nicotinic acid; enc., encapsulated (microencapsulated NA).

4.7 Systemic effects on human host metabolism under dosage with delayed-release niacin microcapsules

Though encapsulated NA and NAM were targeted to be available to the gut microbiome, it was entirely possible that they induce systemic metabolic effects on human metabolism, since several studies indicate a linkage of gut microbiota and host metabolism. Thus, metabolic parameters were determined in the course of proof-of-concept and safety study. Body weight was measured at the beginning of each study week (hour 0) and BMI was calculated to monitor nutritional status during the study. As expected, body weight remained stable in the course of proof-of-concept and safety study in both groups (**Figure 19**). To evaluate lipid and glucose metabolism in the course of the study, the relevant parameters were measured at baseline and at the end of each dosage week (**Figure 20&21**). Parameters of lipid and glucose metabolism remained stable mainly within normal range under different doses of NA or NAM administration.

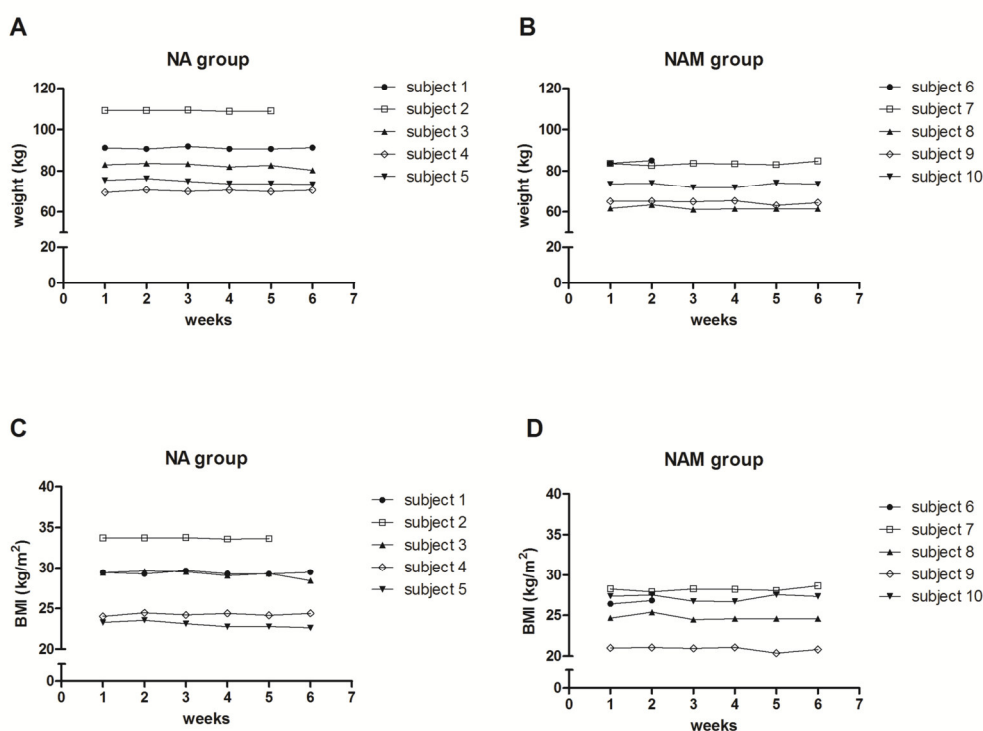


Figure 19: Body weight and BMI in the course of proof-of-concept and safety study

Body weight (A&B) and BMI (C&D) of NA (subject 1–5) and NAM (subject 6–10) group, measured at hour 0 in each week within the course of proof-of-concept study. Abbreviations: NA, nicotinic acid; NAM nicotinamide; BMI, body mass index.

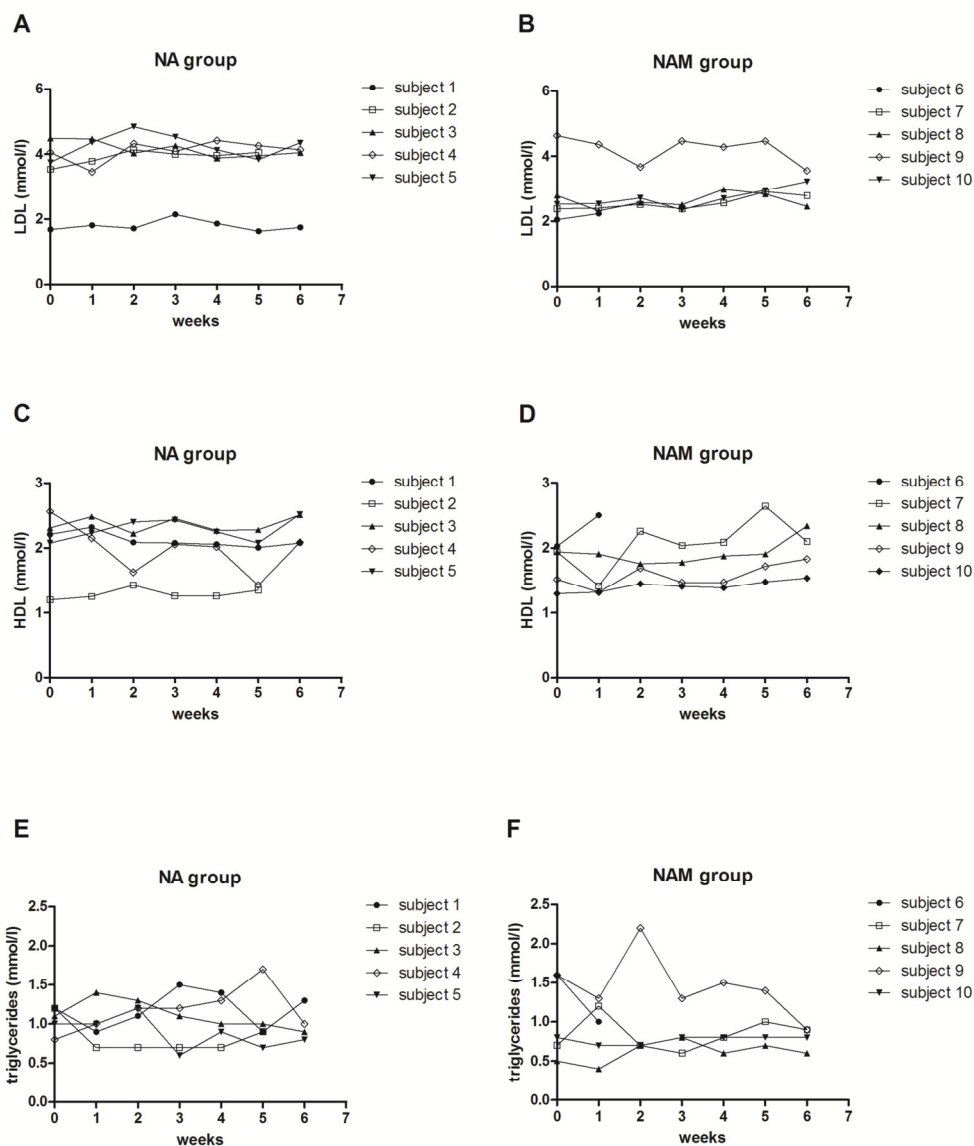


Figure 20: Parameters of lipid metabolism in the course of proof-of-concept and safety study

Parameters of lipid metabolism were measured at baseline (hour 0/ week 0) and at the end of each dosage week (hour 72) in NA group (subject 1–5) and NAM group (subject 6–10). Values of low density lipoprotein (A&B), high density lipoprotein (C&D) and triglycerides (E&F) are shown. Abbreviations: NA, nicotinic acid; NAM, nicotinamide; LDL, low density lipoprotein; HDL, high density lipoprotein.

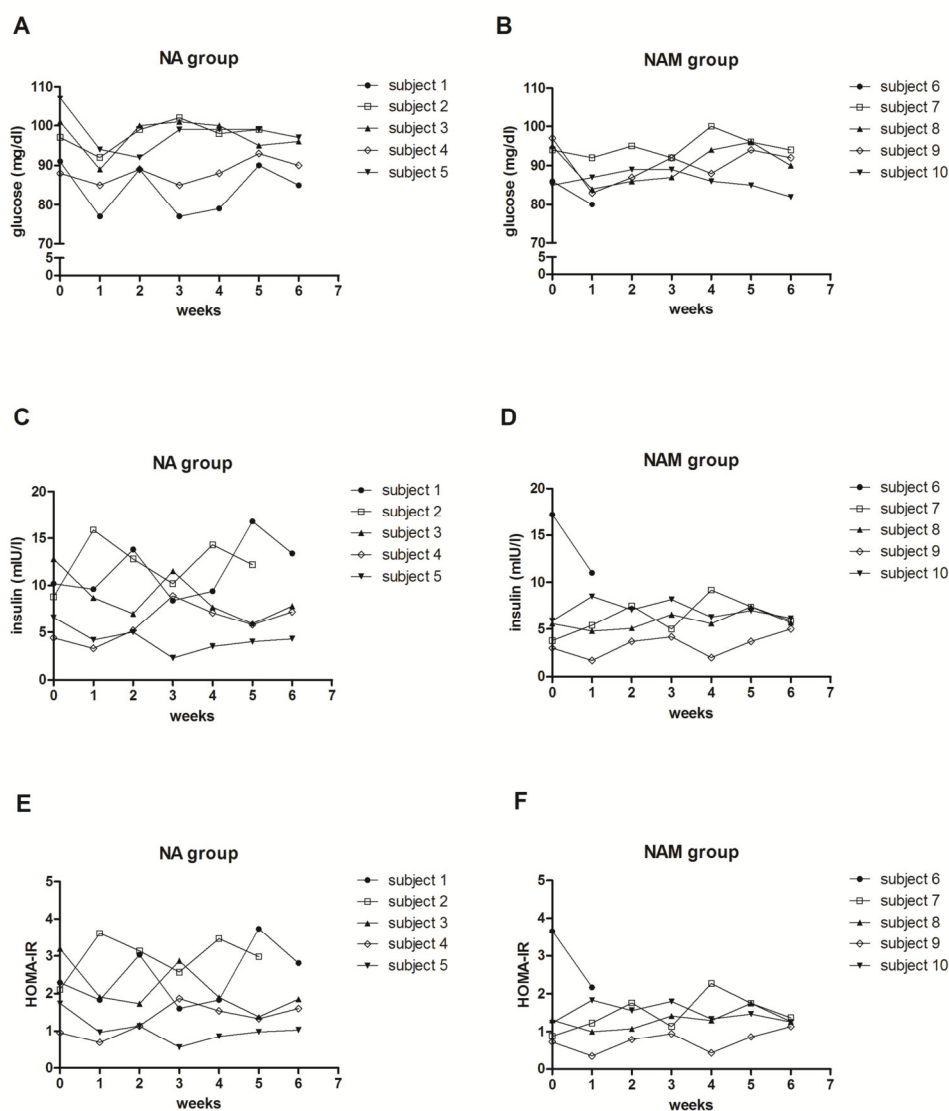


Figure 21: Parameters of glucose metabolism in the course of proof-of-concept and safety study

Parameters of glucose metabolism were measured at baseline (hour 0/ week 0) and at the end of each dosage week (hour 72) in NA group (subject 1–5) and NAM group (subject 6–10). Values of glucose (A&B), insulin (C&D) and HOMA insulin resistance index (E&F) are shown. Abbreviations: NA, nicotinic acid; NAM, nicotinamide; HOMA; homeostasis model assessment insulin resistance index.

In addition to the measured parameters of lipid and glucose metabolism, several more sensitive biomarkers for human metabolism were determined, representing different tissues in humans. Biomarkers were measured in serum samples of NA and NAM group of proof-of-concept study using ELISAs. **Table 16** shows values of these biomarkers at the beginning of each study week

(baseline/hour 0) before dosage and at the end of each NA dosage (hour 72). Myostatin, decreased under application of microencapsulated (30 mg and 60 mg) NA, but not under free NA (30 mg). Similar results were found for fetuin-A, which was significantly decreased under dosage of 60 mg microencapsulated NA. Besides examining circulating factors related to systemic insulin sensitivity, osteopontin levels were also measured as a marker for metabolic inflammation. Osteopontin concentrations significantly decreased under application of microencapsulated NA (60 mg). During dosage in week four to six no significant changes of biomarkers were observed within NA group. Further, no effect on the adipokines adiponectin and FGF-21 occurred under NA application and none of the microencapsulated NAM doses induced significant changes of the measured biomarkers (**Supplemental Information, Table S1**).

Table 16: Biomarkers of human metabolism at the beginning and end of each week within NA group of proof-of-concept and safety study

Tissue		Muscle	Liver	WAT	BAT	Innate Immunsystem
Target		Myostatin (ng/ml)	Fetuin-A (ng/ml)	Adiponectin (ng/ml)	FGF-21 (pg/ml)	Osteopontin (ng/ml)
30 mg free NA	h0	1.76 ± 0.67	2.49 ± 0.72	0.77 ± 0.09	46.29 ± 2.62	2.04 ± 0.74
	h72	1.67 ± 0.80	2.41 ± 1.68	0.75 ± 0.11	48.19 ± 1.56	2.29 ± 1.05
30 mg enc. NA	h0	1.84 ± 0.69	3.21 ± 1.19	0.77 ± 0.11	48.34 ± 1.82	2.37 ± 0.79
	h72	1.51 ± 0.50	2.62 ± 0.58	0.77 ± 0.10	48.88 ± 3.49	2.23 ± 0.80
60 mg enc. NA	h0	1.78 ± 0.67	3.29 ± 1.10	0.77 ± 0.08	47.23 ± 1.66	2.77 ± 1.39
	h72	1.55 ± 0.48	2.66 ± 0.95	0.74 ± 0.02	59.50 ± 27.34	2.09 ± 0.78
90 mg enc. NA	h0	1.77 ± 0.53	3.24 ± 1.59	0.85 ± 0.07	49.49 ± 8.08	2.62 ± 1.20
	h72	1.67 ± 0.46	2.60 ± 0.41	0.75 ± 0.06	51.38 ± 8.83	2.19 ± 0.87
195 mg enc. NA	h0	1.86 ± 1.01	2.47 ± 0.59	0.83 ± 0.18	47.03 ± 7.94	2.38 ± 1.17
	h72	1.67 ± 0.57	2.44 ± 0.58	0.84 ± 0.16	36.74 ± 21.70	2.42 ± 1.39
300 mg enc. NA	h0	1.64 ± 0.73	2.78 ± 1.08	0.84 ± 0.12	47.13 ± 9.24	2.52 ± 1.75
	h72	1.68 ± 0.79	2.71 ± 0.71	0.88 ± 0.16	37.26 ± 19.15	2.22 ± 1.52

^amean ± sd (all such values)

in bold: significant change of the target during the week (from h0 to h72) tested with Wilcoxon-test and significance level set at p<0.05.

Abbreviations: NA, nicotinic acid, enc.= encapsulated (microencapsulated NA); h0, hour 0 (baseline); h72, hour 72 (after dosage); FGF21= fibroblast growth factor 21, WAT= white adipose tissue, BAT= brown adipose tissue

5 Discussion

5.1 Tryptophan and niacin nutritional intake and serum concentrations in relation to obesity, type 2 diabetes and gut microbial composition

Analyses of nutritional intake of Trp, niacin and NE in subjects with different metabolic phenotypes revealed comparable nutritional intake of these parameters in lean and obese groups. In contrast underweight group exhibited significantly lower intake levels than the other groups, most likely due to lower food intake in underweight subjects. Nonetheless, nutritional intake of niacin equivalents of all underweight subjects was adequate according to intake recommendations of the German Nutrition Society [28], suggesting sufficient oral supply of all subjects according to nutritional aspects. Oral supply of niacin within the German population has been evaluated by the Max-Rubner-Institute (MRI, 2008). Researchers of the MRI evaluated dietary intake of 15,371 subjects using Diet-History method. Results of their work showed intake of niacin to be higher than intake recommendations in all age groups, and niacin intake to be insufficient in only 1% of the male and 2% of the female population [33], matching results within the present FoCus subset population.

Despite comparable nutritional intake of the groups, obese subjects especially with T2D exhibited lower serum levels of NAM compared to healthy volunteers, indicating altered niacin metabolism. In a mouse model it has been shown, that obese and type 2 diabetic mice have lower hepatic levels of NAD and NADP [17]. NAM serum levels were not measured in that experiment, but due to low NAD levels in the liver, NAM might be absorbed by the liver in order to serve as a precursor for NAD and maintain NAD homeostasis. This could explain low NAM serum level in obese and type 2 diabetic subjects. Due to the high metabolic turnover from NA to NAM via NAD, NAM is the principal circulating form of niacin and NA serum levels are difficult to determine [24, 29]. Thus, NA serum levels of almost all subjects were below the detection limit (12 µg/l) within the FoCus subset.

The results on nutritional intake and serum levels were also reflected by correlation analyses, since nutritional intake levels correlated with Trp but not with NAM serum levels. But it has to be mentioned that correlation of nutritional intake levels and Trp serum levels were weak. Moreover, it should be considered that over- as well as underreporting can be a general problem of food-frequency questionnaires that can lead to bias [65]. Furthermore, the performed FFQ was a twelve months retrospective method and serum levels were measured once at a specific time point. Though subjects were advised to come to the study center in fasted state for blood sample

collection, individual behaviour can cause bias between twelve months retrospective FFQ and serum level measurement.

Still an altered niacin metabolism in obese and type 2 diabetic seems to be indicated. Though it was aimed to develop niacin microcapsules that deliver niacin into the colon, minimal systemic resorption might be possible but seems tolerable according to altered niacin metabolism in obese.

In addition to niacin status, gut microbiome of the subjects with different metabolic phenotypes was analysed by 16S rRNA gene sequencing. Analyses showed reduced alpha-diversity in obese and obese and type 2 diabetic subjects compared to lean and underweight subjects. Reduced alpha-diversity in obese was detected independent from the measure of alpha-diversity, since differences were found on genera, OTU and phylodiversity level. These findings are consistent with other studies. Turnbaugh and co-workers showed obesity to be associated with reduced bacterial diversity in their twin study [6]. Also Le Chatelier et al. observed in their cohort of 292 obese and non-obese of the Danish population, that individuals with low bacterial gene richness were characterised by more overall adiposity and insulin resistance [11]. Moreover, individuals with reduced microbial gene richness present more pronounced insulin-resistance, dyslipidaemia and low-grade inflammation, and thus an increased risk of obesity associated co-morbidities [10].

Besides reduced bacterial diversity, analyses of gut microbiome on phylum level revealed reduced *Bacteroidetes* abundance in obese FoCus subjects, one of the major phyla in mouse and human gut microbiome [3, 4, 45]. In mouse models a reduced abundance of *Bacteroidetes* in obese mice has been shown, too. Further an increase in *Firmicutes*, and thus an increased *Firmicutes/Bacteroidetes* ratio is commonly observed in mice [4]. Also several studies in humans showed similar findings of an altered *Bacteroidetes* and/or *Firmicutes* abundance in obese [3, 5, 6, 66]. However this alteration could not be observed by all studies [8, 9], with the inverse also reported [67]. Alterations in abundance of *Firmicutes* were also not observed in the FoCus subcohort. Surprisingly, reduced *Bacteroidetes* abundance was only observed in metabolic healthy obese, but not in obese with T2D. In contrast Louis et al. observed a higher *Firmicutes/Bacteroidetes* abundance ratio in obese with metabolic syndrome compared to healthy obese [68].

Mechanisms of the involvement of the gut microbiota in the development of obesity are not yet completely understood, but several studies prove the link between gut microbial composition and obesity and examined the mechanisms of the gut-host interaction. For instance, results of a mouse model indicated the obese microbiota to have an increased capacity to harvest energy from the diet [2]. The microbiota for example can ferment complex polysaccharides resulting in short chain fatty acids and monosaccharides, which are absorbed by the host [1, 41, 69]. Thus, the caecum of obese

subjects has an increased concentration of short-chain fatty acids [2]. Further Turnbaugh and co-workers showed, that obesity-enriched genes were mainly from *Actinobacteria* and *Firmicutes*, whereas lean-enriched genes were mainly from *Bacteroidetes*. The obesity-enriched genes were indicated to be involved in carbohydrate, lipid and amino acid metabolism [6]. For example the obese microbiome was enriched for Phosphotransferase systems [6], whose genes can be found in *Firmicutes* [69]. The Phosphotransferase system plays a role in the uptake and phosphorylation of carbohydrates [70].

Taken together the results indicate, that an amelioration of the altered gut microbiome in obese can possibly exert beneficial effects in the prevention and/or treatment of obesity and its comorbidities.

Having shown alterations in gut microbiota, the association of niacin status and gut microbiota was further analysed. Both, NAM serum levels and niacin nutritional intake were positively associated with alpha-diversity measures, especially in obese subjects. Furthermore a positive correlation of niacin intake and *Bacteroidetes* abundance was shown exclusively in obese subjects. The correlations were not found in obese subjects with T2D, similar to the result on *Bacteroidetes* abundance shown and discussed above. Nevertheless the positive correlation of niacin status with gut microbiota especially in obese, gives first hints on a beneficial effect of niacin on gut microbial composition. Firstly, because bacterial diversity is reduced in obese (shown above) and could be increased by niacin treatment. Further, the *Bacteroidetes* abundance is also reduced in obese (shown above) and could also be increased by niacin treatment according to the observed correlation of niacin intake and *Bacteroidetes* in obese. Still it has to be mentioned, that correlation-coefficients for alpha-diversity measures and niacin intake varied between 0.268–0.306 in obese and for *Bacteroidetes* abundance and niacin intake variables they varied between 0.191–0.198. But niacin intake was calculated by retrospective FFQ from the usual diet of the subjects and therefore niacin will probably not reach the colon in high amounts. With our gut-targeted formulation of niacin it is expected to exert greater effects on microbial composition through niacin intake and thus probably stronger association can be observed.

The impact of nutrition on the composition of gut microbiota was revealed by several other studies. They showed alterations of bacterial diversity [10] as well as changes on phylum level due to specific diets [8, 10, 12, 52, 71-74], matching the present results. Most of the studies focused on nutritional load of the diet [8, 10, 12, 71], or macronutrients and dietary patterns [52, 72-74], but little is known about the association of vitamins and gut microbiome. Thus, the present approach connecting niacin micro-nutrition and the human gut microbiome is novel.

5.2 Bioavailability of delayed-release niacin from microcapsules in healthy human subjects

Appearance and composition of NA and NAM microcapsules after gastrointestinal passage in the human volunteer were first analysed in order to determine the release of NA and NAM from the microcapsules. Thus, SEM pictures of the digested NA and NAM capsules were taken. In addition, SEM pictures of undigested capsules served as controls.

Before digestion NAM microcapsules showed a smooth and tight coating, with no cracks and a NAM core inside. Digested capsules appeared as open and empty (no core inside) coating shells with a porous and spongy structure instead, indicating dissolution of the pH-dependent coating during passage in the human volunteer as it was predicted by *in vitro* dissolution tests. The porous and open structure of coating shells allows gastrointestinal water to enter and solve NAM, which is highly water-soluble [24]. Since the core of NAM microcapsules consisted of granulated NAM, empty coating shells indicate the whole NAM core to be dissolved. Thus the release of NAM from administered NAM microcapsules is assumed. Further, no cores showing same crystalline surface structure as uncoated NAM-cores (granula) occurred within analysed stool samples.

NA microcapsules showed the same smooth surface structure before digestion as NAM microcapsules, since the same (shellac) coating was used. Also the coating of NA microcapsules appeared as an open coating shell with a porous and spongy structure after digestions, similar to digested NAM capsules. Thus, the coating of NA microcapsules also seems to be dissolved after passage in the human volunteer, allowing gastrointestinal water to enter and solve NA, which is moderately soluble in water [24]. In contrast to digested NAM microcapsules, coating shells of digested NA microcapsules were not empty, but showed a core inside. Surface structure of the core was higher magnified and compared with surface structure of undigested and uncoated cellulose-cellets and undigested cellulose-cellets coated with NA (NA cores). The NA-cores showed crystalline surface structure and the uncoated cellulose-cores showed a fibrous surface structure instead, which resembled surface structure of observed cores within stool samples. Therefore it is assumed, that the core observed within the open coating shells of stool samples was an indigestible cellulose core, without NA-coating.

Taken together, these findings indicate that both, NA and NAM microcapsules opened during the gastrointestinal passage to release active ingredients.

Having shown the release of NA and NAM from administered microcapsules, systemic exposure profile was determined within pilot and main bioavailability studies. In both studies NA and NAM serum concentrations were measured in specific time intervals for twelve hours after administration of different doses of microencapsulated and free niacin.

Administration of reference dose free NAM led to a rapid increase in NAM serum level with the serum peak occurring 1 h after dosage. This was observed in all subjects of pilot and main bioavailability study and shows the immediate resorption of free NAM as it was expected, since it is commonly known that niacin can be absorbed through the stomach lining and in the small intestine very rapidly [24, 27]. In contrast NAM serum level peaks occurred 4 h after ingestion of the same dose of microencapsulated NAM, indicating the delayed release of NAM from novel microcapsules. The same was indicated by NAM serum peaks observed under higher doses of microencapsulated NAM, but inter-individual variance increased with dose. NA serum levels under dosage with NAM were mostly under the detection limit in pilot and main bioavailability study, due to rapid metabolism (as described above; s. chapter 5.1). Only under dosage with free NAM and under highest microencapsulated NAM dose (3000 mg), NA serum levels were measurable, indicating that NAM can also be metabolized to NA. In fact, it has been reported that large doses of NAM can cause an induction of deamidase enzymes, leading to formation of NA [24, 75]. Nicotinamide deamidase enzymes convert NAM to NA for Preiss-Handler Pathway [24] and are found in some bacteria of gut microflora [24, 76]. Bender et al. also reported these enzymes to be present in the liver of rats [75], whereas Gazzangia et al. stated in their review, that vertebrates are lacking genes for nicotinamidase [76]. In the present study NA serum levels above detection limit were only measured at time points also showing highest NAM serum levels. Therefore, it can be hypothesized that conversion of NAM to NA was performed after gastrointestinal resorption, for example through deamidase enzyme activity in the liver. However, appearance of NA serum levels above detection limit either didn't occur under microencapsulated NAM (900 mg and 1500 mg) or peaked at later time points than under dosage of free NAM. Taken together NA and NAM serum levels measured under NAM application, indicate the delayed-release of NAM from microcapsules in comparison to reference dose of free NAM.

As reviewed by Pišlar et al. (2015), the median time of gastric emptying after ingestion of non-disintegrating tablets accounts to approx. 5 min, the small intestinal transit time to 215 min (min/max: 60/544) and the colon arrival time to 254 min (min/max: 117/604) [77]. Thus, taking the findings of Pišlar and co-workers into account, our bioavailability curves of microencapsulated niacin with serum peak levels after 4–8 h indeed suggest a pH-dependent release of niacin in the ileo-colonic region. It has to be mentioned, that the gastro-intestinal transit times reported by Pišlar et al. were examined in a group of subjects, receiving the first meal at 4 h post dose, whereas meals

given earlier (after 35–120 min) can increase the time of gastric emptying or shorten small intestine transit time [77]. During our NA and NAM bioavailability study the first meal was given 3 h post dose. Nevertheless, transit times seem comparable to the ones reported, because pellets smaller than 2 mm are emptied from the stomach very rapidly and are unaffected from the digestive state of the subjects [78].

Within NA group, NAM serum concentrations were also measured, since all NA serum levels were below the detection limit. NAM serum levels only showed minimal fluctuations on a high background level, but no consistent time point for serum peak level was recognisable. Further, no consistent and dose-dependent increase in NAM serum levels occurred, despite 10-fold dose increase, proofing minimal systemic resorption. This becomes particularly evident, comparing serum levels with measured NAM serum levels under dosage with reference dose free NAM. If NA was released from microcapsules immediately, higher NAM serum levels would be expected at least under 10-fold dose increase, since NA would be rapidly absorbed in the stomach and small intestine (discussed above) and was further reported to be rapidly metabolized with peak plasma concentrations after 30–60 min [40]. Thus, the delayed release of NA from microcapsules is still assumed, despite the lack of clear time points for NAM serum level peaks. The results indicate, that microencapsulation of NA is able to reduce systemic resorption and induce the delayed release of NA into the ileo-colonic region.

In order to evaluate total systemic resorption of NAM within NAM group, area under the curves were calculated. As expected and already indicated by NAM serum level curves, total systemic resorption under 900 mg and 1500 mg microencapsulated NAM dose was significantly lower than systemic resorption under reference dose free NAM. Thus, NAM serum levels within NAM group not only proof the delayed release (discussed above), but also minimal systemic resorption of NAM from microcapsules. Even under 3000 mg microencapsulated NAM no significant difference to the AUC of 900 mg free NAM dose occurred. But it has to be mentioned, that NA serum levels above detection limit could be measured under 3000 mg dose (discussed above) and serum levels after twelve hours did not match baseline serum levels. Therefore, serum level curves were extrapolated to match baseline serum levels, and NA serum levels that appeared under 3000 mg dose were included in calculation of AUCs. Nevertheless, no significant difference occurred.

Still, AUCs calculated with extrapolated values are only estimated, and a trend of higher AUCs under 3000 mg dose compared to AUCs of reference dose was observed in some subjects. Further inter-individual variance increased with dose. But to ensure avoidance of negative side effects, which were not observed in the performed study, total systemic resorption should not be higher than under reference dose of free NAM. Thus, it might be considered to evaluate total systemic niacin exposure under 3000 mg dose in a higher sample size and/or in a time extended study

design. Also body weight of the subjects was not respected in these analyses or rather in definition of dose but could be considered in evaluation of NAM serum levels and systemic exposure, too. However, there were also other studies performed on pharmacokinetics of niacin, which did not adjust administered doses for weight [79-81]. Bernier and co-workers showed a trend towards decreasing NAM serum levels in heavier subjects, but correlation was weak [79]. Of importance, BMI range of the study population of the present bioavailability study was small and within normal range. Thus, fixed doses of niacin still seem to be reasonable. Moreover, only serum levels of niacin, but not the metabolites of NA and NAM were measured in the present intervention. Though some studies have looked to the metabolites, it was discussed not to be common [79]. Furthermore serum levels under dosage with microencapsulated niacin were compared to a reference dose of free niacin in the present study, expecting comparable serum levels also leading to comparable levels of the metabolites. In contrast, other studies did not include a reference dose.

Pharmacokinetics of diverse NA and NAM formulations showed faster and higher systemic exposure than the novel microencapsulation formulation [79-85]. Dragovic and co-workers evaluated the pharmacokinetics of different doses of NAM in twelve patients with cancer. They observed dose-dependent maximum plasma concentrations, with the plasma concentration increasing with dose, and peak plasma concentrations to occur at later time points with increasing dose. Under dosage with 10 g NAM, for example, maximum plasma concentrations were observed after 2–4 h, whereas 3 or 4 g doses induced plasma peak levels already after 30 min [80]. Also Bernier and co-workers observed plasma peak concentrations after 1 h in only 54% of the examined subjects dosed with 6 g NAM [79]. However, in the present study maximum administered dose of NAM was 3000 mg (microencapsulated). Serum peak levels under this dose occurred at later time points (4–8 h) not only in comparison to the peak levels of reference dose of 900 mg free NAM (1 h), but also in comparison to serum peak levels (0.5-1 h) observed under the same dose (3000 mg) free NAM, as it was shown in other studies [80, 81]. Further, highest plasma concentrations under 3000 mg free NAM observed in other studies [80] were far beyond those observed under 3000 mg microencapsulated NAM in the present investigation. Similarly to the present study design, Petley et al. not only examined the pharmacokinetic of free NAM, but also of a long-acting formulation. Maximum systemic NAM levels measured under 2 g dose of their long-acting NAM formulation [82] were higher than NAM serum levels measured under the comparable dose of 1500 mg microencapsulated NAM in the present study. Even under a higher dose of 3000 mg microencapsulated NAM, serum levels appeared to be lower in most subjects than those measured in the study of Petley and co-workers. Moreover, they compared AUCs, and thus, total systemic exposure of (approx. 2 g) free and long-acting NAM. AUCs for free NAM appeared to be 1.7 times higher than those for long-acting NAM [82]. In contrast, in our approach mean AUC of

free NAM was 18 times higher as mean AUC of the same dose of microencapsulated NAM, indicating lower systemic resorption with the present formulation.

In the case of NA, different sustained- and extended-release formulations have been developed in the past due to the beneficial effects of NA on lipid metabolism. But in contrast to the present approach, they all aimed systemic bioavailability to treat dyslipidaemia. Hence, administration of 1500 mg of an extended-release NA formulation for example led to NA plasma peak concentrations of 4220 µg/l in patients with chronic kidney disease [83]. A dose of 2 g NA in healthy volunteers led to NA plasma peak concentrations of 9300 µg/l [84]. It has to be noticed, that the present delayed-release formulation did not even lead to measurable NA values, probably due to much lower doses administered. It has been reported that low oral doses of NA can be completely metabolized [86]. Thus only minimal fluctuations in NAM serum level but not NA serum level were observed, as discussed above. Neuvonen and co-workers also evaluated pharmacokinetics of lower NA doses. They compared 500 mg of two different slow release formulations with 500 mg of unchanged NA and proofed lower bioavailability of slow release formulations [85]. Still, NA serum levels were measurable in their study and the administered dose was higher than doses used in the present study.

Taken together, findings of bioavailability study show that the developed microcapsules are able to deliver high amounts of NA or NAM into the colon, without significant increase in total systemic NA/NAM uptake in comparison to systemic uptake under application of reference dose free NA/NAM or other NA/NAM formulations.

5.3 Safety profile of delayed-release niacin microcapsules

Safety parameters were monitored during both, bioavailability and proof-of-concept and safety study. During bioavailability study no safety signals occurred according to determined laboratory parameters. Further, NAM serum levels measured during bioavailability study mostly reached baseline levels after 12 h also under higher dosage with microencapsulated niacin. These findings suggest, that no accumulation would be expected in the case of daily dosage (every 24 hours) with microencapsulated niacin. Even under highest NAM dose, extrapolated NAM serum levels curves mostly indicated NAM serum levels to reach baseline within 24 h.

Also within proof-of-concept and safety study no accumulation of NA and NAM in the course of the study weeks could be observed. Apart from one subject of NA group, showing elevated AST-levels under second highest NA dose, no safety signals occurred according to safety laboratory

parameters under different doses of microencapsulated niacin applied for several days. Besides that, neither group exhibited clinical symptoms as nausea and vomiting or flush phenomenon.

In the past, different niacin formulations to treat lipid disorders have been reported by independent researchers and pharma companies that showed several side effects. For instance it is commonly known, that NA doses higher than 30–50 mg can induce facial flush, occasionally associated with pain [23, 30, 87]. Flush symptomatic was reported to occur 75 min (mean) after ingestion of immediate-release NA [88] and to last between 30 min and 2 hours [40, 88, 89]. Though higher doses than 30–50 mg NA were applied in the present niacin interventions, none of the subjects within bioavailability study or proof-of-concept and safety study showed flush phenomenon, proofing delayed release and reduced systemic resorption. Of importance, flush as a negative side effect under NA treatment also appeared under dosage with extended-release formulations [90-92]. Doses of 300–2000 mg NA can cause nausea and vomiting as well as other unspecific gastrointestinal symptoms [23, 30, 89, 91, 93, 94]. Gille and co-workers, for example, reported heart-burn, indigestion, nausea, diarrhoea and stomach pain [89]. Further, administration of high NA doses can lead to liver-disorders or even hepatitis and liver failure in both, immediate release and extended- or sustained-release formulations [23, 40, 90, 93, 95-98]. McKenney and co-workers compared increasing doses up to 3000 mg sustained-release and immediate-release formulations in 46 subjects. Subjects under sustained-release formulation showed increases in level of liver aminotransferase, often associated with symptoms of hepatic dysfunction. Instead none of the subjects taking the immediate release NA formulation developed hepatotoxic effects [93]. Elevation of liver enzymes under dosage with extended-or sustained-release formulations were also observed by other authors [90, 94]. This goes in line with the observations in the present study, since one subject showed an elevation of transaminase level. But of importance, changes in liver enzymes were reversible in the present and other investigations. Clinical symptoms of hepatitis under time-release niacin developed shortly after few days to few weeks of dosage [95, 96]. Instead, under dosage with free NA they were reported to occur under prolonged release [95]. The hepatotoxic effects have been reported to be more common under dosage with sustained- or extended-release formulations compared to free NA formulations [23, 30, 40, 94]. As an explanation it is assumed, that sustained- and immediate-release formulations are metabolized through different pathways. There are two hepatic pathways described for NA metabolism. One pathway is a low-affinity and high-capacity pathway generating metabolites associated with flushing. And the second pathway is a high-affinity, low capacity pathway leading to metabolites that are associated with hepatotoxicity. High doses of immediate-release NA will quickly saturate the low-capacity pathway and thus be metabolized through high-capacity pathway resulting in flush. Instead, long-acting niacin formulations will be preferentially metabolized through low-

capacity pathway [99, 100]. On the other hand, Scientific Committee on Food and the German Federal Institute for risk assessment both discussed in their statements, that administration of high doses of NA can lead to hepatotoxic effects independently of the formulation [23, 30], and thus, such high doses of NA should not be used unmonitored [23, 93, 96]. Further, daily doses of 3000 mg NA can induce impaired glucose tolerance and an increase in uric acid [23, 30, 101]. Of importance, doses administered in the present study are far below those used in the studies discussed. And in complete contrast to other formulations, a topical exposure of NA or NAM in the colon with minimal systemic resorption was aimed. Still, it has to be considered that also under dosage with our microencapsulated NA an elevation of transaminase level can occur, even though it is not expected.

Compared to NA, side effects of NAM are fewer and only appear under doses ≥ 3000 mg [23, 102]. Doses up to 5 g NAM didn't induce nausea or vomiting, whereas doses of 6 or 10 g NAM led to those symptoms, which occurred 30 min to 1 h after ingestion and lasted for several hours up to 24 h [80]. Stratford et al. also observed nausea and vomiting under dosage with 6 g NAM, but they couldn't detect a clear association of high peak levels, longer half-lives or greater AUCs for patient exhibiting nausea and vomiting compared to those with no symptoms. Thus no measures were identified, that will predict toxicity [81]. In contrast, Knip and co-workers reviewed NAM to be well tolerated using pure preparations [102], matching the present observations, whereby administered doses in the present study were far below those leading to nausea and vomiting in other studies. Knip et al. also reviewed liver toxicity of NAM. On the one hand they reported studies showing a negative effect on liver enzymes but on the other hand they reported studies not showing a noteworthy hepatotoxicity. They discussed these effects to depend on purity and solely administration of NAM [102]. However, the described side effects were not observed in the present invention.

Taken together, the novel NA and NAM formulations show a preferable safety profile, especially compared to prior developed NA and NAM formulations. But if side effects would be expected, they would probably occur under high dosage with NA, but not NAM due to fewer side effects in NAM. Nevertheless also NA microcapsules of the present investigation show a preferable safety profile under aimed doses.

5.4 Effects of delayed-release niacin microcapsules on gut microbial composition and human systemic metabolism

Though the present niacin interventions were performed as a pilot project to evaluate novel niacin microcapsules *in vivo*, stool samples of proof-of-concept and safety study were analysed using high-throughput sequencing technologies in order to get a first impression of effects on gut microbial composition through niacin microcapsules. Volunteers of NA group showed a significant increase in *Bacteroidetes* over the time period of six weeks and a trend of decrease in *Firmicutes* abundance under dosage with microencapsulated NA. Since a reduced abundance of *Bacteroidetes* and an increased abundance of *Firmicutes* have been linked to obesity as discussed above (Chapter 5.1), the observed effects represent an amelioration of the gut microbial composition with respect to obesity. Study subjects of proof-of-concept study were slightly overweight in mean but not obese. Thus, it can be hypothesized, that administration of microencapsulated NA might induce even greater effects on microbial composition in obese subjects with significantly reduced *Bacteroidetes* and increased *Firmicutes* abundance. The observed effects also go in line with results of the analyses of the FoCUS subset since dietary niacin intake and *Bacteroidetes* abundance were positively correlated, but correlation was weak (Chapter 5.1). Of importance, most of dietary niacin is expected to be already absorbed in the upper gastro-intestinal tract, whereas in the proof-of-concept study a gut microbiome targeted niacin intervention was performed, showing alterations of gut microbial composition. In contrast to the observed changes on phylum level, the bacterial diversity did not change during intervention with microencapsulated NA. In the past, other studies evaluated the impact of a dietary intervention on gut microbiome and also observed an impact on the abundance of phyla but not bacterial diversity [12, 73]. Simões et al. suggested a strong host effect on the composition of the fecal bacterial population to be causative for that [12]. In contrast to NA, significant increase of *Bacteroidetes* was neither observed under intervention with free nor with microencapsulated NAM, suggesting that NA and NAM differently affect gut bacteria.

Bacteria have been classified by virtue of their ability to synthesize NAD, a central co-factor of metabolism, *de novo* from aspartic acid and/or the vitamin precursors NAM, NA and NR [76, 103]. The ability to use NAM was predicted on the basis of possession of homologs of nicotinamidase (PncA) and nicotinamide phosphoribosyltransferase (NadV). Remarkably, *Bacteroidetes* were reported to be deficient in both genes resulting in the inability to metabolize NAM [76]. This finding might explain the specificity of the increase in *Bacteroidetes* abundance to the delayed-release NA intervention found in the present investigation. The trend of decrease in *Firmicutes* abundance might be compensatory to the increase in *Bacteroidetes*, since bacteria can also affect each other. But the effects of NA intervention on specific bacteria have to be further examined to clearly identify molecular mechanisms involved in the alterations of gut microbiota composition.

Diet during proof-of-concept study was not standardized. Also no standards on physical activity were defined in the study design. Thus, it was entirely possible that the observed alterations in gut microbial composition are mediated through changes in diet or physical activity of the subjects, since both, diet (discussed above) and physical activity [104, 105] can impact gut microbial composition. Of importance, subjects were advised not to change their usual diet and life style and nutritional diaries were kept in order to monitor these confounders. Furthermore, the study design did not include a control group. Two intervention groups were included instead, whereby one group (NAM group) did not exert an effect on gut microbial composition but the other group did (NA). Thus, NAM group can be regarded as a “control group” in the present intervention.

Having shown beneficially alterations of the gut microbiota, effects on human systemic metabolism were further examined, since the gut microbiome has been linked to host metabolism. Because subjects were advised not to change their usual nutritional behavior or lifestyle, body weight and BMI remained stable in the course of the study. Further, measures of glucose (glucose, insulin, HOMA-IR) and lipid (LDL, HDL, triglycerides) metabolism were determined. Since healthy subjects with metabolic parameters within normal range were included in the study, no amelioration of these measures occurred under delayed-release niacin application. Adverse effects on metabolic parameters were neither observed. An impairment of glucose tolerance under NA application has been reported in the past (discussed above, chapter 5.3), but was not expected in the present intervention, since bioavailability study proofed minimal systemic resorption. Hence, neither beneficial nor adverse effects on host metabolism occurred after examination of the mentioned parameters. Therefore, more sensitive biomarkers were measured in serum samples of the subjects and their change during each study week was determined.

Myostatin, which is expressed in skeletal muscle [106, 107], is associated with insulin resistance. A decrease in myostatin was observed only for microencapsulated (30 and 60 mg) but not for free (30 mg) NA, suggesting an indirect beneficial effect on skeletal muscle insulin sensitivity [108, 109]. Similar results were found for fetuin-A, a factor being expressed in the liver [110], shown to be a natural inhibitor of insulin receptor tyrosine-kinase [111, 112] and to be associated with insulin resistance in human subjects [113]. Fetuin-A was significantly decreased under dosage of 60 mg microencapsulated NA. Thus, both the reduction in myostatin and the reduction in fetuin-A levels found in the present study suggest an improvement of systemic insulin sensitivity due to the delayed-release NA microcapsules. However, these effects seem to be specific for skeletal muscle and liver, since no effect on the adipokines adiponectin and FGF-21 could be observed, which are both expressed in adipose tissue [114, 115]. In analogy to fetuin-A, a decrease of osteopontin under dosage with microencapsulated NA (60 mg) has been observed, suggesting a reduction of obesity

associated adipose tissue inflammation [116, 117]. The chronic low-grade inflammation in obese can be seen as a link between obesity and insulin resistance [118]. Hence, Kiefer and co-workers showed that an antibody-mediated neutralization of osteopontin decreased adipose-tissue inflammation and also significantly decreased insulin resistance in obese mice [116]. Nomiya et al. also concluded from their study in mice that osteopontin may play a role in linking obesity to the development of insulin resistance by promoting inflammation [117]. Changes in the measured biomarkers were only found under dosage with delayed-release NA. This specificity of the host physiological parameters to delayed-release NA but not delayed-release NAM or systemic NA suggests that the improvement in *Bacteroidetes* to be capable of driving improvements in human health and warrant testing in people with conditions such as obesity and T2D in future studies.

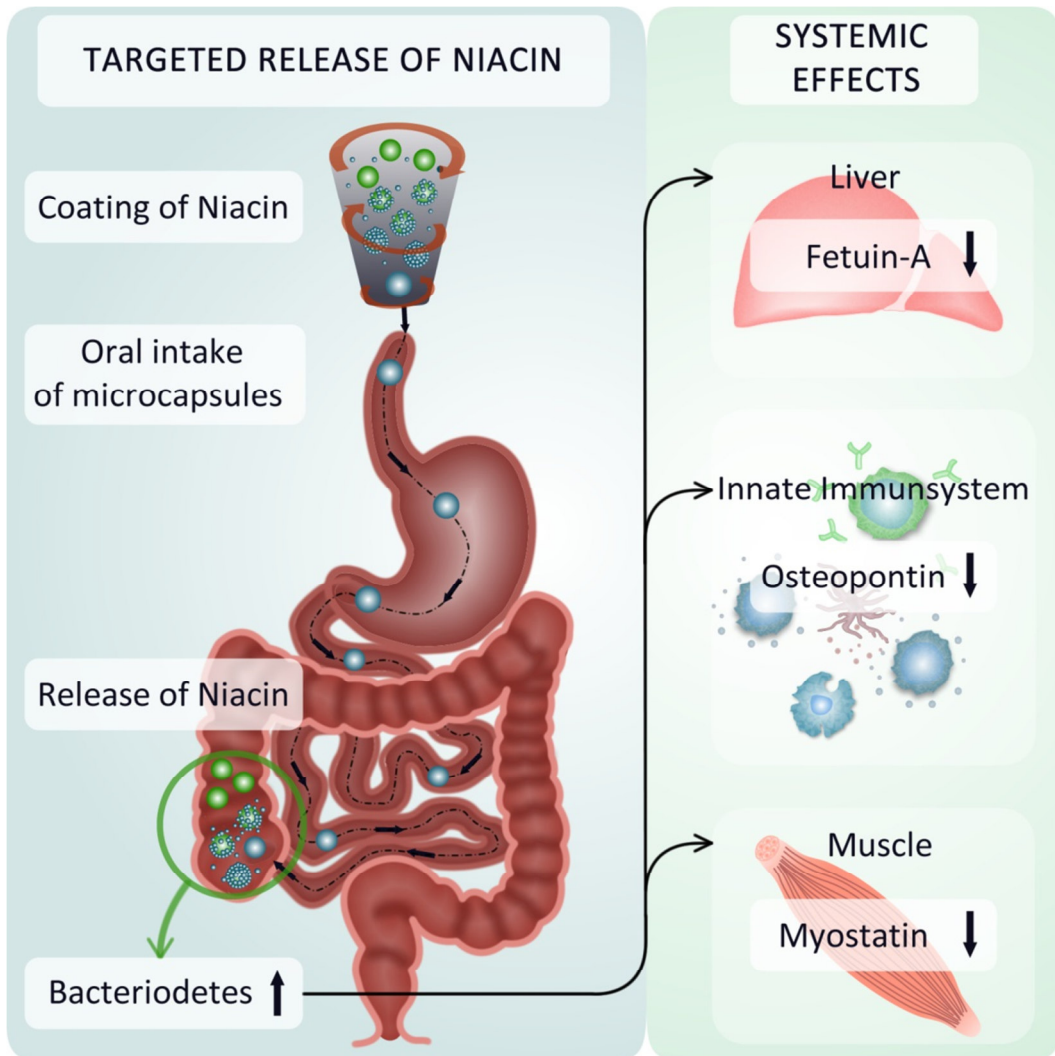
Several other studies showed an association of gut microbiota and insulin sensitivity [11, 119-121]. Caricilli and co-workers concluded in their review, that an increased proportion of *Firmicutes* and a decreased proportion of *Bacteroidetes* have been associated with increased LPS (lipopolysaccharide), leading to an activation of inflammatory pathways and thus to insulin resistance [42]. The hypothesis of metabolic endotoxemia promoting insulin resistance was underscored by other authors [122, 123]. Further the production of short-chain fatty acids (e.g. butyrate) by specific gut bacteria can improve gut epithelial barrier function [121, 124] and thus avoid translocation of endotoxic compounds derived from gut microbiota [125]. Moreno-Indias et al. observed an increase of *Firmicutes* abundance in obese with high insulin resistance compared to obese with low insulin resistance. In addition a loss of butyrate-producing bacteria has been reported in obese with high insulin resistance [120]. Still the mechanism of linkage between gut microbiota and host metabolic disorders and the issue of a “healthy” gut microbiome needs to be further explored in future.

It has to be mentioned that the effects on host biomarkers for insulin sensitivity could not be observed under delayed-release NA dosage in study week 4–6. Probably the effect might be saturable, especially in healthy subjects. Furthermore, sample size during this proof-of-concept and safety study within the pilot project was small. Nevertheless results show a beneficial alteration in gut microbial composition through delayed-release NA and also give a first hint on host metabolic effects mediated through these gut microbial changes and not primarily and directly since (I) the observed effects were specific for delayed-release NA (II) and the improvement of insulin sensitivity and metabolic inflammation was indicated by three independent parameters.

6 Conclusion and outlook

The present thesis presents evidence that NAD metabolism is altered in human obesity with reduced serum concentrations especially in subjects with T2D and that these alterations are correlated with reduced *Bacteroidetes* abundance found in obesity. Therefore novel gut targeted niacin formulations were developed using food-grade microencapsulation procedure. These novel delayed-release niacin formulations deliver NA and NAM into the ileo-colonic region while minimizing systemic resorption and induction of severe side effects. Preliminary safety and efficacy in producing an increase in *Bacteroidetes* abundance was shown in five healthy subjects in a manner that depend on targeted NA to the human gut with corresponding improvements in host biomarkers of insulin sensitivity and metabolic inflammation. To the best of our knowledge this is the first report showing a targeted microbiome intervention in humans using a micronutrient approach, thereby making possible mechanistic testing of the causative roles of dysbiosis in metabolic diseases and enabling clinical testing of microencapsulated NA for the prevention of obesity and/or T2D. Therefore, delayed-release NA formulations should be further evaluated in a higher sample size and obese and type 2 diabetic subjects to proof results of the pilot project and gain further insights in the potential of novel delayed-release NA in future obesity treatment and/or prevention.

Graphical abstract



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Supplemental Information

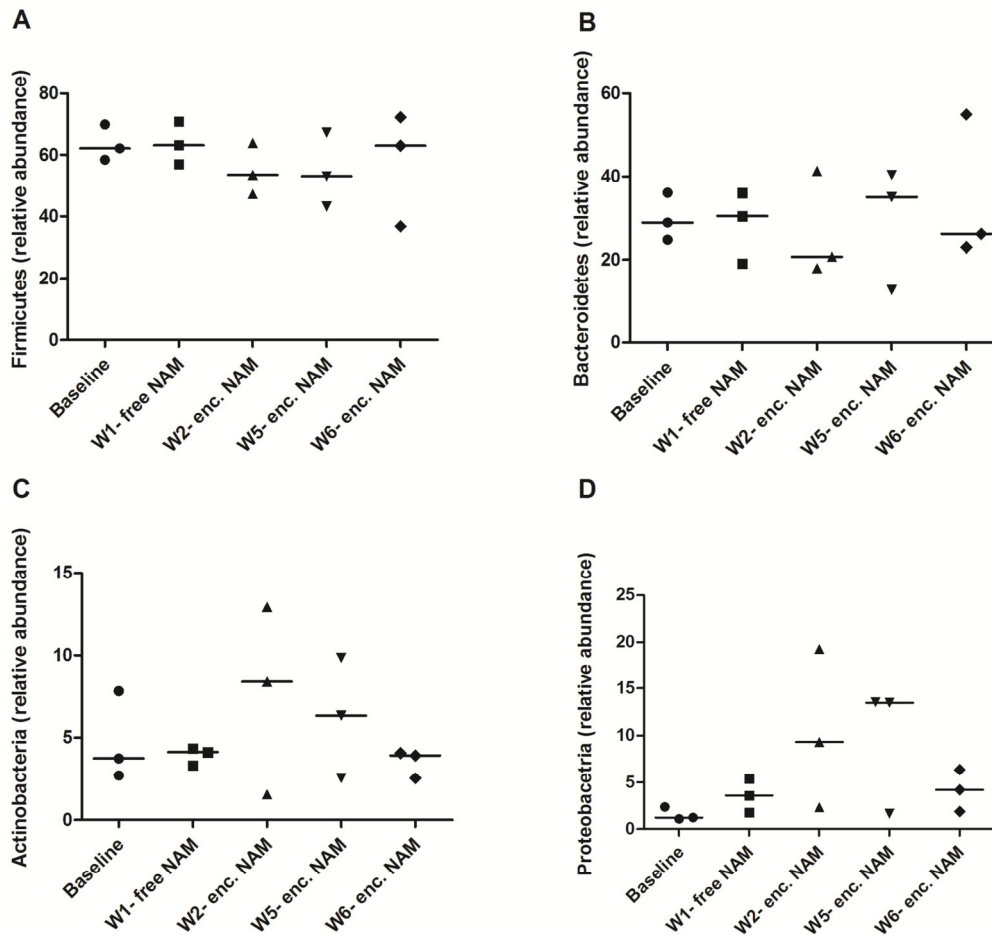


Figure S1: Relative abundance of the gut microbial phyla of NAM group in the course of proof-of-concept and safety study

Relative abundance of *Firmicutes* (A), *Bacteroidetes* (B), *Actinobacteria* (C) and *Proteobacteria* (D) of NAM group in the course of proof-of-concept and safety study showing no significant change. To determine significant changes in the microbial composition under niacin intervention over time repeated ANOVA and paired t-test were used. Statistical significance was set at $p < 0.05$ for ANOVA and at $p < 0.0125$ for paired t-test due to Bonferroni adjustment. One subject dropped out in the course of the study and thus values are not shown and values of one subject are missing. Abbreviations: NAM, nicotinamide; enc., encapsulated (microencapsulated NAM).

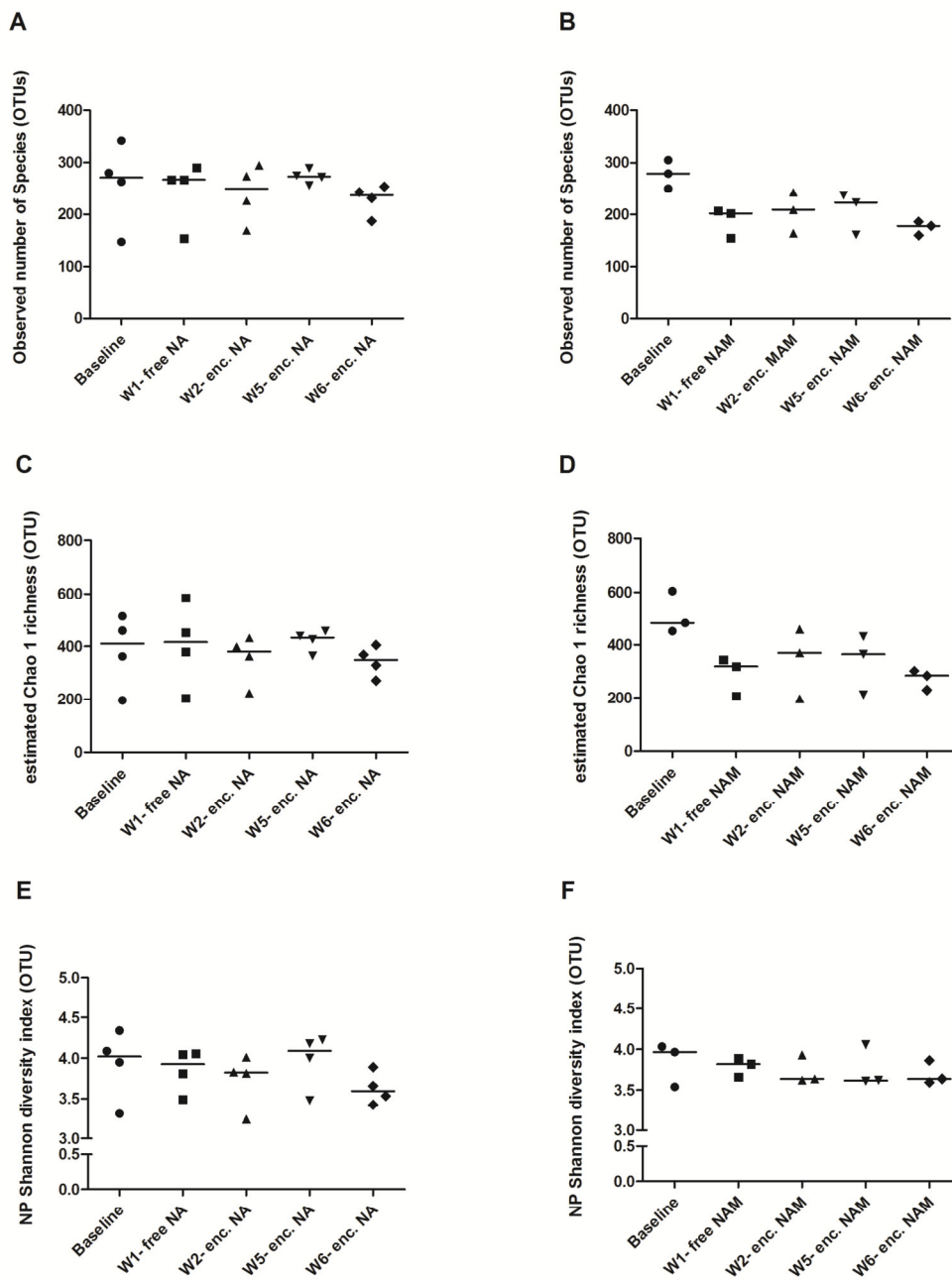


Figure S2: Alpha-diversity of the gut microbiota of NA and NAM group in the course of proof-of-concept and safety study

No significant change of alpha-diversity measures OTU_Number, Chao 1_OTU and Shannon_OTU under dosage with NA (A,C,E) or NAM (B,D,F) in the course of proof-of-concept and safety study. To determine significant changes in the microbial composition under niacin intervention over time repeated ANOVA and paired t-test were used. Statistical significance was set at $p < 0.05$ for ANOVA and at $p < 0.0125$ for paired t-test due to Bonferroni adjustment. Abbreviations: NA, nicotinic acid; NAM, nicotinamide; enc., encapsulated (microencapsulated NA/NAM).

Table S1: Biomarkers of human metabolism at the beginning and end of each week within NAM group of proof-of-concept and safety study

Tissue		Muscle	Liver	WAT	BAT	Innate Immunsystem
Target		Myostatin (ng/ml)	Fetuin-A (ng/ml)	Adiponectin (ng/ml)	FGF-21 (pg/ml)	Osteopontin (ng/ml)
900 mg free NAM	h0	1.86 ± 0.62	3.09 ± 2.09	0.73 ± 0.06	33.45 ± 20.24	1.84 ± 0.46
	h72	2.17 ± 0.47	3.73 ± 3.62	0.80 ± 0.09	41.66 ± 20.15	1.88 ± 0.52
900 mg enc. NAM	h0	1.85 ± 0.43	3.04 ± 1.56	0.75 ± 0.08	31.38 ± 22.11	1.91 ± 0.54
	h72	1.82 ± 0.27	4.06 ± 4.00	0.72 ± 0.05	32.77 ± 22.53	1.88 ± 0.55
1200 mg enc. NAM	h0	1.65 ± 0.35	3.15 ± 2.07	0.75 ± 0.05	31.18 ± 21.30	1.98 ± 0.55
	h72	1.73 ± 0.55	3.61 ± 2.85	0.74 ± 0.03	37.44 ± 25.23	1.78 ± 0.48
1500 mg enc. NAM	h0	1.78 ± 0.34	2.95 ± 1.65	0.77 ± 0.06	42.96 ± 24.00	2.03 ± 0.62
	h72	1.74 ± 0.41	4.23 ± 4.38	0.78 ± 0.12	31.59 ± 22.78	1.82 ± 0.34
2250 mg enc. NAM	h0	1.66 ± 0.41	3.19 ± 2.29	0.77 ± 0.05	37.35 ± 17.53	1.95 ± 0.32
	h72	1.63 ± 0.28	2.53 ± 0.96	0.80 ± 0.09	37.00 ± 24.81	1.82 ± 0.58
3000 mg enc. NAM	h0	1.58 ± 0.38	2.81 ± 1.59	0.76 ± 0.09	37.38 ± 22.00	1.82 ± 0.54
	h72	1.71 ± 0.74	3.24 ± 2.15	0.73 ± 0.07	38.26 ± 25.83	1.73 ± 0.42

^amean ± sd (all such values)

in bold: significant change of the target during the week (from h0 to h72) tested with Wilcoxon-test and significance level set at p<0.05. None of the microencapsulated NAM doses induced a significant change in the examined biomarkers. Significant effects are only mediated directly through dosage with free and systemic-available NAM.

Abbreviations: NAM, nicotinamide, enc.= encapsulated (microencapsulated NAM); h0, hour 0 (baseline); h72, hour 72 (after dosage); FGF-21= fibroblast growth factor 21, WAT= white adipose tissue, BAT= brown adipose tissue

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