The NAD salvage pathway during the progression of non-alcoholic fatty liver disease

Von der Fakultät für Biowissenschaften, Pharmazie und Psychologie

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Dissertation

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Non-alcoholic fatty liver disease (NAFLD) is a major chronic liver disease and thus a main reason for liver-related morbidities and mortality. NAFLD covers a wide range of diseases starting with steatosis and frequently progressing to non-alcoholic steatohepatitis (NASH), which is an independent predictor for the development of the hepatocellular carcinoma (HCC). Nicotinamide phosphoribosyltransferase (NAMPT), the key enzyme of the mammalian NAD salvage pathway, recycles nicotinamide to nicotinamide mononucleotide (NMN), which is further converted to nicotinamide adenine dinucleotide (NAD). NAD is not only an important redox partner but also a crucial co-substrate for NAD-dependent enzymes such as sirtuin 1 (SIRT1). Thus, NAD metabolism might be involved in the progression of NAFLD by regulating many cellular processes, such as apoptosis, *de novo* lipogenesis, glycolysis and gluconeogenesis, in the liver. Interestingly, tumor cells have a high NAD turnover due to their rapid proliferation and high activity of NAD-dependent enzymes. For these reasons, I hypothesized that the NAD salvage pathway is dysregulated during the progression of non-alcoholic fatty liver disease.

Therefore, the first study of the present work deals with the role of the NAD salvage pathway in a diet-induced mouse model of hepatic steatosis. In mice fed a high-fat diet for 11 weeks hepatic NAMPT mRNA, protein abundance and activity as well as NAD levels were increased. Additionally, SIRT1 protein abundance was upregulated indicating a higher SIRT1 activity. This could be confirmed by detecting decreased acetylation or transcription of SIRT1 targets. For example, p53 and nuclear factor κB (NF-κB) were less acetylated demonstrating lower activity of key regulators of apoptosis and inflammation, respectively.

In the second study of this thesis NAMPT activity was inhibited by applying its specific inhibitor FK866 in hepatocarcinoma cells to investigate whether or not NAMPT inhibition could be a potential novel therapeutic approach in HCC treatment. Hepatocarcinoma cells were more sensitive to NAMPT inhibition by FK866 than primary human hepatocytes, presenting a high number of apoptotic cells after FK866 treatment. FK866 induced NAD and ATP depletion which was associated with activation of the key regulator of energy metabolism 5'-AMP-activated protein kinase (AMPK) and decreased activity of its downstream target mammalian target of rapamycin (mTOR).

This thesis shows that the NAD salvage pathway is involved in hepatic steatosis and HCC. During hepatic steatosis NAD metabolism is upregulated to potentially protect against adverse effects of the massive hepatic lipid accumulation. To repress the progression to NASH it might be useful to maintain the hepatic NAD levels during early disease stages by administration of NAD precursors, such as NMN. However, hepatocarcinoma cells have a higher activity of NAMPT and NAD-dependent enzymes. NAMPT inhibition by FK866 could be a potential therapeutic approach in HCC, especially due to the fact that NAD depletion is selectively induced in hepatocarcinoma cells, but not in primary human hepatocytes.

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Abbreviations

2-OAADPR	2’-O-acetyl-ADP-riboside
4E-BP1	4E binding protein 1
ac	acetyl-group
ACC	acetyl-coenzyme A-carboxylase
ADP	adenosine diphosphate
AIRg	acute insulin response to glucose
AKT	protein kinase B
ALT	alanine transaminase
ATP	adenosine monophosphate
AMPK	5’-AMP-activated protein kinase
An	Annexin
AST	aspartate transaminase
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMAL1	aryl hydrocarbon receptor translocator-like protein 1
CAD	coronary artery disease
cADPR	cyclic ADP-ribose
CLOCK	circadian locomoter output cycles protein kaput
CPT	carnitine palmitoyltransferase
CRP	C-reactive protein
CVD	cardiovascular disease
CXCL16	chemokine (C-X-C motif) ligand 16
eNAMPT	extracellular NAMPT
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
FOXO1	forkhead box protein O1
FPG	fasting plasma glucose
FRD	fructose-rich diet
G6P	glucose-6-phosphatase
GSIS	glucose-stimulated insulin secretion
HCC	hepatocellular carcinoma
HFD	high-fat diet
HIF1α	hypoxia inducible factor 1α
HOMA	homeostatic model assessment
IL-6	Interleukin-6

iNAMPT	intracellular NAMPT
ITT	insulin tolerance test
MART	mono (ADP-ribosyl) transferase
MCP-I	monocyte chemoattractant protein 1
MnSOD	mitochondrial superoxide dismutase
mTOR	mammalian target of rapamycin
NA	nicotinic acid
NAAD	nicotinic acid adenine dinucleotide
NAD	nicotinamide adenine dinucleotide
NADS	nicotinamide adenine dinucleotide synthase
NAFLD	non-alcoholic fatty liver disease
NAM	nicotinamide
NAMN	nicotinic acid mononucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NAPRT	nicotinate phosphoribosyltransferase
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor κB
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide/nicotinic acid mononucleotide adenylyltransferase
NR	nicotinamide riboside
NRK	nicotinamide riboside kinase
NSPC	neuronal stem and/or progenitor cell
OGTT	oral glucose tolerance test
P38 MAPK	mitogen-activated protein kinase P38
p70S6K	70S ribosomal protein S6 kinase
PARP	poly(ADP-ribose) polymerase
PBEF	pre-b cell colony-enhancing factor
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1α
PHH	primary human hepatocytes
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PNPLA3	patatin-like phospholipase domain containing 3
PRPP	5′-phosphoribosyl-1-pyrophosphate
ROS	reactive oxygen species
SCDI	stearoyl-CoA-desaturase 1
SIRT	sirtuin

SREBP-1c	sterol regulatory element-binding protein 1c
STAT	signal transducer and activator of transcription
T1D	type 1 diabetes
T2D	type 2 diabetes
TAG	triacylglyceride
TDO	tryptophan 2,3-dioxygenase
TGFβ	transforming growth factor β
TNFα	tumour necrosis factor α
TSC	tuberous sclerosis complex
UCP2	mitochondrial uncoupling protein 2
VAT	visceral adipose tissue
WAT	white adipose tissue
WHR	waist–hip ratio
α-SMA	α-smooth muscle actin
γ-GT	γ-glutamyltransferase

Summary

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in western countries and therefore a major cause for liver-related morbidities and mortality e.g. by progression to the hepatocellular carcinoma (HCC). About 15-20 % of female and 30-40 % of male population are affected by NAFLD with an increasing number in patients with type 2 diabetes (T2D) and obesity. Thus, NAFLD is closely associated with the metabolic syndrome and related diseases like cardiovascular disorders, T2D and chronic kidney diseases. While the risk factors for NAFLD are well established (age >50 years, obesity, insulin resistance, T2D, elevated ferritin levels, *patatin-like phospholipase domain containing 3* (*PNPLA3*) *1148M* polymorphism), the pathological mechanisms are not well understood.

Hyperinsulinemia and hyperglycaemia, which occur with the development of insulin resistance, are involved in the initiation of NAFLD, the so called hepatic steatosis, which is defined by an increased accumulation of triacylglycerides (TAGs) in the liver. Several mechanisms underlie the development of steatosis. Insulin is no longer able to suppress the hormone-sensitive lipase in the insulin-resistant visceral adipose tissue (VAT) leading to an efflux of free fatty acids into the portal vein to the liver, a central organ in lipid metabolism. High serum insulin levels lead to an inhibition of hepatic β-oxidation and an increase of transcriptions factors for the *de novo* lipogenesis, for example sterol-regulatory binding protein 1c (SREBP-1c). In parallel, the excess blood glucose is absorbed by the liver and metabolized to acetyl-CoA which is important for the *de novo* lipogenesis. Lipolysis in VAT as well as decreased hepatic β-oxidation and increased *de novo* lipogenesis are major drivers in hepatic steatosis, which in 30-45 % of cases progresses to non-alcoholic steatohepatitis (NASH) due to inflammatory processes, increased apoptosis of hepatocytes and increased levels of reactive oxygen species (ROS). NASH can further progress to cirrhosis or HCC.

A large number of studies deal with the association of nicotinamide phosphoribosyltransferase (NAMPT), also called visfatin or pre-B cell colony-enhancing factor (PBEF), and NAFLD. Human NAMPT consists of 491 amino acids, has a theoretical molecular mass of 55.5 kDA and is highly expressed in leucocytes, liver, skeletal muscle and adipose tissue. NAMPT is the key enzyme in the mammalian nicotinamide adenine dinucleotide (NAD) salvage pathway starting from nicotinamide. There are several precursors of NAD such as vitamin B3 (nicotinic acid, nicotinamide riboside and nicotinic acid riboside) and tryptophan as the starting point for the *de novo* biosynthesis of NAD. NAMPT catalyzes the reaction of nicotinamide with 5'-phosphoribosyl-1-pyrophosphate (PRPP), generating nicotinamide mononucleotide (NMN) and releasing pyrophosphate (PP_i). Subsequently, this 5'mononucleotide is converted to NAD by nicotinamide mononucleotide-adenylyltransferases 1-3 (NMNAT1-3) and concomitant cleavage of ATP to ADP (Figure 1). In animal studies it has been shown that NAMPT activation protects against steatosis, inflammation and

glucose intolerance. The review of *Chapter 1* summarises, among other things, of how NAMPT and NAFLD are associated. As a key enzyme of NAD biosynthesis, NAMPT can act through NAD-dependent biochemical reactions. NAD serves as a co-substrate for poly(ADP-ribose) polymerases (PARP), cyclic (ADP-ribose) hydrolases such as CD38 and class-III-NAD-dependent deacetylases (sirtuins (SIRT)) or as a redox partner in a wide range of metabolic pathways, for example glycolysis.

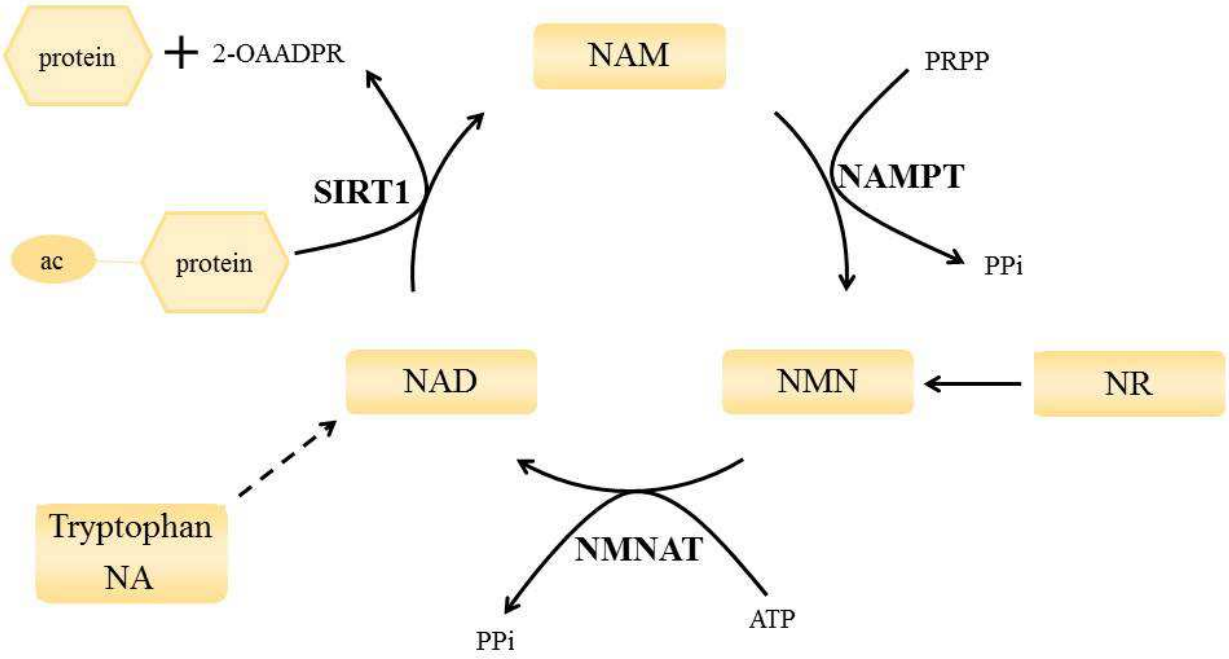

Fig. 1 Mammalian NAD salvage pathway.
Abbreviations: 2-OAADPR: 2'-O-acetyl-ADP-riboside; ac: acetyl-group; ATP: adenosine triphosphate; NA: nicotinic acid; NAM: nicotinamide; NAD: nicotinamide adenine dinucleotide; NAMPT: nicotinamide phosphoribosyltransferase; NMN: nicotinamide mononucleotide; NMNAT: nicotinamide mononucleotide adenylyltransferase; NR: nicotinamide riboside; PPi: pyrophosphate; PRPP: 5'-phosphoribosyl-1-pyrophosphate; SIRT1: sirtuin 1

Up to 70 % of the total cellular NAD pool is located in the mitochondria, the place of metabolic processes reducing NAD to NADH such as tricarboxylic acid cycle or β-oxidation. NADH is necessary in the mitochondria as an electron donor for oxidative phosphorylation producing ATP. As mentioned above, NAD is also an important co-substrate for protein modifications (e.g. deacetylation), which influences the activity, stability and localisation of proteins. Sirtuins (SIRT) are NAD-dependent deacetylases, which are located in the mitochondria (SIRT3, 4, 5), in the nucleus (SIRT1, 6, 7) and in the cytoplasm (SIRT2). For example, SIRT1 can deacetylate and thus, suppress the transcriptional activity of SREBP-1c. NAD is cleaved to ADP-ribose and nicotinamide, which can be recycled by NAMPT. SIRT1 transfers the acetyl-group from SREBP-1c to the 2'-OH group of ADP-ribose producing 2'-O-acetyl-ADP-riboside (2-OADDPR) (Figure 1). Constant NAD salvage is absolutely necessary especially for tumor cells due to their special characteristics, such as rapid cell growth and proliferation as well as a dysregulated cellular energy metabolism. Thus, tumor cells have an increased NAD turnover. A high rate of NAD consuming enzymatic reactions would lead to an

accumulation of nicotinamide, a natural feedback inhibitor of NAD-dependent enzymes. This makes a higher NAMPT activity essential for NAD replenishment and recycling of nicotinamide in cancer cells. It has already been shown that different malignant tumor cells have increased NAD levels and overexpress NAMPT. Increased NAMPT levels correlate with tumor growth, metastases, cellular dedifferentiation and a poor prognosis.

By regulating intracellular NAD levels NAMPT is essential for many cellular processes. On the one hand, the NAD/NADH ratio represents the energy status of the cell; on the other hand NAD regulates NAD-dependent enzymes linking the metabolic status to signalling processes. Consequently, the NAD salvage pathway is involved in many processes associated with the progression of NAFLD. Therefore, I hypothesized that the NAD salvage pathway is dysregulated during the progression of non-alcoholic fatty liver disease. My hypothesis, aims and scientific questions are described in detail in *Chapter 2*. In *Chapter 3*, the role of NAD metabolism in a murine model of diet-induced hepatic steatosis was investigated. In *Chapter 4* it was examined whether or not NAMPT inhibition could be a potential therapeutic approach for HCC.

The role of the NAD salvage pathway in NAFLD was analysed in the animal study described in *Chapter 3*. Mice were fed with a high-fat diet (HFD) for 11 weeks. These mice gained weight due to an increase of fat body mass, showed an impaired oral glucose tolerance test and stored more TAGs in the liver without any indications for inflammation (*interleukin*-6 (*IL-6), tumor necrosis factor α (TNFα)*), macrophage infiltration (C*D68*), cirrhosis (*α-smooth muscle actin (α-SMA)*) and fibrosis (*fibrinogen, collagen I*) compared to control mice. Therefore, the HFD-fed mice were considered a model for hepatic steatosis, the initial stage of NAFLD. Surprisingly, NAMPT mRNA, protein abundance and activity as well as intracellular NAD levels were increased in the HFD-fed mice. In the literature it has been shown that NAD-dependent SIRTs contribute to the progression of NAFLD by regulating processes like hepatic gluconeogenesis, mitochondrial biogenesis and fatty acid synthesis. Liver-specific SIRT1 and whole-body SIRT3 knockout mice showed exacerbated hepatic steatosis and insulin resistance under a HFD while global transgenic SIRT1 overexpressing mice were protected against the negative impact of a HFD. In the present work, hepatic SIRT3 was unchanged and only SIRT1 protein levels were enhanced after 11 weeks on a HFD compared to the control group. Deacetylation and consequently inhibition of nuclear factor κB (NF-κB) at lysine 310 and p53 at lysine 382 indicated a higher activity of SIRT1 and a down regulation of the activity of two key enzymes of hepatic inflammation and apoptosis, respectively. The increased activity of SIRT1 was also represented by a decreased mRNA expression of its transcriptional target *mitochondrial uncoupling protein 2* (*UCP2*) indicating that oxidative stress was not present in the liver samples of HFD-fed mice. Transcriptional activity of SRREBP-1c as a key regulator of the *de novo* lipogenesis can be also inhibited due to deacetylation by SIRT1. In this study, *SREBP-1c* mRNA expression was increased, but its acetylation status was unchanged in the HFD- and control group. However, its downstream targets *fatty acid synthase* (*FAS*), *acetyl-CoA carboxylase* (*ACC*) and *stearoyl-CoA*

desaturase-1 (*SCD1*) were downregulated indicating a lower transcriptional activity of SREBP-1c. The increased SIRT1 activity could cause a decreased activity of the SIRT1 target forkhead box protein O1 (FOXO1). This is supported by measuring a reduced expression of its downstream targets *glucose-6-phosphatase* (*G6Pc*) and *phosphoenolpyruvate carboxykinase* (*PEPCK*), key regulators in gluconeogenesis. Taken together, it has been shown in *Chapter 3* that the hepatic NAD salvage pathway is improved in mice with hepatic steatosis which could be a compensatory mechanism to protect against the negative impact of hepatic lipid accumulation.

NASH is an independent predictor of HCC, which is the sixth most common cancer and the second cause of cancer-related mortality worldwide. Hepatocarcinoma cells have a higher activity of NAMPT and NAD-dependent enzymes. Thus, hepatoccarcinoma cells are expected to be more sensitive to NAD depletion. Since sorafenib is to date the only potential medication in HCC, the effect of the specific NAMPT inhibitor FK866 on hepatocarcinoma cells compared to non-cancerous hepatocytes was investigated in *Chapter 4***.** FK866 decreased intracellular NAMPT activity and NAD levels in hepatocarcinoma cells. These cellular responses were not present in non-cancerous hepatocytes even after incubation with a 10-fold enhanced dose of FK866. In hepatocarcinoma cells, ATP levels were decreased after stimulation with FK866 which was associated with an induction of apoptosis. FK866-induced reduction of NAD and ATP indicates that NAMPT is involved in cellular energy metabolism. In further experiments the effect of NAMPT inhibition on the key regulator of energy metabolism, 5'-AMP-activated protein kinase (AMPK) and its downstream target mammalian target of rapamycin (mTOR), was investigated. In the literature it has been shown that AMPK is less active in HCC with a consequently upregulated activity of mTOR. This fact could be confirmed in the present study. Furthermore, inhibition of NAMPT by FK866 led to a higher phosphorylation and activation of AMPK with simultaneous dephosphorylation and deactivation of mTOR. This could be further confirmed due to decreased phosphorylation and inhibition of mTOR downstream targets 70S ribosomal protein S6 kinase (p70S6K) and 4E binding protein 1 (4E-BP1), key regulators of protein translation and cellular growth, respectively. To investigate whether or not the effects on NAD and ATP levels as well as on the AMPK/mTOR signalling pathway were NAMPT-dependent, hepatocarcinoma cells were co-stimulated with FK866 and NMN. This led to a normalisation of the cellular responses to control levels. These results suggest FK866 as a potential new therapy option for HCC by reducing NAMPT activity and NAD levels and thus disturbing energy homeostasis in hepatocarcinoma cells while not affecting non-cancerous cells.

To sum up, this thesis demonstrates that the hepatic NAD salvage pathway is involved in NAFLD and HCC. It has been shown that in the phase of hepatic steatosis an enhanced NAD salvage pathway counteracts the negative impact of hepatic lipid accumulation. It could be assumed that during further disease progression the NAD salvage pathway is down regulated leading to a lower SIRT1 activity. This supports further pathogenic pathways like hepatic apoptosis, inflammation or increased ROS levels. In HCC, NAD turnover is massively increased to support tumor growth. Hence

inhibition of the NAD salvage pathway by FK866 induced apoptosis due to decreased NAD and ATP levels as well as downregulation of the AMPK/mTOR pathway (Figure 2). This works suggests the importance of the time point at which the NAD salvage pathway is enhanced or down regulated during disease progression. In early stages of hepatic steatosis it could be important to maintain NAD metabolism to delay disease progression e.g. by the administration of NAD precursors, such as NMN. Inhibition of NAMPT activity by FK866 could be useful to suppress tumor growth in HCC. Therefore, the NAD salvage pathway represents a novel potential target for both, hepatic steatosis and HCC (Figure 2).

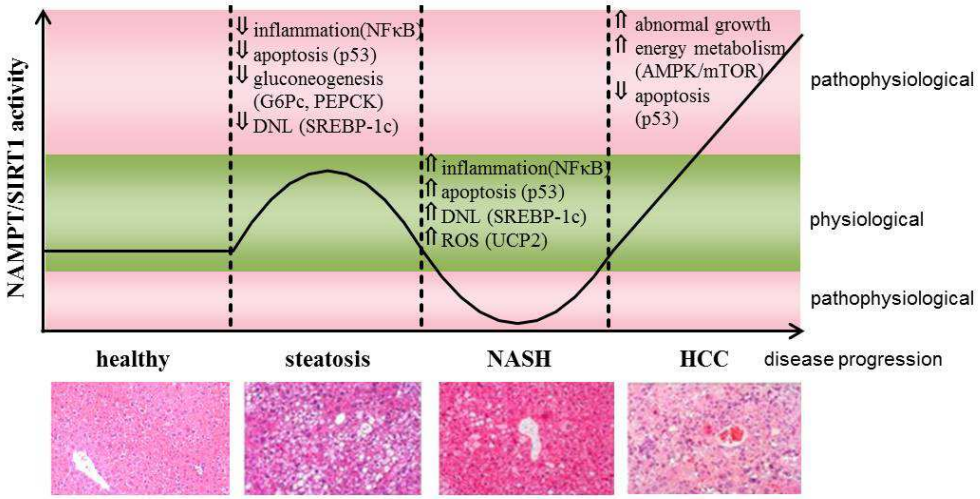

Fig. 2 Hypothetic scheme of alterations in the NAD salvage pathway during disease progression of non-alcoholic fatty liver disease.

Abbreviations: AMPK: 5'-AMP-activated protein kinase; DNL: *de novo* lipogenesis; G6Pc: glucose-6-phosphatase; HCC hepatocellular carcinoma; mTOR: mammalian target of rapamycin; NAD: nicotinamide adenine dinucleotide; NAMPT: nicotinamide phosphoribosyltransferase; NASH: non-alcoholic steatohepatitis; NF-κB: nuclear factor κB; PEPCK: phosphoenolpyruvate carboxykinase; SREBP-1c: sterol regulatory binding protein-1c; UCP2: mitochondrial uncoupling protein 2

Zusammenfassung

In den westlichen Industriestaaten gehört die nicht-alkoholbedingte Fettlebererkrankung (NAFLD) zu einer der häufigsten hepatischen Erkrankungen. Da diese zur Progression des hepatozellulären Karzinoms (HCC) beiträgt, ist die NAFLD eine Hauptursache für erhöhte leberbedingte Morbiditäten und Mortalität. Etwa 15-20 % der weiblichen und 30-40 % der männlichen Bevölkerung sind gefährdet an der NAFLD zu erkranken. Dabei kommt es zu einer erhöhten Inzidenz bei Patienten mit Typ 2 Diabetes (T2D) und Adipositas. NAFLD ist stark assoziiert mit dem metabolischen Syndrom und Komorbiditäten, wie z.B. kardiovaskulären Erkrankungen, T2D und chronischen Nierenerkrankungen. Während die Risikofaktoren weitgehend bekannt sind (Alter >50 Jahre, Adipositas, Insulinresistenz, T2D, erhöhte Ferritin-Spiegel, *patatin-like phospholipase domain containing 3* (*PNPLA3*) *1148M* Polymorphismus), ist bis heute nicht vollständig geklärt, welche pathologischen Mechanismen an der Progression der NAFLD beteiligt sind.

Hyperinsulinämie und Hyperglykämie, die einhergehen mit Insulinresistenz, führen zu Beginn der NAFLD zu einer verstärkten Einlagerung von Triacylglyceriden (TAGs) in der Leber, der sogenannten hepatischen Steatose. Dadurch kann Insulin die Lipasen des insulinresistenten Fettgewebes nicht mehr hemmen. Folglich kommt es zu einer verstärkten Freisetzung von freien Fettsäuren in die Portalvene, die zur Leber führt, dem zentralen Organ im Lipidmetabolismus. Hohe Insulinspiegel führen zu einer Inhibierung der hepatischen β-Oxidation und Aktivierung von Transkriptionsfaktoren der *de novo* Lipogenese, wie z.B. dem *sterol regulatory element-binding protein 1c* (SREBP-1c). Parallel dazu wird die überschüssige Blutglukose von der Leber aufgenommen und zu acetyl-CoA abgebaut, das essentiell für die *de novo* Lipogenese ist. Lipolyse im Fettgewebe sowie verringerte β-Oxidation und erhöhte *de novo* Lipogenese in der Leber sind entscheidende Prozesse in der Entstehung der hepatischen Steatose. In 30-45 % der Fälle entwickelt sich die Steatose durch inflammatorische Prozesse, verstärkter Apoptose der Hepatozyten und einem Anstieg der reaktiven Sauerstoffspezies (ROS) zur nicht-alkoholbedingten Steatohepatitis (NASH). Diese fördert die Entstehung der Zirrhose oder des HCCs.

In den letzten Jahren hat sich eine Vielzahl an Studien mit der Assoziation von Nikotinamidphosphoribosyltransferase (NAMPT), auch Visfatin oder *pre-B cell colony-enhancing factor* (PBEF) genannt, und NAFLD beschäftigt. Humanes NAMPT besteht aus 491 Aminosäuren und ist 55,5 kDA groß. Es kommt hauptsächlich in der Leber, in den Leukozyten, im Skelettmuskel und Fettgewebe vor. NAMPT ist das Schlüsselenzym der Nikotinamidadenindinukleotid (NAD)-Biosynthese aus Nikotinamid in Säugetieren, dem sogenannte *NAD salvage pathway*. Weitere Vorstufen von NAD sind u.a. Vitamin B3 (Nikotinsäure, Nikotinamidribosid, Nikotinsäureribosid) und Tryptophan, welches für die *de novo* Biosynthese von NAD benötigt wird. NAMPT katalysiert die Reaktion zwischen Nikotinamid und 5‘-Phosphoribosyl-1-Pyrophosphat (PRPP) zu

Nikotinamidmononukleotid (NMN) unter Freisetzung von Pyrophosphat (PPi). Die Umsetzung des 5'-Mononukleotids zu NAD wird unter Spaltung von ATP durch die Nikotinamidmononukleotidadenylyltransferasen 1-3 (NMNAT1-3) katalysiert (Abbildung 1). In Tierstudien wurde bereits gezeigt, dass eine Aktivierung von NAMPT gegen hepatische Steatose, entzündliche Prozesse und Glukoseintoleranz schützt. In dem Review von *Kapitel 1* wird u.a. ein detaillierter Überblick darüber gegeben, wie NAMPT und NAFLD assoziiert sind. Als Schlüsselenzym der NAD-Biosynthese kann NAMPT durch viele NAD-abhängige Stoffwechselwege auf den zellulären Metabolismus Einfluss nehmen. NAD ist sowohl ein wichtiges Co-Substrat für Poly-(ADP-Ribose)-Polymerasen (PARPs), zyklische ADP-Ribose-Hydrolasen, z.B. CD38, und Klasse III-NAD-abhängige Deacetylasen (Sirtuine (SIRT)), als auch ein wichtiger Redoxpartner in vielen Stoffwechselwegen, wie der Glykolyse.

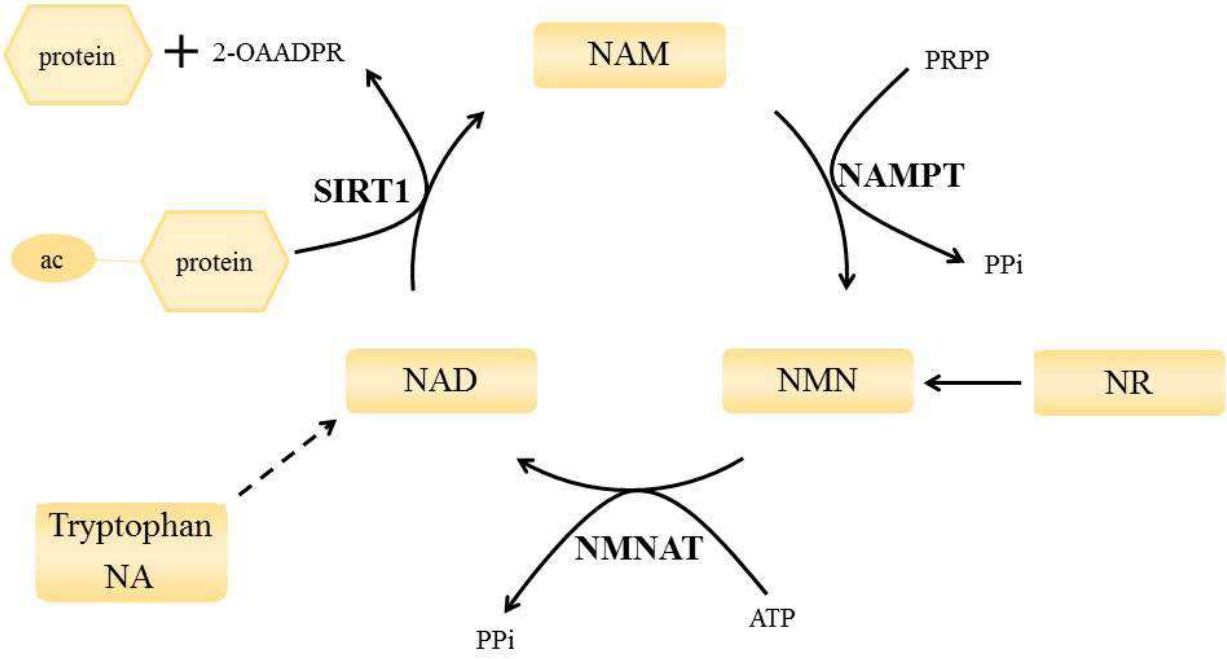

Abb 1. Der *NAD salvage pathway* in Säugetieren.
Abkürzungen: 2-OAADPR: 2'-O-Acetyl-ADP-Ribosid; ac: acetyl-Gruppe; ATP: Adenosintriphosphat; NA: Nikotinsäure; NAM: Nikotinamid; NAD: Nikotinamidadenindinukleotid; NAMPT: Nikotinamid-phosphoribosyltransferase; NMN: Nikotinamidmononukleotid; NMNAT: Nikotinamidmononukleotid-adenylyltransferase; NR: Nikotinamidribosid; PPi: Pyrophosphat; PRPP: 5'-Phosphoribosyl-1-Pyrophosphat; SIRT1: Sirtuin 1

Etwa 70 % des intrazellulären NAD-Reservoirs sind in den Mitochondrien lokalisiert, welche ein zentraler Ort vieler metabolischer Prozesse sind. Während des Citratzyklus oder der β-Oxidation wird NAD zu NADH reduziert, das für die Herstellung von ATP durch oxidative Phosphorylierung benötigt wird. Wie bereits oben erwähnt, ist NAD auch ein wichtiges Co-Substrat für Proteinmodifikationen, z.B. Deacetylierung, die die Aktivität, Stabilität und Lokalisation von Enzymen beeinflussen können. Sirtuine sind NAD-abhängige Deacetylasen, die im Mitochondrium (SIRT3, 4, 5), Zellkern (SIRT1, 6, 7) und im Zytosol (SIRT2) lokalisiert sind. SIRT1 kann z.B. die transkriptionelle Aktivität von SREBP-1c durch Deacetylierung inhibieren. NAD wird dabei durch NAD in ADP-Ribose und Nikotinamid gespalten. Letzteres kann von NAMPT recycelt werden. Die Acetylgruppe von SREBP-1c wird durch SIRT1 auf die 2'-OH-Gruppe der ADP-Ribose übertragen, wobei 2'-O-Acetyl-ADP-Ribosid (2-OAADPR) entsteht (Abbildung 1). Das Regenerieren von NAD ist besonders in Tumorzellen essentiell, um deren kanzerogene Eigenschaften, z.B. ein rapides Zellwachstum, –proliferation und einen dysregulierteren Energiemetabolismus, aufrechtzuerhalten. Daher besitzen Tumorzellen einen deutlich erhöhten NAD-Verbrauch im Vergleich zu gesunden Zellen. Eine verstärkte Aktivität von NAD-abhängigen Enzymen würde zu einer Akkumulation von Nikotinamid führen, einem natürlichen *feedback* Inhibitor von NAD-abhängigen Enzymen. Deswegen ist es für Tumorzellen essentiell, dass NAMPT verstärkt aktiv ist, um Nikotinamid zu recyceln und so das NAD-Reservoir der Zelle wieder aufzufüllen. In malignen Tumoren wurde bereits gezeigt, dass erhöhte NAMPT-Spiegel mit dem Tumorwachstum, der Metastasierung, der zellulären Entdifferenzierung und einer schlechteren Prognose korrelieren.

NAMPT ist für die Zelle durch die Regulation des intrazellulären NAD-Spiegels und somit vieler zellulärer Stoffwechselwege von großer Bedeutung. Einerseits wird durch das NAD/NADH-Verhältnis der Energiestatus der Zelle widergespiegelt, andererseits wird die Aktivität von NAD-abhängigen Enzymen gesteuert. Es lässt sich schlussfolgern, dass der NAD-Metabolismus eine wichtige Rolle in der Pathogenese der NAFLD spielt. Dies führt mich zu der Hypothese meiner Doktorarbeit, dass der *NAD salvage pathway* in der Progression der nicht-alkoholbedingten Fettlebererkrankung dysreguliert ist. Die Hypothese, Ziele und Fragestellungen dieser Arbeit werden detailliert in *Kapitel 2* beschrieben. In *Kapitel 3* wurde der NAD-Metabolismus in einem Mausmodell für Diät-induzierte hepatische Steatose untersucht. In *Kapitel 4* wurde geprüft, ob eine NAMPT-Inhibierung in Hepatokarzinomzellen einen potentiellen neuen Ansatz in der Therapie des HCC darstellen könnte.

In der Studie in *Kapitel 3* wurden Mäuse für 11 Wochen mit einer Hochfett-Diät gefüttert, um die Rolle des NAD-Metabolismus in der NAFLD zu untersuchen. Die Mäuse nahmen an Gewicht zu, vor allem durch einen Anstieg der Fettkörpermasse, zeigten einen verschlechterten Glukosetoleranztest und lagerten im Vergleich zur Kontrollgruppe verstärkt TAGs in der Leber ein. Dabei konnte man keine Veränderungen in der Expression von Markergenen der Inflammation (*Interleukin-6* (*IL-6*), *Tumornekrosefaktor α* (*TNFα*)), Makrophageninfiltration (*CD68*), und Fibrose

(*α-smooth muscle actin* (*α-SMA*), Fibrinogen, Kollagen I) erkennen. Interessanterweise kam es zu einem Anstieg der NAMPT mRNA-Expression, Proteinsynthese und Aktivität sowie zu erhöhten intrazellulären NAD-Spiegeln in den steatotischen Mauslebern. In der Literatur wurde gezeigt, dass es unter Fütterung einer Hochfett-Diät vor allem in leber-spezifischen SIRT1- und in Ganzkörper-SIRT3-knockout-Mäusen zu einer Verstärkung der hepatischen Steatose und Insulinresistenz kommt. Eine SIRT1-Überexpression unter Fütterung einer Hochfett-Diät führte dagegen zu einer Verbesserung der hepatischen Steatose durch die Regulation der hepatischen Glukoneogenese, mitochondrialer Biogenese oder der Fettsäurebiosynthese. In der hier vorliegenden Studie war die hepatische SIRT3-Proteinsynthese unverändert, während das SIRT1-Protein nach einer 11-wöchigen Hochfett-Diät anstieg. Zudem kam es zu einer Deacetylierung von *nuclear factor κB* (NF-κB) am Lysin 310 und vom Tumorsuppressorprotein p53 am Lysin 382 und damit zur Inhibierung der Aktivität von zwei Schlüsselenzymen der Inflammation und Apoptose. Der Nachweis der verringerten Acetylierung dieser Proteine deutet auf eine verstärkte SIRT1-Aktivität hin. Dies wurde auch durch die verringerte mRNA-Expression eines Zielgens von SIRT1, des *mitochondrial uncoupling protein 2* (*UCP2*) gezeigt. Zudem ist eine verringerte *UCP2* mRNA-Expression ein Hinweis darauf, dass zum untersuchten Zeitpunkt kein oxidativer Stress in den Zellen vorlag. Die *SREBBP-1c* mRNA-Expression war in den steatotischen Lebern stark erhöht, während dessen Zielgene *Fettsäuresynthase* (*FAS*), *Acetyl-CoA-Carboxylase* (*ACC*) und *Stearoyl-CoA-Desaturase-1* (*SCD1*) weniger stark exprimiert waren. Daraus könnte man schlussfolgern, dass SREBP-1c aufgrund einer verstärkten Deacetylierung durch SIRT1 weniger transkriptionell aktiv ist. Dies konnte in der vorliegenden Studie jedoch nicht bestätigt werden. Allerdings ist die Genexpression der Schlüsselenzyme der Glukoneogenese, *Glukose-6-Phosphatase* (*G6Pc*) und *Phosphoenolpyruvat-Carboxykinase* (*PEPCK*) verringert, was ebenfalls auf eine verstärkte SIRT1-Aktivität hindeutet. SIRT1 kann das *forkhead box protein O1* (FOXO1) deacetylieren, welches gleichzeitig phosphoryliert wird und somit die Expression der *G6Pc* und *PEPCK* inhibiert. Zusammenfassend kann man sagen, dass in den steatotischen Mauslebern der *NAD salvage pathway* verbessert war. Dies könnte ein zeitiger kompensatorischer Mechanismus der Leber gegen die negativen Auswirkungen massiver hepatischer Fetteinlagerungen sein.

NASH ist ein unabhängiger Prädiktor des HCCs, welches die sechsthäufigste Krebsart und zweithäufigste tumorbedingte Todesursache ist. In der Literatur wurde bereits gezeigt, dass in Hepatokarzinomazellen NAD-abhängige Enzyme und die NAMPT-Aktivität stark erhöht sind. Tumorzellen könnten deswegen sensibler auf eine Inhibierung von NAMPT reagieren. Sorafenib ist aktuell das einzig verfügbare Medikament gegen HCC. Deswegen wird in *Kapitel 4* untersucht, welche Effekte der spezifische NAMPT-Inhibitor FK866 auf Hepatokarzinomzellen im Vergleich zu gesunden Hepatozyten hat. In der hier vorliegenden Studie reduzierte FK866 die intrazelluläre NAMPT-Aktivität und NAD-Spiegel in Hepatokarzinomzellen. In gesunden Hepatozyten kam es auch mit einer 10-fach erhöhten Dosis an FK866 nicht zu einer Verringerung der NAMPT-Aktivität und der

NAD-Spiegel. Zudem führte FK866 in Hepatokarzinomzellen zu einem verminderten ATP-Spiegel, der mit einer steigenden Anzahl an apoptotischen Zellen assoziiert war. Da FK866 die intrazelluläre NAD- und ATP-Spiegel reduzierte, konnte schlussgefolgert werden, dass eine Inhibierung von NAMPT den zellulären Energiemetabolismus entscheidend stört. In den folgenden Experimenten wurde deswegen der Fokus auf 5'-AMP-aktivierte Proteinkinase (AMPK), ein zentrales Enzym des Energiemetabolismus, und dessen Zielprotein *mammalian target of rapamycin* (mTor) gelegt. In der Literatur wurde bereits beschrieben, dass AMPK weniger und mTOR verstärkt aktiv im HCC-Gewebe ist. Das wurde in der hier vorliegenden Studie bestätigt. Zudem führte eine Inhibierung von NAMPT in Hepatokarzinomzellen zu einer verstärkten Phosphorylierung von AMPK, sowie Dephosphorylierung und damit Inhibierung von mTOR. Die Zielproteine von mTOR 70S ribosomales Protein S6-Kinase (p70S6K) und 4E-Bindeprotein 1 (4E-BP1), die eine entscheidende Aufgabe sowohl in der Proteintranslation wie auch im zellulären Wachstum haben, waren ebenfalls weniger phosphoryliert. Um sicher zu stellen, dass die zellulären Effekte von FK866 spezifisch auf einer NAMPT-Inhibierung beruhen, wurden die Hepatokarzinomzellen mit dessen Enzymprodukt NMN co-stimuliert. NMN normalisierte alle FK866-induzierten Effekte. Diese Studie zeigt, dass FK866 eine potentielle neue Therapiemöglichkeit des HCC sein könnte, da die FK866-vermittelten Effekte spezifisch in Hepatokarzinomzellen auftraten, jedoch nicht in gesunden humanen Hepatozyten.

Die hier vorliegende Arbeit zeigt, dass der NAD-Metabolismus in der hepatischen Steatose und des HCC involviert ist. Im frühen Stadium der hepatischen Steatose kann ein verbesserter *NAD salvage pathway* gegen den negativen Einfluss hepatischer Lipidakkumulation schützen. Während der weiteren Progression der hepatischen Steatose zur NASH wird eventuell der *NAD salvage pathway* und damit auch SIRT1 gehemmt. Dadurch werden u.a. inflammatorische und apoptotische Prozesse in der Leber weiter gefördert. Im HCC dagegen wird für das Tumorwachstum massiv NAD benötigt. Deswegen stellt eine Hemmung von NAMPT mit FK866 eine potentielle Therapieoption dar, da selektiv in Hepatokarzinomzellen Apoptose induziert wird (Abbildung 2). Diese Arbeit unterstützt die Theorie, dass es wichtig ist, zu welchem Zeitpunkt in der Progression der NAFLD der NAD-Metabolismus verändert ist. Zu Beginn der NAFLD kann es durchaus förderlich sein, den NAD-Metabolismus durch Gabe von NAD-Vorstufen, z.B. NMN, aufrecht zu erhalten. Dadurch könnte möglicherweise die Progression der hepatischen Steatose zu einer NASH entgegengewirkt werden. Bei HCC-Patienten dagegen könnte eine Therapie mit FK866 dazu führen, dass der zelluläre NAD-Spiegel gesenkt wird und somit nicht ausreichend Energie für das Wachstum und die Proliferation der Krebszellen zur Verfügung steht. Damit stellt der *NAD salvage pathway* einen neuen potentiellen Ansatzpunkt in der Therapie von hepatischer Steatose und HCC dar (Abbildung 2).

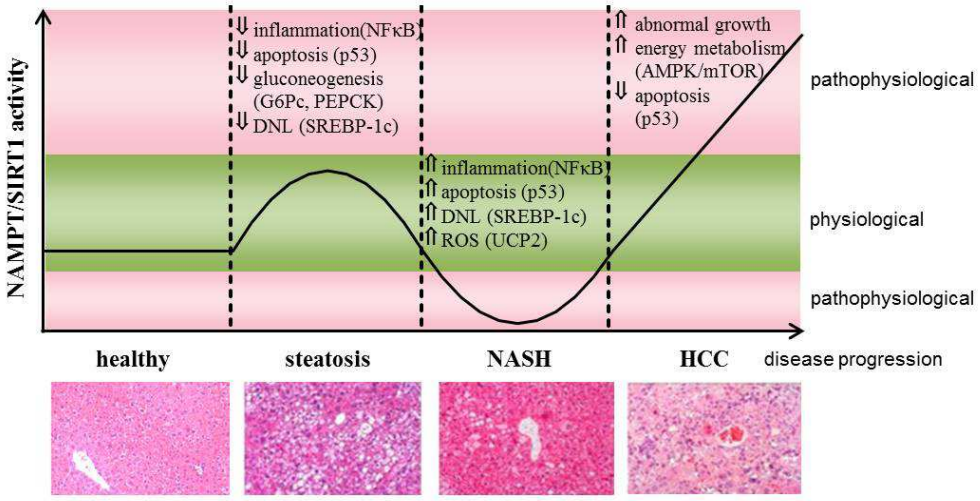

Abb. 2 Hypothetische Veränderungen des *NAD salvage pathway* während der Progression der nicht-alkoholbedingten Fettlebererkrankung.
Abkürzungen: AMPK: 5'-AMP-aktivierte Proteinkinase; DNL: *de novo* Lipogenese; G6Pc: Glukose-6-Phosphatase; HCC hepatozelluläres Karzinom; mTOR: *mammalian target of rapamycin*; NAD: Nikotinamidadenindinukleotid; NAMPT: Nikotinamidphosphoribosyltransferase; NASH: Nicht-alkoholbedingte Steatohepatitis; NF-κB: *nuclear factor κB*; PEPCK: Phosphoenolpyruvat-Carboxylase; SREBP-1c: *sterol regulatory binding protein 1*; UCP2: *mitochondrial uncoupling protein 2*

Chapter 1

Physiological and pathophysiological roles of NAMPT and NAD metabolism

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1.1 Abstract

Nicotinamide phosphoribosyltransferase (NAMPT) is a regulator of the intracellular nicotinamide adenine dinucleotide (NAD) pool. NAD is an essential coenzyme involved in cellular redox reactions and a substrate for NAD-dependent enzymes. NAD concentrations are decreased in various metabolic disorders and during ageing. Through its NAD-biosynthetic activity, NAMPT influences the activity of NAD-dependent enzymes, thereby regulating cellular metabolism. In addition to its enzymatic function, extracellular NAMPT (eNAMPT) possesses cytokine-like activity. Abnormal levels of eNAMPT are associated with various metabolic disorders. NAMPT is able to modulate processes in the pathogenesis of obesity and related disorders, such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) by influencing the oxidative stress response, apoptosis, lipid and glucose metabolism, inflammation and insulin resistance. NAMPT also has a crucial role in cancer cell metabolism, is often overexpressed in tumour tissues and an experimental target for antitumor therapies. In this review, we discuss current understanding of the functions of NAMPT and highlight progress made in identifying the physiological role of NAMPT and its relevance in various human diseases and conditions, such as obesity, NAFLD, T2DM, cancer and ageing.

1.2 Physiological role of NAMPT

1.2.1. Mechanism of action

NAD metabolism and NAMPT enzyme activity

NAD is an essential coenzyme involved in cellular redox reactions and a substrate for NAD-dependent enzymes. In mammals, NAD can be synthesized *de novo* from tryptophan or NAD precursors such as nicotinamide (NAM), nicotinic acid, nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) (Figure 1). NAM rather than nicotinic acid is thought to be the predominant NAD precursor in mammals [1–3]. NAM is also a product of deacetylation and ADP-ribosylation reactions, which are catalysed by NAD-dependent enzymes [4–6]. NAMPT activity generates NMN from NAM and 5′-phosphoribosyl-1-pyrophosphate (PRPP), catalysing the rate-limiting step in the mammalian NAD salvage pathway from NAM [7,8]. NMN, together with ATP, is then converted into NAD by nicotinamide/nicotinic acid mononucleotide adenylyltransferases 1-3 (NMNAT1-3). NR is converted into NMN by nicotinamide riboside kinase (NRK) [9], which enters the NAD salvage pathway. Evidence of extracellular conversion of NAD and NMN to NR by the ectocellular enzymes CD38 and CD73 also exists (Figure 2) [10–12].

By analysing tissue sources of 719 cDNA clones, NAMPT was found to be expressed in nearly all organs, tissues, and cells examined [13,14]. This ubiquitous expression of NAMPT suggests pleiotropic functions of the protein in human physiology. NAMPT occurs intracellularly (mainly in the cytoplasm and nucleus) [15], and extracellularly [16]; NAMPT has also been reproted to reside in mitochondria [17], although this finding has been disputed [18]. Significant sequence homology exists

in NAMPT among prokaryotic organisms, primitive metazoans such as marine sponges, and humans [19], which suggests a crucial role for NAMPT in cellular metabolism and survival. Several groups have characterized the structural and enzymological features of mammalian NAMPT [20–27]. Structural and mutagenesis studies have shown that mutations in NAMPT which impair dimerization, attenuate enzymatic activity [22]; furthermore, Asp219 is important in defining the substrate specificity of NAMPT [25], which does not include nicotinic acid [8,28]. Autophosphorylation of NAMPT (using ATP) at H247 [20,22] creates a reaction intermediate that is hydrolysed during each catalytic cycle, which increases the affinity of NAMPT for NAM and its enzymatic activity up to 1000-fold [20].

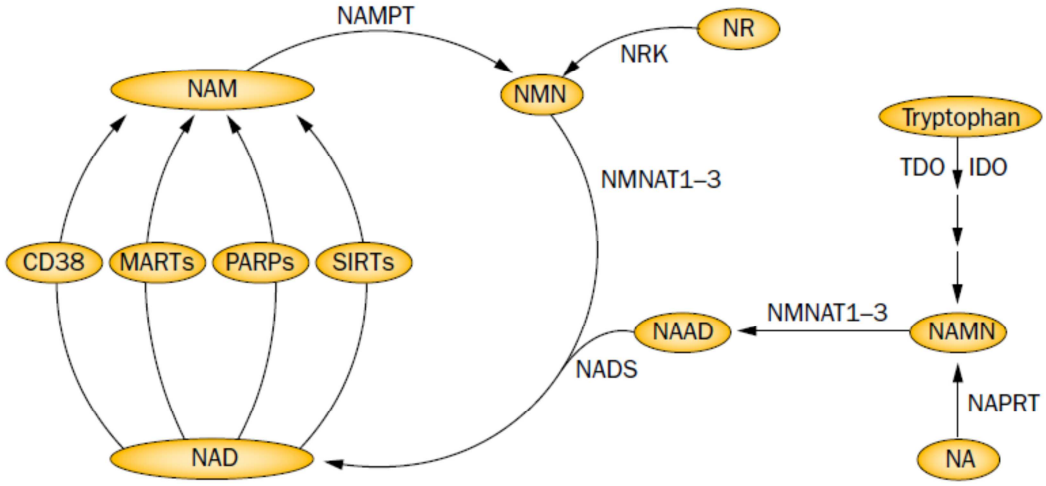

Fig.1. Mammalian NAD metabolism. The NAD *de novo* kynurenine pathway is comprised of several steps. The first rate-limiting step is catalysed by TDOs (mainly in the liver) or IDOs. By multiple reactions, L-tryptophan is converted to NAMN. At this stage, NA enters the pathway and is phosphoribosylated by NAPRT. NAMN is then converted to NAAD by NMNAT1-3. Finally, NAAD is amidated by NADS to yield NAD. NAM is another NAD precursor and a product of deacetylation and ADP-ribosylation reactions, which are catalysed by NAD-dependent enzymes such as SIRTs, PARPs, MARTs and ADP-ribosyl cyclases such as CD38. NAMPT catalyses the formation of NMN from NAM and 5′-phosphoribosyl-1-pyrophosphate (PRPP), which is the rate-limiting step in the NAD salvage pathway from NAM. NMN is then converted into NAD by NMNAT1-3. NR enters the NAD biosynthesis pathway by phosphorylation to NMN, which is catalysed by NRKs. Abbreviations: IDOs, indoleamine 2,3-dioxygenases; MART, mono(ADP-ribosyl) transferase; NA, nicotinic acid; NAAD, nicotinic acid adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; NADS, nicotinamide adenine dinucleotide synthase; NAM, nicotinamide; NAMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, nicotinate phosphoribosyltransferase; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide/nicotinic acid mononucleotide adenylyltransferase; NR, nicotinamide riboside; NRKs, nicotinamide riboside kinases; PARP, poly(ADP-ribose) polymerase; SIRT, sirtuin; TDOs, tryptophan 2,3-dioxygenases.

NAMPT is one of the regulators of the intracellular NAD pool [7,8]. Through its NAD-biosynthetic activity, NAMPT influences the activity of NAD-dependent enzymes, such as sirtuins (SIRTs) [8,17,29–32] and poly (ADP-ribose) polymerase (PARP)-1 [33], and thereby regulates cellular metabolism, mitochondrial biogenesis [34–36] and adaptive stress responses, which include inflammatory, oxidative, proteotoxic, and genotoxic stress [37]. Further information on the roles of NAD-dependent enzymes in cellular metabolism can be found elsewhere [38–42]. Mono-ADP-ribosyl transferases [43] and ADP-ribosyl cyclases [44] are involved in several physiological processes, which include calcium signalling and DNA repair. As these enzymes also use NAD as substrate and are also inhibited by the product NAM, NAMPT activity probably influences their functions. Interestingly, the *NAMPT* gene is regulated by the circadian locomoter output cycles protein kaput (CLOCK)- aryl hydrocarbon receptor translocator-like protein 1 (BMAL1, also known as ARNTL) core clock machinery, which is responsible for circadian rhythmicity and leads to a circadian oscillation of NAD levels *in vivo* [45,46]. SIRT1 is recruited to the *NAMPT* promoter and contributes to the circadian synthesis of NAMPT. This completes a transcriptional-enzymatic feedback loop that connects cellular metabolism and circadian rhythms [45,46].

Extracellular NAMPT

eNAMPT has been reported in the human [47] and the mouse [16] circulation, in human cerebrospinal fluid [48] and seminal plasma [49], and in the supernatant of numerous cell types including differentiated adipocytes [16,50,51], hepatocytes [52,53], leukocytes [14], cardiomyocytes [54], neurons [55,56], amniotic epithelial cells [57], pancreatic β cells [58] and LPS-activated monocytes [59,60] as well as spermatozoa (S. Thomas, personal communication). Several studies indicated that eNAMPT might function as a growth factor [13,61–65], however, the mechanism of NAMPT secretion and its physiological function in the extracellular space is far from certain. Another uncertainty is whether NAMPT occurs in the same form and configuration inside the cell as it does in the extracellular space. Some investigations of the action of eNAMPT have used recombinant NAMPT expressed from bacterial sources, which might not accurately represent the endogenous form of mammalian eNAMPT. A 2015 study reported that SIRT1 deacetylates intracellular NAMPT (iNAMPT), thereby predisposing NAMPT to secretion from adipocytes [51]. Several studies have measured enzymatic activity of eNAMPT [14,16,52,58,66] or detected the enzyme product (NMN) in the extracellular space [11,16,67]; however, it has been reported that eNAMPT is not enzymatically active in mouse plasma owing to the low concentration of PRPP and ATP [68]. This observation raises the question as to whether enzymatic activity of eNAMPT is linked to pathophysiological conditions in which plasma PRPP and ATP levels increase due to cell death. Interestingly, the presence of a significant nicotinate phosphoribosyltransferase activity has been reported in human plasma, which suggests the existence of nicotinic acid mononucleotide (NAMN) in the human circulation [66]. As a receptor for eNAMPT has not been discovered yet, the mechanism of eNAMPT signal transduction continues to be the subject of research (Figure 2), owing to its importance in targeting eNAMPT in

various pathological conditions. Improved understanding of the mechanism of action of eNAMPT is a prerequisite for exploitation of eNAMPT-related pathways as a therapeutic approach in relevant diseases.

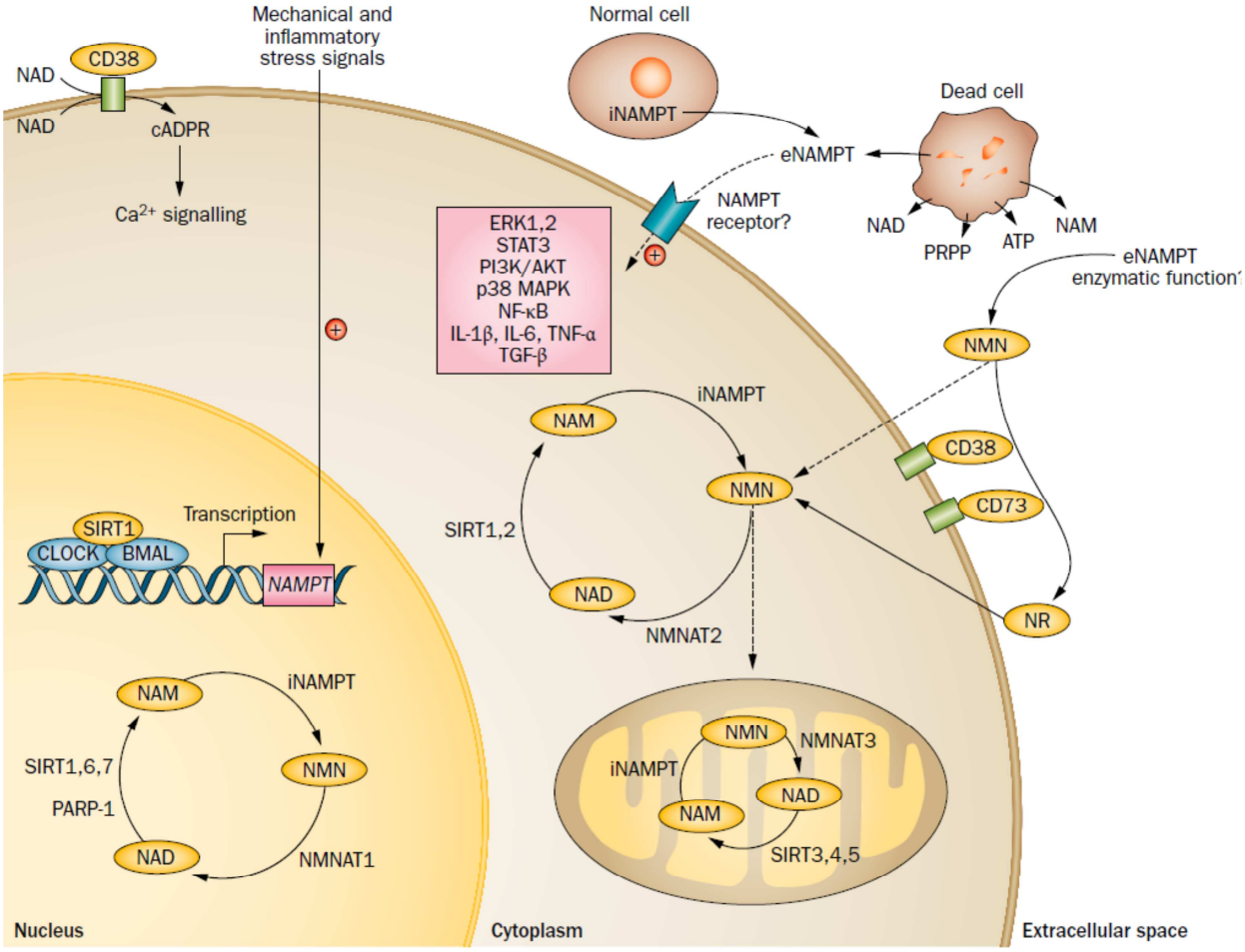

Figure 2 Physiological actions of NAMPT. iNAMPT is found in the cytoplasm, nucleus and mitochondria. NAMPT exerts its effects by maintaining intracellular levels of NAD and recycling NAM, which is produced by the action of NAD-dependent enzymes such as SIRT1,6,7 and PARP-1 in the nucleus, SIRT1,2 in the cytoplasm and SIRT3,4,5 in mitochondria. The ectoenzyme CD38 produces cADPR and regulates intracellular Ca^{2+} signalling. NAMPT expression is induced by the circadian regulators CLOCK and BMAL1 in complex with SIRT1. Other stimulators of NAMPT expression include mechanical stress and pro-inflammatory cytokines (such as TNFα, IL-1β and IFNγ). NAMPT is secreted from different cell types and is probably released from dying cells together with ATP and PRPP. Extracellular production of NMN therefore, potentially, occurs and NMN might subsequently enter the cell, possibly after conversion to NR by the ectocellular enzymes CD38 and CD73. Apart from enzymatic activity, eNAMPT acts as a pro-inflammatory or anti-inflammatory cytokine in multiple signalling pathways, such as ERK1,2, IL-6–STAT3, PI3K–AKT, p38 MAPK and NF-κB, which influence the expression of several cytokines (such as TNFα, IL-1β and TGFβ). A receptor for eNAMPT has not yet been identified. Abbreviations: AKT, protein kinase B; BMAL1, aryl hydrocarbon receptor translocator-like protein 1; cADPR, cyclic ADP-ribose; CLOCK, circadian locomoter output cycles protein kaput; eNAMPT, extracellular nicotinamide phosphoribosyltransferase; ERK, extracellular signal-regulated kinase; iNAMPT, intracellular nicotinamide phosphoribosyltransferase; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NF-κB, nuclear factor κB; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide/nicotinic acid mononucleotide adenylyltransferase; NR, nicotinamide riboside; NRKs, nicotinamide riboside kinases; P38 MAPK, mitogen-activated protein kinase P38; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; PRPP, 5′-phosphoribosyl-1-pyrophosphate; SIRT, sirtuin; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α.

1.3 Pathophysiological role of NAMPT

1.3.1. NAMPT and obesity

White adipose tissue (WAT) operates as a functional endocrine unit [69]. Ever since eNAMPT was first described as an adipocytokine (more commonly known as adipokine) with insulin-mimetic effects [70], the role of NAMPT in obesity and obesity-related disorders has been the subject of debate. Different adipocyte models including preadipocyte cell lines 3T3-L1 and SGBS as well as primary human adipocytes have been shown to secrete NAMPT into the supernatant via a non-classical pathway [16,50,71], thereby identifying adipose tissue as one of the major sources of eNAMPT. A number of SNPs in *NAMPT* are associated with obesity [72] and obesity-related co-morbidities such as coronary artery disease [73] as well as glucose and lipid parameters [74]. However, several studies have reported that genetic variation in NAMPT does not have a major role in the development of obesity or T2DM [75–77]. The relevance of genetic variations in *NAMPT* in disease development might depend on whether the active site, assembly of the active NAMPT dimer or gene expression of *NAMPT* is affected.

A meta-analysis, which included human studies on the association between eNAMPT and obesity parameters, reportedthat levels of eNAMPT were generally increased in individuals with obesity [78]. Several human studies investigating the association of NAMPT with parameters of obesity have been published during the past five years (Table 1). Discrepancies between these studies, which have reported either a positive association or no association between circulation levels of eNAMPT and obesity-related parameters, might be due to differences in study populations, sample handling or systems to detect NAMPT [47]. Several metabolic factors whose levels are increased in individuals with obesity have been shown to modulate NAMPT gene and protein expression and/or release (Table 2). Both glucose and oxidized low density lipoprotein stimulate NAMPT gene and protein expression and release in human adipocytes via the PI3-kinase/AKT pathway [79–81]. In addition, glucose administration in humans led to increased levels of eNAMPT [14]. *In vitro* studies have shown that expression of *NAMPT* mRNA increases during adipogenesis [82] and is stimulated by insulin resistance-inducing factors such as IL-6, dexamethasone, growth hormone, tumor necrosis factor (TNF)α and isoproterenol [83–85]. NAMPT is also upregulated in adipocytes under hypoxic conditions [86]. The macrophage population in visceral WAT in individuals with obesity is another source of eNAMPT [87]. Whether eNAMPT exhibits pro-inflammatory or anti-inflammatory activity is still the subject of debate [16,54–56,88,89]. However, several studies have reported pro-inflammatory effects of eNAMPT on different cell types, which include iNOS induction [90], ERK1,2 activation [62], NF-κB activation [90,91] and production of cytokines, such as TNFα, IL-6, IL-1β [91,92],transforming growth factor β [93] and monocyte chemoattractant protein 1 [94]. Production of inflammatory cytokines and *NAMPT* gene expression in adipocytes, thus, seem to be regulated by a positive feedback activation loop. Furthermore, incubation with eNAMPT increased expression of lipoprotein lipase and peroxisome proliferator-activated receptor γin preadipocytes and fatty acid

synthase in differentiated adipocytes, which suggests that eNAMPT is a regulator of lipid metabolism [95].

Table 1. Reported associations between levels of NAMPT and obesity or NAFLD

NAMPT in human studies	**NAMPT correlation with**	**References**
↑ levels of NAMPT in serum* and liver‡ of women with obesity vs normal-weight women ↑ levels of NAMPT in serum* and liver‡ of women with obesity and NAFLD vs women with obesity without NAFLD	Levels of IL-6*, CRP*, resistin‡ and TNFα‡	Auguet *et al*. (2013) [96]
No change in serum levels of NAMPT in patients with NAFLD vs health individuals	TNFα levels	Genc *et al*. (2013) [97]
↓ levels of NAMPT in serum after 6-week weight reduction	None	Hosseinzadeh-Attar *et al*. (2013) [98]
No change in serum levels of NAMPT in healthy women with obesity vs normal-weight women	Carbohydrate intake	Saboori *et al*. (2013) [99]
↑ serum levels of NAMPT in children with obesity vs normal-weight children	BMI and levels of IL-6 (boys)	Li *et al*. (2013) [100]
↑ serum levels of NAMPT in children and adolescents with obesity vs normal-weight children and adolescents	None	Jaleel *et al*. (2013) [101]
↑ serum levels of NAMPT in patients with NAFLD vs individuals without NAFLD	None	Akbal *et al*. (2012) [102]
↑ serum levels of NAMPT in women with morbid obesity vs lean women* ↓ serum levels of NAMPT after bariatric surgery‡ ↑ levels of NAMPT in subcutaneous and visceral adipose tissue§ in women with morbid obesity vs lean women	Levels of IL-6*‡§, CRP* and TNFα‡§	Terra *et al*. (2012) [103]
↑ serum levels of NAMPT in adolescents with exogenous pubertal obesity vs healthy adolescents	Body weight, BMI, WHR, levels of serum triglycerides, LDL cholesterol, insulin and C-peptide, insulin resistance as defined by HOMA and glucosose:insulin ratio	Taskesen *et al*. (2012) [104]

↑ levels of NAMPT in serum and peripheral blood cells[‡] in individuals with obesity vs lean individuals	BMI*[‡], WHR*[‡], % body fat[‡], levels of ALT*, AST*, γ-GT* and levels of HIF1α mRNA[‡]	Catalan *et al*. (2011) [105]
↑ serum levels of NAMPT in children with obesity vs lean children	Leucocyte counts	Friebe *et al*. (2011) [14]
↑ serum levels of NAMPT in adult individuals with obesity vs normal-weight adult individuals	BMI, waist circumference, hip circumference and insulin resistance as defined by HOMA	Reda *et al*. (2011) [106]
↑ serum levels of NAMPT in children and adolescents with obesity vs lean children and adolescents	Waist circumference, BMI, whole body insulin sensitivity and levels of IL-6 and thiobarbituric-acid-reactive substances (boys)	Krzystek-Korpacka *et al*. (2011) [107]
↑ serum levels of NAMPT in prepubertal children with obesity vs prepubertal lean children	Levels of resistin and IL-6	Martos-Moreno *et al*. (2011) [108]
↓ levels of NAMPT in serum and liver in patients with NAFLD vs healthy controls	None	Dahl *et al*. (2010) [109]
↓ levels of NAMPT in visceral adipose tissue in patients with NAFLD vs individuals without NAFLD	None	Gaddipati *et al*. (2010)[110]
↑ levels of NAMPT in liver in patients with fibrosis vs individuals with NAFLD without fibrosis	Stage of fibrosis	Kukla *et al*. (2010) [111][187]
No change in serum levels of NAMPT in women with obesity vs lean women	None	Ersoy *et al*. (2010)[112]
↑ serum levels of NAMPT in individuals with obesity vs healthy, normal-weight individuals	Age, WHR and HbA_{1c} levels	Kaminska *et al*. (2010)[113]
↓ levels of NAMPT in subcutaneous tissue in individuals with obesity vs normal-weight individuals	None	Barth *et al*. (2010) [114]
Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein; γ-GT, γ-glutamyltransferase; HIF1α, hypoxia inducible factor 1α; HOMA, homeostatic model assessment; NAMPT, nicotinamide phosphoribosyltransferase; TNFα, tumour necrosis factor α; WHR, waist–hip ratio.		

Table 2. Metabolic factors that influence levels of NAMPT

Factor	**Effect**	**References**
Glucose restriction	↑ NAMPT levels in skeletal muscle ↑ NAMPT levels in hepatocytes ↑ NAMPT levels in human preadipocytes and adipocytes	Costford *et al*. (2010) [115] Dahl *et al*. (2010) [109] Friebe *et al*. (2011) [84][85]
Glucose	↑ NAMPT levels in human adipocytes	Haider *et al*. (2006) [79]
Fructose	↑ NAMPT levels in BAT and WAT of FRD-fed mice ↓ NAMPT levels in circulation of FRD-fed mice	Caton *et al*. (2011) [116]
Oxidized LDL	↑ NAMPT levels in 3T3-L1 adipocytes	Chen *et al*. (2013) [81]
Growth hormone	↓ NAMPT levels in 3T3-L1 adipocytes	Kralisch *et al*. (2005) [82]
IL-6	↑ NAMPT levels in rheumatoid arthritis ↓ NAMPT levels in 3T3-L1 adipocytes	Nowell *et al*. (2006) [117] Kralisch *et al*. (2005) [82]
TNFα	↑ NAMPT levels in adipose tissue ↓ NAMPT levels in 3T3-L1 adipocytes	Hector *et al*. (2007) [92] Kralisch *et al*. (2005) [82]
Palmitate	↑ NAMPT levels in HepG2 cells	Choi *et al*. (2011) [118]
Leucine	↑ NAMPT levels in HFD-fed mice	Li *et al*. (2012) [119]
Dexamethasone	↑ NAMPT levels in human preadipocytes and adipocytes ↑ NAMPT levels in 3T3-L1 adipocytes	Friebe *et al*. (2011) [84][85] Kralisch *et al*. (2005) [82]
Isoproterenol	↑ NAMPT levels in human preadipocytes ↓ NAMPT levels in 3T3-L1 adipocytes	Friebe *et al*. (2011) [84] Kralisch *et al*. (2005) [82]
Hypoxia	↑ NAMPT levels in 3T3-L1 adipocytes ↑ NAMPT levels in MCF-7 breast cancer cells	Segawa *et al*. (2006) [86] Bae *et al*. (2006)[120]

Abbreviations: BAT, brown adipose tissue; FRD, fructose-rich diet; HFD, high-fat diet; NAMPT, nicotinamide phosphoribosyltransferase; TNFα, tumour necrosis factor α; WAT, white adipose tissue.

1.3.2. NAMPT and non-alcoholic fatty liver disease

NAFLD, which is the most common liver disorder in the West, encompasses a wide range of pathophysiological conditions, ranging from simple steatosis (hepatic lipid accumulation) to inflammation (non-alcoholic steatohepatitis), which frequently leads to fibrosis and cirrhosis accompanied by a higher risk for hepatocellular carcinoma and eventually the need for liver transplantation [121]. Hepatocytes have been identified as a source of eNAMPT [52]. Furthermore, plasma concentrations of eNAMPT correlate with portal inflammation in individuals with NAFLD [122]. Several, partly conflicting, studies investigating the association of NAMPT with severity of NAFLD in humans have been published during the past few years (Table 1), with upregulation and downregulation of NAMPT both observed in animal models or patients with NAFLD [96,109,111,123–125]. In mouse models of diet-induced obesity, upregulation of NAMPT by different strategies has proven beneficial in protecting against steatosis, inflammation, and glucose intolerance [126–128]. Both supplementation with troxerutin, a trihydroxyethylated derivative of the natural bioflavonoid rutin [126], and leucine [127] enhanced levels of NAMPT protein and NAD and, consequently, SIRT1 activity. In another mouse model of hepatic triglyceride accumulation induced by combined liver specific knockout of *Foxo1*, *Foxo3* and *Foxo4*, levels of NAMPT were downregulated, which indicates that NAMPT expression is regulated by Foxo transcription factors in the mouse liver [128]. In the same study, overexpression of NAMPT markedly reduced hepatic triglycerides *in vivo* [128]. Another regulator of NAMPT is miRNA-34a which has been shown to be increased in individuals with obesity and to substantially reduce NAD levels and SIRT1 activity in the liver by directly targeting *NAMPT* mRNA expression [129]. In diet-induced obese mice inhibition of miR-34 restored levels of NAMPT and NAD and improved steatosis, inflammation, and glucose intolerance [130]. In patients with NAFLD, hepatic NAMPT expression was shown to be down regulated, possibly via activation of peroxisome proliferator-activated receptor α [109]. Conversely, overexpression of NAMPT improved apoptosis in stress-exposed rat hepatocytes, which suggests an anti-apoptotic effect of NAMPT in NAFLD [109]. In contrast to the beneficial effects of NAMPT overexpression in the liver, NAMPT overexpression in mouse skeletal muscle did not improve mitochondrial biogenesis or function [131]. Furthermore, no change in hepatic NAD levels and *NAMPT* gene expression was found in mice fed a HFD for 4 weeks, even though SIRT1 activity was attenuated [132]. These findings implicate factors besides NAD levels in contributing to the regulation of sirtuin activity.

A pro-inflammatory action of NAMPT has been reported in HepG2 cells [118]. When treated with palmitate, a time-and dose-dependent increase in NAMPT gene and protein expression as well as in levels of of IL-6 and TNFα was found, whereas down regulation of NAMPT counteracted the inflammatory response [118]. By inhibition of NF-κB, iNAMPT protein levels were normalized after stimulation with palmitate, which indicates that NAMPT might have a role in palmitate-induced inflammation in hepatocytes through the NF-κB pathway [118]. Another *in vitro* study using HepG2

cells showed that incubation with eNAMPT activated gluconeogenesis via activation of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, independent of SIRT1 activation [61].

Taken together, NAMPT is able to modulate processes that are involved in the pathogenesis of NAFLD by regulating oxidative stress (or mitochondrial biogenesis), apoptosis, lipid and glucose metabolism, inflammation and insulin resistance.

1.3.3. NAMPT and type 2 diabetes mellitus

Increased abdominal adiposity is associated with low-grade inflammation, abnormal hormone secretion and various metabolic disturbances that contribute to the genesis of insulin resistance [133,134]. Of late, interest has grown about the role of different adipokines in the pathogenesis of metabolic complications related to obesity [135,136]. In particular, in the large spectrum of adipokines, eNAMPT represents one of the most promising and interesting molecules that seems directly implicated in the regulation of glucose-stimulated insulin secretion (GSIS) in pancreatic β cells [16]. Accumulating evidence shows a possible association between levels of eNAMPT and T2DM in individuals with and without obesity [137–139]. Several studies have reported that patients with T2DM present with significantly higher levels of eNAMPT than healthy controls, independent of BMI [140–142]. In addition, all these studies confirmed, by use of multiple logistic regression analysis, that eNAMPT can be considered as an independent factor for T2DM, even after adjustment for other risk factors [138,140,141]. Subsequent studies showed that eNAMPT is not only associated with T2DM but also with diabetes complications [135], such as endothelial dysfunction [143], diabetic nephropathy and impairment of lipid metabolism [139]. A possible association between different *NAMPT* polymorphisms and T2DM or T2DM-related complications has been reported [73,144]. Many human studies have reported an association between eNAMPT and T2DM (Table 3). Although some animal and *in vitro* studies have attributed the effects of NAMPT on glucose metabolism to the, still controversial, insulin-mimetic actions of NAMPT [70,145], other studies attribute the effects to NAMPTs role as a NAD biosynthetic enzyme. In particular, a mouse model with β-cell-specific overexpression of *Sirt1* (BESTO) had improved glucose tolerance that disappeared with age [146]. Circulating levels of the NAMPT product NMN and consequently NAD levels in pancreatic β cells and islets were significantly lower in old BESTO mice than in young BESTO mice [146]. Furthermore, administration of NMN restored the positive effect of Sirt1 on glucose tolerance and GSIS in aged BESTO female mice [146]. In heterozygous NAMPT knock out ($Nampt^{+/-}$) mice, a defect in NAD biosynthesis and GSIS was found in pancreatic islets [16]. Female $Nampt^{+/-}$ mice had impaired glucose tolerance which was ameliorated by administration of the Nampt enzyme product NMN [16]. Moreover, administration of FK866, a potent NAMPT inhibitor, significantly inhibited NAD biosynthesis and GSIS in wild type mice [16]. Overall, these findings strongly suggest that NAMPT is able to control the regulation of insulin secretion by enhancing NAD concentrations in pancreatic β cells [16]. In confirmation of this hypothesis, NAMPT and NMN were shown to induce

increased insulin secretion compared to glucose alone in human islets after 1 h incubation with high glucose concentrations [147]. In a HFD-induced mouse model of obesity, a direct effect of Nampt not only on glucose metabolism but also on the pathogenesis of T2DM was found [148]. Nampt mediated NAD biosynthesis was impaired in HFD-fed mice with T2DM compared with that in regular chow-fed controls, whereas administration of NMN ameliorated glucose intolerance by restoring NAD levels [148]. In addition, NMN augmented hepatic insulin sensitivity and other biological pathways associated with oxidative stress, inflammation and lipid metabolism [148]. Mice fed a fructose-rich diet had increased levels of NAMPT in brown and WAT, yet significantly lower levels of eNAMPT [116]. Administration of NMN abolished the suppressive effects of the fructose-rich diet on insulin secretion [116]. NMN also demonstrated protective effects against pro-inflammatory cytokine-mediated islet dysfunction [116]. Furthermore, insulin secretion in islets cultured with pro-inflammatory cytokines was restored by NMN; the anti-inflammatory effects of NMN were partially blocked by inhibition of Sirt1 [116].

Table 3. Reported associations between levels of NAMPT and diabetes mellitus

NAMPT in human studies	**NAMPT correlation with**	**References**
↑ NAMPT levels in patients with obesity and T2DM vs normal-weight patients with T2DM vs healthy individuals	BMI, WHR, FPG, insulin resistance as defibed by HOMA, and levels of triglycerides, total cholesterol, IL-6 and vaspin	El-Mesallamy *et al*. (2011) [140]
↑ NAMPT levels in patients with obesity and T2DM vs healthy individuals	T2DM (after controlling for anthropometric variables such as blood pressure, lipid profile and smoking status)	Chen *et al*. (2006) [141]
↑ NAMPT levels in patients with long-standing T1DM[*] and in patients with T2DM[‡] vs healthy individuals[§] ↑ NAMPT levels in patients with long-standing T1DM vs patients with T2DM	[*]Age, no correlation with HbA_{1c} levels [‡]HbA1c levels [§]AIRg, fasting insulin levels, insulin sensitivity and 30-min OGTT (insulin secretion)	López-Bermejo et al. (2006) [142]
↑ NAMPT levels in patients with T2DM and minor or severe proteinuria vs healthy individuals ↑ NAMPT levels in patients with T2DM and severe proteinuria vs patients with T2DM and minor proteinuria	Proteinuria, flow-mediated dilatation	Yilmaz *et al*. (2008) [143]
↑ NAMPT levels in patients with T2DM and CAD vs patients with T2DM without CAD (No change in NAMPT levels in individuals with CAD vs healthy individuals) ↑ frequency of T allele of *NAMPT* polymorphism rs9770242 in Brazilian cohort vs North-American, non-Hispanic white cohort	CAD	Saddi-Rosa *et al*. (2013) [73]

↑ NAMPT levels in patients with T2DM vs healthy individuals ↑ NAMPT levels in patients with T2DM, CVD and T allele of *NAMPT* polymorphism rs9770242 vs patients with T2DM and CVD without *NAMPT* polymorphism rs9770242 *NAMPT* –948G/G genotypes and G alleles in patients with T2DM	T2DM CVD	Motawi *et al*. (2014) [144]
Abbreviations: AIRg, acute insulin response to glucose; CAD, coronary artery disease; CVD, cardiovascular disease; FPG, fasting plasm glucose; HOMA, homeostatic model assessment; NAMPT, nicotinamide phosphoribosyltransferase; OGTT, oral glucose tolerance test; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; WHR, waist–hip ratio.		

Considering all the evidence from *in vitro* and *in vivo* studies, NAMPT seems to be involved in the pathogenesis of T2DM and in the development of diabetes-related complications. More interestingly, *in vitro* studies indicate a possible role for the NAD intermediates, NMN and NR, in amelioration of β-cell function and cellular homeostasis, glucose metabolism and stress responses.

1.3.4. NAMPT and ageing

Sirtuins have been comprehensively investigated as mediators of longevity [149]. NAMPT as a regulator of sirtuin function has been shown to delay cellular senescence by increasing resistance to oxidative stress in human vascular smooth muscle cells [150]. By contrast, eNAMPT induces telomere damage and premature *senescence* in human endothelial cells by activation of NADPH oxidase [151]. Reduced levels of NAMPT and NAD have been detected in peripheral tissue of ageing mice, such as pancreas, WAT, and skeletal muscle [148]. In this study administration of the NAMPT enzyme product, NMN, was shown to be an effective intervention to treat the pathophysiology of age-induced T2DM [148]. In line with these results, rats treated with the NAMPT enzyme inhibitor FK866 failed to exhibit increased Sirt1 and Sirt3 activity in response to caloric restriction - a widely-used strategy to ameliorate age-associated diseases in animal models. Consequently, NAMPT inhibition abolished caloric-restriction-induced mitochondrial biogenesis and enhanced insulin sensitivity [152]. Interestingly, NAMPT is involved in the molecular mechanisms that lead to declining neuronal stem and/or progenitor cell (NSPC) numbers during aging [153]. NSPCs possess the ability to proliferate and differentiate into major cells of the brain, such as neurons, oligodendrocytes, and astrocytes. Ageing is one of the strongest negative regulators of adult NSPC proliferation [153]. One study raised the possibility that long-term administration of NMN might counteract age-related declines in NSPC functionality [154]. A study on young and old Wistar rats demonstrated that Nampt levels were lower in the aged group than in the young group [31]. Interestingly, the age-associated decreases in Nampt and NAD levels were reversed with regular exercise, which increased the specific activity of SIRT1 [31]. A clinical study on a large population of elderly people investigated the relationships between

levels of eNAMPT, nutritional status, and insulin resistance. Levels of eNAMPT declined with age and were associated with nutritional status, visceral obesity, and inflammation [155]. Several theories have been postulated to explain the decline in levels of NAMPT and NAD with ageing. As discussed earlier, NAMPT is a major product of the action of the circadian transcription factors BMAL1 and CLOCK [45]. One hypothesis is that a decline in central and peripheral circadian function during ageing results in a deficit in the production of NAMPT and NAD. Furthermore, ageing is accompanied by a state of chronic, low-grade inflammation [156], which is a major contributor to the development of many age-related chronic disorders. In this context, TNFα, one of the major inflammatory cytokines, and oxidative stress markedly reduced levels of NAMPT and NAD levels in primary hepatocytes [148]. TNFα also suppresses CLOCK-BMAL1-mediated functions in mice [157], which might also contribute to a reduction in NAMPT-mediated NAD biosynthesis during ageing. As mentioned earlier, chronic inflammation, oxidative stress and DNA damage are crucial factors associated with ageing. Activation of NAD-dependent PARPs is induced immediately after DNA damage to facilitate repair and maintenance of genomic integrity. Thus, acute DNA damage can induce a sudden depletion of NAD levels owing to PARP activation. During ageing, damaged DNA accumulates in the nucleus, which causes activation of PARP and might be another possible explanation for age-induced reduction in NAD levels.

1.3.5. NAMPT in cancer

Cancer cells possess a high glucose uptake and an increased rate of glycolysis even in the presence of oxygen (the so-called Warburg effect [158]). The metabolic alterations leading to this phenotype require increased amounts of the redox co-factor NAD. NAD functions in many critical cellular processes that are necessary for cancer cell growth, including transcriptional regulation, cell-cycle progression, anti-apoptosis, DNA repair, regulation of chromatin dynamics and telomerase activity [38,159]. NAMPT is essential for the replenishment of the intracellular NAD pool, as NAD is rapidly consumed by NAD-dependent enzymes in cancer cells and converted to NAM [28,160–162]. The development of many cancers including colorectal, ovarian, breast, gastric, prostrate, well-differentiated thyroid, and endometrial carcinomas, myeloma, melanoma and astrocytomas is, thus, associated with increased *NAMPT* gene expression [163]. NAMPT is differentially expressed in hepatocarcinoma cell lines compared to non-cancerous primary human hepatocytes, and can be regulated by resveratrol [53]. In a meta-analysis of genome-wide expression data, which identified genes influenced by NAMPT, a reduced *NAMPT* gene expression was found to be strongly associated with dysregulation of cancer signalling pathways [164]. Cancer cells are more susceptible to NAMPT inhibition than normal cells [165,166].

Clinical studies have also demonstrated that serum or blood levels of eNAMPT are increased in patients with cancer and a positive correlation exists between either tissue or eNAMPT levels and the stage of cancer progression [167–171]. However, the molecular pathways of eNAMPT signaling in carcinogenesis are far from clear. eNAMPT affects redox adaptive responses and promotes tumour

proliferation in human malignant melanoma cells [172], and influences resting monocytes, polarizing them toward a tumour-supporting M2 macrophage phenotype [171]. By this latter mechanism, eNAMPT induces an immunosuppressive and tumour-promoting microenvironment in chronic lymphocytic leukemia [171]. Furthermore, eNAMPT stimulates vascular endothelial growth factor by activating the MAPK/ERK pathway and promote angiogenesis, which is a crucial process during tumour growth and expansion [173]. Cell lines overexpressing NAMPT are considerablymore resistant to chemotherapeutic agents than control cells [174]. By contrast, stable knock-down of NAMPT renders cells more sensitive to such treatment than controls [17]. Targeting NAMPT activity, thus, represents a novel therapeutic strategy for treating human cancers. For example, the specific NAMPT inhibitor FK866 has been evaluated in a broad variety of tumours, including solid tumours and leukemias [161,175,176], both *in vitro* and in nude mouse xenografts [27,175,177–179], in which FK866 was able to reduce or attenuate tumour growth. NAMPT inhibition also attenuates glycolysis in conjunction with reduced NAD levels, which leads to blockade of the pentose phosphate pathway, serine biosynthesis, and the tricarboxylic acid cycle [179]. FK866 has been shown to induce delayed energy stress in hepatocarcinoma cells that triggers the activation of AMPKα and downregulation of mTOR signalling which was associated with increased cancer cell death. Non-cancerous human hepatocytes were less sensitive to FK866 [160]. In contrast to the strategy of NAD depletion by inhibition of NAD-producing enzymes, restoration of the pool of NAD with NR prevented DNA damage and tumour formation in a mouse model of hepatocarcinoma [180]. Clinical trials using NAMPT inhibitors as monotherapy for the treatment of solid tumours (for example melanoma), lymphomas and leukaemias have so far not been promising [181–188]. One possible explanation could be the action of CD38 or CD73 reversing cell death induced by NAMPT inhibition through the supply of ectocellular NAD precursors [12]. However, combining FK866 or other NAMPT inhibitors with antineoplastic agents, chemotherapy or radiotherapy might enhance their therapeutic efficacy [189].

1.4 Conclusion

Levels of NAD are decreased in various metabolic disorders and during ageing. By recycling NAM to NAD, NAMPT is involved in regulating cellular energy metabolism by providing substrate for NAD-dependent enzymes. The role of NAMPT in various metabolic disorders is not completely known. One reason might be the difficulty in differentiating between the intracellular NMN-producing action of NAMPT and the extracellular NMN-producing and/or cytokine-like action of eNAMPT. Several *in vitro* and *in vivo* studies have shown that NAD precursors are successful in augmenting NAD levels and ameliorating the negative effects of pathophysiological conditions and ageing.

In the circulation, abnormal levels of eNAMPT are associated with metabolic disorders and cancer progression. However, association studies on eNAMPT levels in NAFLD are still controversial. This may, in part, be explained by the wide range of disease phenotypes in NAFLD and perhaps a dependency of eNAMPT levels on liver disease progression. In addition, NAMPT has a crucial role in cancer cell metabolism and is often overexpressed in tumour tissues. NAMPT inhibition

and NAD depletion have been applied in *in vitro* studies and in animal studies to reduce tumour growth. However, clinical trials on NAMPT inhibitors as monotherapy have so far failed to show significant antitumour action.

Several questions, therefore, remain unanswered. Firstly, does NAMPT expression and function depend on disease progression and severity in metabolic disorders? Secondly, is NAMPT a pathogenetic factor in the development of NAFLD? Thirdly, how s NAMPT secretion regulated in different cell types and what is the role of eNAMPT? Fourthly, does the secreting cell type influence eNAMPT function? Fifthly, under which conditions is eNAMPT enzymatically active *in vivo*? Sixthly, what are the molecular differences between eNAMPT and iNAMPT besides differential acetylation? Seventhly, are there any adverse effects of long-term NAD precursor treatment? Eighthly, why does NAMPT inhibition lead to tumour cell death *in vitro* and tumour remission in animal models, but not in clinical trials? Finally, is a combination therapy with chemotherapeutics useful for patients? Answers to these questions are eagerly awaited.

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1.6 References

[1] V. Mori, A. Amici, F. Mazzola, M. Di Stefano, L. Conforti, G. Magni, et al., Metabolic Profiling of Alternative NAD Biosynthetic Routes in Mouse Tissues., PLoS One. 9 (2014) e113939. doi:10.1371/journal.pone.0113939.

[2] S.J. Yang, J.M. Choi, L. Kim, S.E. Park, E.J. Rhee, W.Y. Lee, et al., Nicotinamide improves glucose metabolism and affects the hepatic NAD-sirtuin pathway in a rodent model of obesity and type 2 diabetes, J. Nutr. Biochem. 25 (2014) 66–72. doi:10.1016/j.jnutbio.2013.09.004.

[3] P.B. Collins, S. Chaykin, The management of nicotinamide and nicotinic acid in the mouse., J. Biol. Chem. 247 (1972) 778–83.

[4] H. Ushiro, Y. Yokoyama, Y. Shizuta, Purification and characterization of poly (ADP-ribose) synthetase from human placenta., J. Biol. Chem. 262 (1987) 2352–7.

[5] X. Guan, P. Lin, E. Knoll, R. Chakrabarti, Mechanism of Inhibition of the Human Sirtuin Enzyme SIRT3 by Nicotinamide: Computational and Experimental Studies., PLoS One. 9 (2014) e107729. doi:10.1371/journal.pone.0107729.

[6] K.J. Bitterman, R.M. Anderson, H.Y. Cohen, M. Latorre-Esteves, D.A. Sinclair, Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1., J. Biol. Chem. 277 (2002) 45099–107. doi:10.1074/jbc.M205670200.

[7] A. Rongvaux, R.J. Shea, M.H. Mulks, D. Gigot, J. Urbain, O. Leo, et al., Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis., Eur. J. Immunol. 32 (2002) 3225–34. doi:10.1002/1521-4141(200211)32:11<3225::AID-IMMU3225>3.0.CO;2-L.

[8] J.R. Revollo, A.A. Grimm, S. Imai, The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells, J. Biol. Chem. 279 (2004) 50754–50763. doi:10.1074/jbc.M408388200.

[9] P. Bieganowski, C. Brenner, Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD+ in fungi and humans., Cell. 117 (2004) 495–502.

[10] A. Nikiforov, C. Dölle, M. Niere, M. Ziegler, Pathways and subcellular compartmentation of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation., J. Biol. Chem. 286 (2011) 21767–78. doi:10.1074/jbc.M110.213298.

[11] S. Garavaglia, S. Bruzzone, C. Cassani, L. Canella, G. Allegrone, L. Sturla, et al., The high-resolution crystal structure of periplasmic Haemophilus influenzae NAD nucleotidase reveals a novel enzymatic function of human CD73 related to NAD metabolism., Biochem. J. 441 (2012) 131–41. doi:10.1042/BJ20111263.

[12] A. Grozio, G. Sociali, L. Sturla, I. Caffa, D. Soncini, A. Salis, et al., CD73 protein as a source of extracellular precursors for sustained NAD+ biosynthesis in FK866-treated tumor cells., J. Biol. Chem. 288 (2013) 25938–49. doi:10.1074/jbc.M113.470435.

[13] B. Samal, Y. Sun, G. Stearns, C. Xie, S. Suggs, I. McNiece, Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor., Mol. Cell. Biol. 14 (1994) 1431–7. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=358498&tool=pmcentrez&rendertype=abstract (accessed April 1, 2015).

[14] D. Friebe, M. Neef, J. Kratzsch, S. Erbs, K. Dittrich, A. Garten, et al., Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans, Diabetologia. 54 (2011) 1200–1211. doi:10.1007/s00125-010-2042-z.

[15] T. Kitani, S. Okuno, H. Fujisawa, Growth phase-dependent changes in the subcellular localization of pre-B-cell colony-enhancing factor., FEBS Lett. 544 (2003) 74–8.

[16] J.R. Revollo, A. Korner, K.F. Mills, A. Satoh, T. Wang, A. Garten, et al., Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme, Cell Metab. 6 (2007) 363–375. doi:10.1016/j.cmet.2007.09.003.

[17] H. Yang, T. Yang, J.A. Baur, E. Perez, T. Matsui, J.J. Carmona, et al., Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival., Cell. 130 (2007) 1095–107. doi:10.1016/j.cell.2007.07.035.

[18] M. Pittelli, L. Formentini, G. Faraco, A. Lapucci, E. Rapizzi, F. Cialdai, et al., Inhibition of nicotinamide phosphoribosyltransferase: cellular bioenergetics reveals a mitochondrial insensitive NAD pool., J. Biol. Chem. 285 (2010) 34106–14. doi:10.1074/jbc.M110.136739.

[19] P.R. Martin, R.J. Shea, M.H. Mulks, Identification of a plasmid-encoded gene from Haemophilus ducreyi which confers NAD independence., J. Bacteriol. 183 (2001) 1168–74. doi:10.1128/JB.183.4.1168-1174.2001.

[20] E.S. Burgos, V.L. Schramm, Weak coupling of ATP hydrolysis to the chemical equilibrium of human nicotinamide phosphoribosyltransferase., Biochemistry. 47 (2008) 11086–96. doi:10.1021/bi801198m.

[21] M.-K. Kim, J.H. Lee, H. Kim, S.J. Park, S.H. Kim, G.B. Kang, et al., Crystal structure of visfatin/pre-B cell colony-enhancing factor 1/nicotinamide phosphoribosyltransferase, free and in complex with the anti-cancer agent FK-866., J. Mol. Biol. 362 (2006) 66–77. doi:10.1016/j.jmb.2006.06.082.

[22] T. Wang, X. Zhang, P. Bheda, J.R. Revollo, S. Imai, C. Wolberger, Structure of Nampt/PBEF/visfatin, a mammalian NAD+ biosynthetic enzyme., Nat. Struct. Mol. Biol. 13 (2006) 661–2. doi:10.1038/nsmb1114.

[23] G.B. Kang, M.-H. Bae, M.-K. Kim, I. Im, Y.-C. Kim, S.H. Eom, Crystal structure of Rattus norvegicus Visfatin/PBEF/Nampt in complex with an FK866-based inhibitor., Mol. Cells. 27 (2009) 667–71. doi:10.1007/s10059-009-0088-x.

[24] E.S. Burgos, M.J. Vetticatt, V.L. Schramm, Recycling nicotinamide. The transition-state structure of human nicotinamide phosphoribosyltransferase., J. Am. Chem. Soc. 135 (2013) 3485–93. doi:10.1021/ja310180c.

[25] J.A. Khan, X. Tao, L. Tong, Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents., Nat. Struct. Mol. Biol. 13 (2006) 582–8. doi:10.1038/nsmb1105.

[26] R. Takahashi, S. Nakamura, T. Yoshida, Y. Kobayashi, T. Ohkubo, Crystallization of human nicotinamide phosphoribosyltransferase., Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun. 63 (2007) 375–7. doi:10.1107/S1744309107006069.

[27] M.K. Christensen, K.D. Erichsen, U.H. Olesen, J. Tj??rnelund, P. Fristrup, A. Thougaard, et al., Nicotinamide phosphoribosyltransferase inhibitors, design, preparation, and structure-activity relationship, J. Med. Chem. 56 (2013) 9071–9088.

[28] M. Hasmann, I. Schemainda, FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis., Cancer Res. 63 (2003) 7436–42.

[29] S.C. Bowlby, M.J. Thomas, R.B. D'Agostino, S.J. Kridel, Nicotinamide phosphoribosyl transferase (Nampt) is required for de novo lipogenesis in tumor cells., PLoS One. 7 (2012) e40195. doi:10.1371/journal.pone.0040195.

[30] S. Bruzzone, F. Fruscione, S. Morando, T. Ferrando, A. Poggi, A. Garuti, et al., Catastrophic NAD+ depletion in activated T lymphocytes through Nampt inhibition reduces demyelination and disability in EAE., PLoS One. 4 (2009) e7897. doi:10.1371/journal.pone.0007897.

[31] E. Koltai, Z. Szabo, M. Atalay, I. Boldogh, H. Naito, S. Goto, et al., Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats., Mech. Ageing Dev. 131 (2010) 21–8. doi:10.1016/j.mad.2009.11.002.

[32] F. Van Gool, M. Gallí, C. Gueydan, V. Kruys, P.-P. Prevot, A. Bedalov, et al., Intracellular NAD levels regulate tumor necrosis factor protein synthesis in a sirtuin-dependent manner., Nat. Med. 15 (2009) 206–10. doi:10.1038/nm.1906.

[33] J.B. Pillai, A. Isbatan, S. Imai, M.P. Gupta, Poly(ADP-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD+ depletion and reduced Sir2alpha deacetylase activity., J. Biol. Chem. 280 (2005) 43121–30. doi:10.1074/jbc.M506162200.

[34] J.T. Rodgers, C. Lerin, Z. Gerhart-Hines, P. Puigserver, Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways., FEBS Lett. 582 (2008) 46–53. doi:10.1016/j.febslet.2007.11.034.

[35] A. van der Horst, L.G.J. Tertoolen, L.M.M. de Vries-Smits, R.A. Frye, R.H. Medema, B.M.T. Burgering, FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1)., J. Biol. Chem. 279 (2004) 28873–9. doi:10.1074/jbc.M401138200.

[36] L. Bordone, M.C. Motta, F. Picard, A. Robinson, U.S. Jhala, J. Apfeld, et al., Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells., PLoS Biol. 4 (2006) e31. doi:10.1371/journal.pbio.0040031.

[37] X. Luo, W.L. Kraus, On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1., Genes Dev. 26 (2012) 417–32. doi:10.1101/gad.183509.111.

[38] L. Mouchiroud, R.H. Houtkooper, J. Auwerx, NAD^+ metabolism: a therapeutic target for age-related metabolic disease., Crit. Rev. Biochem. Mol. Biol. 48 397–408. doi:10.3109/10409238.2013.789479.

[39] C. Dölle, R.H. Skoge, M.R. Vanlinden, M. Ziegler, NAD biosynthesis in humans--enzymes, metabolites and therapeutic aspects., Curr. Top. Med. Chem. 13 (2013) 2907–17.

[40] H.-C. Chang, L. Guarente, SIRT1 and other sirtuins in metabolism., Trends Endocrinol. Metab. 25 (2014) 138–45. doi:10.1016/j.tem.2013.12.001.

[41] S. Imai, W. Kiess, Therapeutic potential of SIRT1 and NAMPT-mediated NAD biosynthesis in type 2 diabetes, Front. Biosci. (Landmark Ed. 14 (2009) 2983–2995.

[42] A. Bürkle, L. Virág, Poly(ADP-ribose): PARadigms and PARadoxes., Mol. Aspects Med. 34 (2013) 1046–65. doi:10.1016/j.mam.2012.12.010.

[43] E.S. Scarpa, G. Fabrizio, M. Di Girolamo, A role of intracellular mono-ADP-ribosylation in cancer biology., FEBS J. 280 (2013) 3551–62. doi:10.1111/febs.12290.

[44] H.C. Lee, R. Aarhus, ADP-ribosyl cyclase: an enzyme that cyclizes NAD+ into a calcium-mobilizing metabolite., Cell Regul. 2 (1991) 203–9.

[45] K.M. Ramsey, J. Yoshino, C.S. Brace, D. Abrassart, Y. Kobayashi, B. Marcheva, et al., Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis., Science. 324 (2009) 651–4. doi:10.1126/science.1171641.

[46] Y. Nakahata, S. Sahar, G. Astarita, M. Kaluzova, P. Sassone-Corsi, Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1., Science. 324 (2009) 654–7. doi:10.1126/science.1170803.

[47] A. Korner, A. Garten, M. Bluher, R. Tauscher, J. Kratzsch, W. Kiess, Molecular characteristics of serum visfatin and differential detection by immunoassays, J. Clin. Endocrinol. Metab. 92 (2007) 4783–4791. doi:10.1210/jc.2007-1304.

[48] M. Hallschmid, H. Randeva, B.K. Tan, W. Kern, H. Lehnert, Relationship between cerebrospinal fluid visfatin (PBEF/Nampt) levels and adiposity in humans., Diabetes. 58 (2009) 637–40. doi:10.2337/db08-1176.

[49] S. Thomas, D. Kratzsch, M. Schaab, M. Scholz, S. Grunewald, J. Thiery, et al., Seminal plasma adipokine levels are correlated with functional characteristics of spermatozoa., Fertil. Steril. 99 (2013) 1256–1263.e3. doi:10.1016/j.fertnstert.2012.12.022.

[50] M. Tanaka, M. Nozaki, A. Fukuhara, K. Segawa, N. Aoki, M. Matsuda, et al., Visfatin is released from 3T3-L1 adipocytes via a non-classical pathway, Biochem. Biophys. Res. Commun. 359 (2007) 194–201. doi:10.1016/j.bbrc.2007.05.096.

[51] I.S. Yoon MJ, Yoshida M, Johnson S, Takikawa A, Usui I, Tobe K, Nakagawa T, Yoshino J, SIRT1-mediated eNAMPT secretion from adipose tissue regulates hypothalamic NAD+ and function in mice, Cell Metab. (n.d.).

[52] a. Garten, S. Petzold, a. Barnikol-Oettler, a. Körner, W.E. Thasler, J. Kratzsch, et al., Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes., Biochem. Biophys. Res. Commun. 391 (2010) 376–81. doi:10.1016/j.bbrc.2009.11.066.

[53] S. Schuster, M. Penke, T. Gorski, S. Petzold-Quinque, G. Damm, R. Gebhardt, et al., Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes, PLoS One. 9 (2014). doi:10.1371/journal.pone.0091045.

[54] V.B. Pillai, N.R. Sundaresan, G. Kim, S. Samant, L. Moreno-Vinasco, J.G.N. Garcia, et al., Nampt secreted from cardiomyocytes promotes development of cardiac hypertrophy and adverse ventricular remodeling., Am. J. Physiol. Heart Circ. Physiol. 304 (2013) H415–26. doi:10.1152/ajpheart.00468.2012.

[55] B. Zhao, M. Zhang, X. Han, X.-Y. Zhang, Q. Xing, X. Dong, et al., Cerebral ischemia is exacerbated by extracellular nicotinamide phosphoribosyltransferase via a non-enzymatic mechanism., PLoS One. 8 (2013) e85403. doi:10.1371/journal.pone.0085403.

[56] Z. Jing, J. Xing, X. Chen, R.A. Stetler, Z. Weng, Y. Gan, et al., Neuronal NAMPT is released after cerebral ischemia and protects against white matter injury., J. Cereb. Blood Flow Metab. 34 (2014) 1613–21. doi:10.1038/jcbfm.2014.119.

[57] S. Ognjanovic, G.D. Bryant-Greenwood, Pre-B-cell colony-enhancing factor, a novel cytokine of human fetal membranes., Am. J. Obstet. Gynecol. 187 (2002) 1051–8.

[58] K. Kover, P.Y. Tong, D. Watkins, M. Clements, L. Stehno-Bittel, L. Novikova, et al., Expression and regulation of nampt in human islets., PLoS One. 8 (2013) e58767. doi:10.1371/journal.pone.0058767.

[59] E. Schilling, S. Hauschildt, Extracellular ATP induces P2X7-dependent nicotinamide phosphoribosyltransferase release in LPS-activated human monocytes., Innate Immun. 18 (2012) 738–44. doi:10.1177/1753425912439614.

[60] R. Van den Bergh, S. Morin, H.J. Sass, S. Grzesiek, M. Vekemans, E. Florence, et al., Monocytes contribute to differential immune pressure on R5 versus X4 HIV through the adipocytokine visfatin/NAMPT., PLoS One. 7 (2012) e35074. doi:10.1371/journal.pone.0035074.

[61] Y.J. Choi, S.E. Choi, E.S. Ha, Y. Kang, S.J. Han, D.J. Kim, et al., Extracellular visfatin activates gluconeogenesis in HepG2 cells through the classical PKA/CREB-dependent pathway, Horm. Metab. Res. 46 (2014) 233–239. doi:10.1055/s-0034-1370907.

[62] S.-R. Kim, S.-K. Bae, K.-S. Choi, S.-Y. Park, H.O. Jun, J.-Y. Lee, et al., Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2., Biochem. Biophys. Res. Commun. 357 (2007) 150–6. doi:10.1016/j.bbrc.2007.03.105.

[63] Y.-H. Bae, M.-K. Bae, S.-R. Kim, J.H. Lee, H.-J. Wee, S.-K. Bae, Upregulation of fibroblast growth factor-2 by visfatin that promotes endothelial angiogenesis., Biochem. Biophys. Res. Commun. 379 (2009) 206–11. doi:10.1016/j.bbrc.2008.12.042.

[64] Y.-S. Kang, M.-K. Bae, J.-Y. Kim, J.-W. Jeong, I. Yun, H.-O. Jang, et al., Visfatin induces neurite outgrowth in PC12 cells via ERK1/2 signaling pathway., Neurosci. Lett. 504 (2011) 121–6. doi:10.1016/j.neulet.2011.09.014.

[65] H.-J. Park, S.-R. Kim, S.S. Kim, H.-J. Wee, M.-K. Bae, M.H. Ryu, et al., Visfatin promotes cell and tumor growth by upregulating Notch1 in breast cancer., Oncotarget. 5 (2014) 5087–99.

[66] F. Zamporlini, S. Ruggieri, F. Mazzola, A. Amici, G. Orsomando, N. Raffaelli, Novel Assay for Simultaneous Measurement of Pyridine Mononucleotides Synthesizing Activities Allows Dissection of NAD(+) Biosynthetic Machinery in Mammalian Cells., FEBS J. (2014). doi:10.1111/febs.13050.

[67] L. Formentini, F. Moroni, A. Chiarugi, Detection and pharmacological modulation of nicotinamide mononucleotide (NMN) in vitro and in vivo., Biochem. Pharmacol. 77 (2009) 1612–20. doi:10.1016/j.bcp.2009.02.017.

[68] N. Hara, K. Yamada, T. Shibata, H. Osago, M. Tsuchiya, Nicotinamide phosphoribosyltransferase/visfatin does not catalyze nicotinamide mononucleotide formation in blood plasma., PLoS One. 6 (2011) e22781. doi:10.1371/journal.pone.0022781.

[69] M. Khan, F. Joseph, Adipose tissue and adipokines: the association with and application of adipokines in obesity., Scientifica (Cairo). 2014 (2014) 328592. doi:10.1155/2014/328592.

[70] A. Fukuhara, M. Matsuda, M. Nishizawa, K. Segawa, M. Tanaka, K. Kishimoto, et al., Visfatin: a protein secreted by visceral fat that mimics the effects of insulin., Science. 307 (2005) 426–430. doi:10.1126/science.1097243.

[71] N.C. Olarescu, T. Ueland, T. Lekva, T.B. Dahl, B. Halvorsen, P. Aukrust, et al., Adipocytes as a source of increased circulating levels of nicotinamide phosphoribosyltransferase/visfatin in active acromegaly., J. Clin. Endocrinol. Metab. 97 (2012) 1355–62. doi:10.1210/jc.2011-2417.

[72] A.I.F. Blakemore, D. Meyre, J. Delplanque, V. Vatin, C. Lecoeur, M. Marre, et al., A rare variant in the visfatin gene (NAMPT/PBEF1) is associated with protection from obesity., Obesity (Silver Spring). 17 (2009) 1549–53. doi:10.1038/oby.2009.75.

[73] P. Saddi-Rosa, C. Oliveira, F. Crispim, F.M.A. Giuffrida, V. de Lima, J. Vieira, et al., Association of circulating levels of nicotinamide phosphoribosyltransferase (NAMPT/Visfatin) and of a frequent polymorphism in the promoter of the NAMPT gene with coronary artery disease in diabetic and non-diabetic subjects., Cardiovasc. Diabetol. 12 (2013) 119. doi:10.1186/1475-2840-12-119.

[74] W.-X. Jian, T.-H. Luo, Y.-Y. Gu, H.-L. Zhang, S. Zheng, M. Dai, et al., The visfatin gene is associated with glucose and lipid metabolism in a Chinese population., Diabet. Med. 23 (2006) 967–73. doi:10.1111/j.1464-5491.2006.01909.x.

[75] P. Paschou, A. Kukuvitis, M.P. Yavropoulou, A. Dritsoula, V. Giapoutzidis, O. Anastasiou, et al., Genetic variation in the visfatin (PBEF1/NAMPT) gene and type 2 diabetes in the Greek population., Cytokine. 51 (2010) 25–7. doi:10.1016/j.cyto.2010.04.006.

[76] A. Körner, Y. Böttcher, B. Enigk, W. Kiess, M. Stumvoll, P. Kovacs, Effects of genetic variation in the visfatin gene (PBEF1) on obesity, glucose metabolism, and blood pressure in children., Metabolism. 56 (2007) 772–7. doi:10.1016/j.metabol.2007.01.009.

[77] Y. Böttcher, D. Teupser, B. Enigk, J. Berndt, N. Klöting, M.R. Schön, et al., Genetic variation in the visfatin gene (PBEF1) and its relation to glucose metabolism and fat-depot-specific messenger ribonucleic acid expression in humans., J. Clin. Endocrinol. Metab. 91 (2006) 2725–31. doi:10.1210/jc.2006-0149.

[78] Y.-H. Chang, D.-M. Chang, K.-C. Lin, S.-J. Shin, Y.-J. Lee, Visfatin in overweight/obesity, type 2 diabetes mellitus, insulin resistance, metabolic syndrome and cardiovascular diseases: a meta-analysis and systemic review., Diabetes. Metab. Res. Rev. 27 (2011) 515–527. doi:10.1002/dmrr.1201.

[79] D.G. Haider, G. Schaller, S. Kapiotis, C. Maier, a. Luger, M. Wolzt, The release of the adipocytokine visfatin is regulated by glucose and insulin, Diabetologia. 49 (2006) 1909–1914. doi:10.1007/s00125-006-0303-7.

[80] D.G. Haider, K. Schindler, G. Schaller, G. Prager, M. Wolzt, B. Ludvik, Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding, J. Clin. Endocrinol. Metab. 91 (2006) 1578–1581. doi:10.1210/jc.2005-2248.

[81] Y. Chen, M. Chen, Z. Wu, S. Zhao, Ox-LDL induces ER stress and promotes the adipokines secretion in 3T3-L1 adipocytes, PLoS One. 8 (2013) e81379. doi:10.1371/journal.pone.0081379.

[82] S. Kralisch, J. Klein, U. Lossner, M. Bluher, R. Paschke, M. Stumvoll, et al., Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes, J. Endocrinol. 185 (2005) R1–8. doi:10.1677/joe.1.06211.

[83] S. Kralisch, J. Klein, U. Lossner, M. Bluher, R. Paschke, M. Stumvoll, et al., Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes., Am. J. Physiol. Endocrinol. Metab. 289 (2005) E586–E590. doi:10.1152/ajpendo.00090.2005.

[84] D. Friebe, D. Loffler, M. Schonberg, F. Bernhard, P. Buttner, K. Landgraf, et al., Impact of metabolic regulators on the expression of the obesity associated genes FTO and NAMPT in human preadipocytes and adipocytes, PLoS One. 6 (2011) e19526. doi:10.1371/journal.pone.0019526.

[85] H.-S. Kim, S.-Y. Han, H.-Y. Sung, S.-H. Park, M.-K. Kang, S.-J. Han, et al., Blockade of visfatin induction by oleanolic acid via disturbing IL-6-TRAF6-NF-κB signaling of adipocytes, Exp. Biol. Med. (Maywood). 239 (2014) 284–292. doi:10.1177/1535370213514511.

[86] K. Segawa, A. Fukuhara, N. Hosogai, K. Morita, Y. Okuno, M. Tanaka, et al., Visfatin in adipocytes is upregulated by hypoxia through HIF1alpha-dependent mechanism., Biochem. Biophys. Res. Commun. 349 (2006) 875–82. doi:10.1016/j.bbrc.2006.07.083.

[87] C. a. Curat, V. Wegner, C. Sengenès, a. Miranville, C. Tonus, R. Busse, et al., Macrophages in human visceral adipose tissue: Increased accumulation in obesity and a source of resistin and visfatin, Diabetologia. 49 (2006) 744–747. doi:10.1007/s00125-006-0173-z.

[88] T. Romacho, C.F. Sánchez-Ferrer, C. Peiró, Visfatin/Nampt: an adipokine with cardiovascular impact., Mediators Inflamm. 2013 (2013) 946427. doi:10.1155/2013/946427.

[89] Y. Li, Y. Zhang, B. Dorweiler, D. Cui, T. Wang, C.W. Woo, et al., Extracellular Nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism., J. Biol. Chem. 283 (2008) 34833–43. doi:10.1074/jbc.M805866200.

[90] T. Romacho, V. Azcutia, M. Vázquez-Bella, N. Matesanz, E. Cercas, J. Nevado, et al., Extracellular PBEF/NAMPT/visfatin activates pro-inflammatory signalling in human vascular smooth muscle cells through nicotinamide phosphoribosyltransferase activity., Diabetologia. 52 (2009) 2455–63. doi:10.1007/s00125-009-1509-2.

[91] A.R. Moschen, R.R. Gerner, H. Tilg, Pre-B cell colony enhancing factor/NAMPT/visfatin in inflammation and obesity-related disorders., Curr. Pharm. Des. 16 (2010) 1913–1920. doi:10.2174/138161210791208947.

[92] J. Hector, B. Schwarzloh, J. Goehring, T.G. Strate, U.F. Hess, G. Deuretzbacher, et al., TNF-alpha alters visfatin and adiponectin levels in human fat., Horm. Metab. Res. 39 (2007) 250–5. doi:10.1055/s-2007-973075.

[93] H.K. Song, M.H. Lee, B.K. Kim, Y.G. Park, G.J. Ko, Y.S. Kang, et al., Visfatin: a new player in mesangial cell physiology and diabetic nephropathy., Am. J. Physiol. Renal Physiol. 295 (2008) F1485–94. doi:10.1152/ajprenal.90231.2008.

[94] G. Sommer, S. Kralisch, N. Kloting, M. Kamprad, K. Schrock, J. Kratzsch, et al., Visfatin is a positive regulator of MCP-1 in human adipocytes in vitro and in mice in vivo., Obesity (Silver Spring). 18 (2010) 1486–1492. doi:10.1038/oby.2009.462.

[95] C.C. Yang, S.J. Deng, C.C. Hsu, B.H. Liu, E.C. Lin, W.T.K. Cheng, et al., Visfatin regulates genes related to lipid metabolism in porcine adipocytes, J. Anim. Sci. 88 (2010) 3233–3241. doi:10.2527/jas.2010-2799.

[96] T. Auguet, X. Terra, J.A. Porras, J.M. Orellana-Gavaldà, S. Martinez, C. Aguilar, et al., Plasma visfatin levels and gene expression in morbidly obese women with associated fatty liver disease., Clin. Biochem. 46 (2013) 202–8. doi:10.1016/j.clinbiochem.2012.11.006.

[97] H. Genc, T. Dogru, M. Kara, S. Tapan, C.N. Ercin, C. Acikel, et al., Association of plasma visfatin with hepatic and systemic inflammation in nonalcoholic fatty liver disease., Ann. Hepatol. 12 (2013) 548–55. http://www.ncbi.nlm.nih.gov/pubmed/23813132.

[98] M.J. Hosseinzadeh-Attar, A. Golpaie, L. Janani, H. Derakhshanian, Effect of weight reduction following bariatric surgery on serum visfatin and adiponectin levels in morbidly obese subjects, Obes. Facts. 6 (2013) 193–202. doi:10.1159/000351162.

[99] S. Saboori, M.J. Hosseinzadeh-Attar, E. Yousefi Rad, M. Hosseini, K. Mirzaei, Z. Ahmadivand, The comparison of serum vaspin and visfatin concentrations in obese and normal weight women, Diabetes Metab. Syndr. (2013). doi:10.1016/j.dsx.2013.10.009.

[100] R.Z. Li, X.Y. Ma, X.F. Hu, S.X. Kang, S.K. Chen, K. Cianflone, et al., Elevated visfatin levels in obese children are related to proinflammatory factors, J. Pediatr. Endocrinol. Metab. 26 (2013) 111–118. doi:10.1515/jpem-2012-0237.

[101] a Jaleel, B. Aheed, S. Jaleel, R. Majeed, a Zuberi, S. Khan, et al., Association of adipokines with obesity in children and adolescents, Biomark Med. 7 (2013) 731–735. doi:10.2217/bmm.13.41.

[102] E. Akbal, E. Koçak, A. Taş, E. Yüksel, S. Köklü, Visfatin Levels in Nonalcoholic Fatty Liver Disease, J. Clin. Lab. Anal. 26 (2012) 115–119. doi:10.1002/jcla.21491.

[103] X. Terra, T. Auguet, I. Quesada, C. Aguilar, A.M. Luna, M. Hernandez, et al., Increased levels and adipose tissue expression of visfatin in morbidly obese women: the relationship with pro-inflammatory cytokines, Clin. Endocrinol. (Oxf). 77 (2012) 691–698. doi:10.1111/j.1365-2265.2011.04327.x.

[104] D. Taşkesen, B. Kirel, T. Us, Serum visfatin levels, adiposity and glucose metabolism in obese adolescents, JCRPE J. Clin. Res. Pediatr. Endocrinol. 4 (2012) 76–81. doi:10.4274/Jcrpe.547.

[105] V. Catalán, J. Gómez-Ambrosi, a. Rodríguez, B. Ramírez, C. Silva, F. Rotellar, et al., Association of increased Visfatin/PBEF/NAMPT circulating concentrations and gene expression levels in peripheral blood cells with lipid metabolism and fatty liver in human morbid obesity, Nutr. Metab. Cardiovasc. Dis. 21 (2011) 245–253. doi:10.1016/j.numecd.2009.09.008.

[106] R. Reda, A. Shehab, D. Soliman, A. Gabr, A. Abbass, Serum visfatin levels in a group of Egyptian obese individuals, Egypt. J. Immunol. 18 (2011) 25–32. http://www.ncbi.nlm.nih.gov/pubmed/23082477.

[107] M. Krzystek-Korpacka, E. Patryn, I. Bednarz-Misa, K. Hotowy, A. Noczynska, Visfatin in juvenile obesity - the effect of obesity intervention and sex, Eur. J. Clin. Invest. 41 (2011) 1284–1291. doi:10.1111/j.1365-2362.2011.02538.x.

[108] G.Á. Martos-Moreno, J. Kratzsch, a Körner, V. Barrios, F. Hawkins, W. Kiess, et al., Serum visfatin and vaspin levels in prepubertal children: effect of obesity and weight loss after behavior modifications on their secretion and relationship with glucose metabolism, Int. J. Obes. 35 (2011) 1355–1362. doi:10.1038/ijo.2010.280.

[109] T.B. Dahl, J.W. Haukeland, A. Yndestad, T. Ranheim, I.P. Gladhaug, J.K. Damås, et al., Intracellular nicotinamide phosphoribosyltransferase protects against hepatocyte apoptosis and is down-regulated in nonalcoholic fatty liver disease, J. Clin. Endocrinol. Metab. 95 (2010) 3039–3047. doi:10.1210/jc.2009-2148.

[110] R. Gaddipati, M. Sasikala, N. Padaki, R.M. Mukherjee, A. Sekaran, M. Jayaraj-Mansard, et al., Visceral adipose tissue visfatin in nonalcoholic fatty liver disease., Ann. Hepatol. Off. J. Mex. Assoc. Hepatol. 9 (2010) 266–270. http://www.ncbi.nlm.nih.gov/pubmed/20720266.

[111] M.M. Kukla, M. Ciupinska-Kajor, M. Kajor, M. Wylezol, K. Zwirska-Korczala, M. Hartleb, et al., Liver visfatin expression in morbidly obese patients with nonalcoholic fatty liver disease undergoing bariatric surgery, Polish J. Pathol. Off. J. Polish Soc. Pathol. 61 (2010) 147–153.

[112] C. Ersoy, G. Sadikoglu, H. Orhan, M. Guclu, E. Sarandol, M.D. Akgun, et al., Body fat distribution has no effect on serum visfatin levels in healthy female subjects, Cytokine. 49 (2010) 275–278. doi:10.1016/j.cyto.2009.11.011.

[113] E. Polska, P. Journal, A. Kamińska, E. Kopczyńska, A. Bronisz, M. Żmudzińska, et al., An evaluation of visfatin levels in obese subjects Ocena stężenia wisfatyny u osób z otyłością, J. Endocrinol. 61 (2010) 169–173. http://www.ncbi.nlm.nih.gov/pubmed/20464702.

[114] S. Barth, P. Klein, T. Horbach, J. Dötsch, M. Rauh, W. Rascher, et al., Expression of neuropeptide Y, omentin and visfatin in visceral and subcutaneous adipose tissues in humans: Relation to endocrine and clinical parameters, Obes. Facts. 3 (2010) 245–251. doi:10.1159/000319508.

[115] S.R. Costford, S. Bajpeyi, M. Pasarica, D.C. Albarado, S.C. Thomas, H. Xie, et al., Skeletal muscle NAMPT is induced by exercise in humans., Am. J. Physiol. Endocrinol. Metab. 298 (2010) E117–26. doi:10.1152/ajpendo.00318.2009.

[116] P.W. Caton, J. Kieswich, M.M. Yaqoob, M.J. Holness, M.C. Sugden, Nicotinamide mononucleotide protects against pro-inflammatory cytokine-mediated impairment of mouse islet function., Diabetologia. 54 (2011) 3083–92. doi:10.1007/s00125-011-2288-0.

[117] M.A. Nowell, P.J. Richards, C.A. Fielding, S. Ognjanovic, N. Topley, A.S. Williams, et al., Regulation of pre-B cell colony-enhancing factor by STAT-3-dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis., Arthritis Rheum. 54 (2006) 2084–95. doi:10.1002/art.21942.

[118] Y.J. Choi, S.E. Choi, E.S. Ha, Y. Kang, S.J. Han, D.J. Kim, et al., Involvement of visfatin in palmitate-induced upregulation of inflammatory cytokines in hepatocytes, Metabolism. 60 (2011) 1781–1789. doi:10.1016/j.metabol.2011.05.003.

[119] H. Li, M. Xu, J. Lee, C. He, Z. Xie, Leucine Supplementation Increases SIRT1 Expression and Prevents Mitochondrial Dysfunction and Metabolic Disorders in High Fat Diet-induced Obese Mice, AJP Endocrinol. Metab. 303 (2012) E1234–44. doi:10.1152/ajpendo.00198.2012.

[120] S.-K. Bae, S.-R. Kim, J.G. Kim, J.Y. Kim, T.H. Koo, H.-O. Jang, et al., Hypoxic induction of human visfatin gene is directly mediated by hypoxia-inducible factor-1., FEBS Lett. 580 (2006) 4105–13. doi:10.1016/j.febslet.2006.06.052.

[121] G. Tarantino, C. Finelli, What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome?, World J. Gastroenterol. 19 (2013) 3375–84. doi:10.3748/wjg.v19.i22.3375.

[122] R. Aller, D. a. De Luis, O. Izaola, M.G. Sagrado, R. Conde, M.C. Velasco, et al., Influence of visfatin on histopathological changes of non-alcoholic fatty liver disease, Dig. Dis. Sci. 54 (2009) 1772–1777. doi:10.1007/s10620-008-0539-9.

[123] H. Genc, T. Dogru, M. Kara, S. Tapan, C.N. Ercin, C. Acikel, et al., Association of plasma visfatin with hepatic and systemic inflammation in nonalcoholic fatty liver disease, Ann. Hepatol. 12 (2013) 548–555.

[124] E. Akbal, E. Kocak, A. Tas, E. Yuksel, S. Koklu, Visfatin levels in nonalcoholic fatty liver disease, J. Clin. Lab. Anal. 26 (2012) 115–119. doi:10.1002/jcla.21491.

[125] R. Gaddipati, M. Sasikala, N. Padaki, R.M. Mukherjee, A. Sekaran, M. Jayaraj-Mansard, et al., Visceral adipose tissue visfatin in nonalcoholic fatty liver disease, Ann. Hepatol. 9 (2010) 266–270.

[126] Z.-F. Zhang, S.-H. Fan, Y.-L. Zheng, J. Lu, D.-M. Wu, Q. Shan, et al., Troxerutin improves hepatic lipid homeostasis by restoring NAD(+)-depletion-mediated dysfunction of lipin 1 signaling in high-fat diet-treated mice, Biochem. Pharmacol. 91 (2014) 74–86. doi:10.1016/j.bcp.2014.07.002.

[127] H. Li, M. Xu, J. Lee, C. He, Z. Xie, Leucine supplementation increases SIRT1 expression and prevents mitochondrial dysfunction and metabolic disorders in high-fat diet-induced obese mice, Am. J. Physiol. Endocrinol. Metab. 303 (2012) E1234–44. doi:10.1152/ajpendo.00198.2012.

[128] R. Tao, D. Wei, H. Gao, Y. Liu, R. a. DePinho, X.C. Dong, Hepatic FoxOs regulate lipid metabolism via modulation of expression of the nicotinamide phosphoribosyltransferase gene., J. Biol. Chem. 286 (2011) 14681–14690. doi:10.1074/jbc.M110.201061.

[129] R.E. Castro, D.M.S. Ferreira, M.B. Afonso, P.M. Borralho, M. V Machado, H. Cortez-Pinto, et al., miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease., J. Hepatol. 58 (2013) 119–25. doi:10.1016/j.jhep.2012.08.008.

[130] S.E. Choi, T. Fu, S. Seok, D.H. Kim, E. Yu, K.W. Lee, et al., Elevated microRNA-34a in obesity reduces NAD+ levels and SIRT1 activity by directly targeting NAMPT, Aging Cell. 12 (2013) 1062–1072. doi:10.1111/acel.12135.

[131] D.W. Frederick, J.G. Davis, A. Dávila, B. Agarwal, S. Michan, M.A. Puchowicz, et al., Increasing NAD Synthesis in Muscle via Nicotinamide Phosphoribosyltransferase Is Not Sufficient to Promote Oxidative Metabolism., J. Biol. Chem. 290 (2015) 1546–58. doi:10.1074/jbc.M114.579565.

[132] C. Escande, C.C.S. Chini, V. Nin, K.M. Dykhouse, C.M. Novak, J. Levine, et al., Deleted in breast cancer-1 regulates SIRT1 activity and contributes to high-fat diet-induced liver steatosis in mice., J. Clin. Invest. 120 (2010) 545–58. doi:10.1172/JCI39319.

[133] S.E. Kahn, R.L. Hull, K.M. Utzschneider, Mechanisms linking obesity to insulin resistance and type 2 diabetes., Nature. 444 (2006) 840–846. doi:10.1038/nature05482.

[134] A.S. Al-Goblan, M.A. Al-Alfi, M.Z. Khan, Mechanism linking diabetes mellitus and obesity., Diabetes. Metab. Syndr. Obes. 7 (2014) 587–91. doi:10.2147/DMSO.S67400.

[135] N.E. Gulcelik, A. Usman, A. Gürlek, Role of adipocytokines in predicting the development of diabetes and its late complications, Endocrine. 36 (2009) 397–403. doi:10.1007/s12020-009-9234-7.

[136] M. Blüher, Adipokines - removing road blocks to obesity and diabetes therapy, Mol. Metab. 3 (2014) 230–240. doi:10.1016/j.molmet.2014.01.005.

[137] C. Hug, H.F. Lodish, Medicine. Visfatin: a new adipokine., Science. 307 (2005) 366–367. doi:10.1126/science.1106933.

[138] V. Varma, A. Yao-Borengasser, N. Rasouli, A.M. Bodles, B. Phanavanh, M.J. Lee, et al., Human visfatin expression: Relationship to insulin sensitivity, intramyocellular lipids, and inflammation, J. Clin. Endocrinol. Metab. 92 (2007) 666–672. doi:10.1210/jc.2006-1303.

[139] H. Hajianfar, A. Bahonar, M.H. Entezari, G. Askari, M. Yazdani, Lipid profiles and serum Visfatin concentrations in patients with type II diabetes in comparison with healthy controls, Int. J. Prev. Med. 3 (2012) 326–331.

[140] H.O. El-Mesallamy, D.H. Kassem, E. El-Demerdash, A.I. Amin, Vaspin and visfatin/Nampt are interesting interrelated adipokines playing a role in the pathogenesis of type 2 diabetes mellitus, Metabolism. 60 (2011) 63–70. doi:10.1016/j.metabol.2010.04.008.

[141] M.-P. Chen, F.-M. Chung, D.-M. Chang, J.C.-R. Tsai, H.-F. Huang, S.-J. Shin, et al., Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus., J. Clin. Endocrinol. Metab. 91 (2006) 295–299. doi:10.1210/jc.2005-1475.

[142] A. López-Bermejo, B. Chico-Julià, M. Fernàndez-Balsells, M. Recasens, E. Esteve, R. Casamitjana, et al., Serum visfatin increases with progressive β-cell deterioration, Diabetes. 55 (2006) 2871–2875. doi:10.2337/db06-0259.

[143] M.I. Yilmaz, M. Saglam, A.R. Qureshi, J.J. Carrero, K. Caglar, T. Eyileten, et al., Endothelial dysfunction in type-2 diabetics with early diabetic nephropathy is associated with low circulating adiponectin, Nephrol. Dial. Transplant. 23 (2008) 1621–1627. doi:10.1093/ndt/gfm828.

[144] Z.M.A.-N. Tarek M.K. Motawi, Olfat G. Shaker, Maha M. El-Sawalhi, No TitleVisfatin −948G/T and resistin −420C/G polymorphisms in Egyptian type 2 diabetic patients with and without cardiovascular diseases, Genome. 57 (2014) 259–266.

[145] H. Xie, S.Y. Tang, X.H. Luo, J. Huang, R.R. Cui, L.Q. Yuan, et al., Insulin-like effects of visfatin on human osteoblasts, Calcif. Tissue Int. 80 (2007) 201–210. doi:10.1007/s00223-006-0155-7.

[146] K.M. Ramsey, K.F. Mills, A. Satoh, S.-I. Imai, Age-associated loss of Sirt1-mediated enhancement of glucose-stimulated insulin secretion in beta cell-specific Sirt1-overexpressing (BESTO) mice., Aging Cell. 7 (2008) 78–88. doi:10.1111/j.1474-9726.2007.00355.x.

[147] R. Spinnler, T. Gorski, K. Stolz, S. Schuster, A. Garten, A.G. Beck-Sickinger, et al., The adipocytokine Nampt and its product NMN have no effect on beta-cell survival but potentiate glucose stimulated insulin secretion., PLoS One. 8 (2013) e54106. doi:10.1371/journal.pone.0054106.

[148] J. Yoshino, K.F. Mills, M.J. Yoon, S. Imai, Nicotinamide Mononucleotide, a Key NAD+ Intermediate, Treats the Pathophysiology of Diet- and Age-Induced Diabetes in Mice, Cell Metab. 14 (2011) 528–536. doi:10.1016/j.cmet.2011.08.014.

[149] S. Imai, L. Guarente, NAD+ and sirtuins in aging and disease., Trends Cell Biol. 24 (2014) 464–71. doi:10.1016/j.tcb.2014.04.002.

[150] E. van der Veer, C. Ho, C. O'Neil, N. Barbosa, R. Scott, S.P. Cregan, et al., Extension of human cell lifespan by nicotinamide phosphoribosyltransferase., J. Biol. Chem. 282 (2007) 10841–5. doi:10.1074/jbc.C700018200.

[151] L.A. Villalobos, A. Uryga, T. Romacho, A. Leivas, C.F. Sánchez-Ferrer, J.D. Erusalimsky, et al., Visfatin/Nampt induces telomere damage and senescence in human endothelial cells., Int. J. Cardiol. 175 (2014) 573–5. doi:10.1016/j.ijcard.2014.05.028.

[152] J. Song, S.-F. Ke, C.-C. Zhou, S.-L. Zhang, Y.-F. Guan, T.-Y. Xu, et al., Nicotinamide phosphoribosyltransferase is required for the calorie restriction-mediated improvements in oxidative stress, mitochondrial biogenesis, and metabolic adaptation., J. Gerontol. A. Biol. Sci. Med. Sci. 69 (2014) 44–57. doi:10.1093/gerona/glt122.

[153] B. Artegiani, F. Calegari, Age-related cognitive decline: can neural stem cells help us?, Aging (Albany. NY). 4 (2012) 176–86.

[154] L.R. Stein, S.I. Imai, Specific ablation of Nampt in adult neural stem cells recapitulates their functional defects during aging., EMBO J. 33 (2014) 1321–40. doi:10.1002/embj.201386917.

[155] M. Olszanecka-Glinianowicz, A. Owczarek, M. Bożentowicz-Wikarek, A. Brzozowska, M. Mossakowska, T. Zdrojewski, et al., Relationship between circulating visfatin/NAMPT, nutritional status and insulin resistance in an elderly population - results from the PolSenior substudy., Metabolism. 63 (2014) 1409–18. doi:10.1016/j.metabol.2014.07.013.

[156] C. Franceschi, M. Bonafè, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani, et al., Inflamm-aging. An evolutionary perspective on immunosenescence., Ann. N. Y. Acad. Sci. 908 (2000) 244–54.

[157] G. Cavadini, S. Petrzilka, P. Kohler, C. Jud, I. Tobler, T. Birchler, et al., TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription., Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 12843–8. doi:10.1073/pnas.0701466104.

[158] O. WARBURG, On respiratory impairment in cancer cells., Science. 124 (1956) 269–70.

[159] A. Chiarugi, C. Dölle, R. Felici, M. Ziegler, The NAD metabolome--a key determinant of cancer cell biology., Nat. Rev. Cancer. 12 (2012) 741–52. doi:10.1038/nrc3340.

[160] S. Schuster, M. Penke, T. Gorski, R. Gebhardt, T.S. Weiss, W. Kiess, et al., FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signalling in hepatocarcinoma cells, Biochem. Biophys. Res. Commun. (2015). doi:10.1016/j.bbrc.2015.01.111.

[161] A. Nahimana, A. Attinger, D. Aubry, P. Greaney, C. Ireson, A. V Thougaard, et al., The NAD biosynthesis inhibitor APO866 has potent antitumor activity against hematologic malignancies., Blood. 113 (2009) 3276–86. doi:10.1182/blood-2008-08-173369.

[162] U.H. Olesen, M.K. Christensen, F. Björkling, M. Jäättelä, P.B. Jensen, M. Sehested, et al., Anticancer agent CHS-828 inhibits cellular synthesis of NAD., Biochem. Biophys. Res. Commun. 367 (2008) 799–804. doi:10.1016/j.bbrc.2008.01.019.

[163] U. Galli, C. Travelli, A. Massarotti, G. Fakhfouri, R. Rahimian, G.C. Tron, et al., Medicinal chemistry of nicotinamide phosphoribosyltransferase (NAMPT) inhibitors., J. Med. Chem. 56 (2013) 6279–96. doi:10.1021/jm4001049.

[164] T. Zhou, T. Wang, J.G.N. Garcia, Expression of nicotinamide phosphoribosyltransferase-influenced genes predicts recurrence-free survival in lung and breast cancers., Sci. Rep. 4 (2014) 6107. doi:10.1038/srep06107.

[165] M. Cea, A. Cagnetta, M. Fulciniti, Y.-T. Tai, T. Hideshima, D. Chauhan, et al., Targeting NAD+ salvage pathway induces autophagy in multiple myeloma cells via mTORC1 and extracellular signal-regulated kinase (ERK1/2) inhibition., Blood. 120 (2012) 3519–29. doi:10.1182/blood-2012-03-416776.

[166] B.K. Thakur, T. Dittrich, P. Chandra, A. Becker, W. Kuehnau, J.-H. Klusmann, et al., Involvement of p53 in the cytotoxic activity of the NAMPT inhibitor FK866 in myeloid leukemic cells., Int. J. Cancer. 132 (2013) 766–74. doi:10.1002/ijc.27726.

[167] K. Zhang, B. Zhou, P. Zhang, Z. Zhang, P. Chen, Y. Pu, et al., Genetic variants in NAMPT predict bladder cancer risk and prognosis in individuals from southwest Chinese Han group., Tumour Biol. 35 (2014) 4031–40. doi:10.1007/s13277-013-1527-z.

[168] W. Tian, Y. Zhu, Y. Wang, F. Teng, H. Zhang, G. Liu, et al., Visfatin, a potential biomarker and prognostic factor for endometrial cancer., Gynecol. Oncol. 129 (2013) 505–12. doi:10.1016/j.ygyno.2013.02.022.

[169] P.S. Reddy, S. Umesh, B. Thota, A. Tandon, P. Pandey, A.S. Hegde, et al., PBEF1/NAmPRTase/Visfatin: a potential malignant astrocytoma/glioblastoma serum marker with prognostic value., Cancer Biol. Ther. 7 (2008) 663–8.

[170] S. Ninomiya, M. Shimizu, K. Imai, K. Takai, M. Shiraki, T. Hara, et al., Possible role of visfatin in hepatoma progression and the effects of branched-chain amino acids on visfatin-induced proliferation in human hepatoma cells., Cancer Prev. Res. (Phila). 4 (2011) 2092–100. doi:10.1158/1940-6207.CAPR-11-0340.

[171] V. Audrito, S. Serra, D. Brusa, F. Mazzola, F. Arruga, T. Vaisitti, et al., Extracellular nicotinamide phosphoribosyltransferase (NAMPT) promotes M2 macrophage polarization in chronic lymphocytic leukemia., Blood. 125 (2015) 111–23. doi:10.1182/blood-2014-07-589069.

[172] R.J. Bułdak, Ł. Bułdak, R. Polaniak, M. Kukla, E. Birkner, R. Kubina, et al., Visfatin affects redox adaptative responses and proliferation in Me45 human malignant melanoma cells: an in vitro study., Oncol. Rep. 29 (2013) 771–8. doi:10.3892/or.2012.2175.

[173] R. Adya, B.K. Tan, A. Punn, J. Chen, H.S. Randeva, Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis., Cardiovasc. Res. 78 (2008) 356–65. doi:10.1093/cvr/cvm111.

[174] U.H. Olesen, J.G. Petersen, A. Garten, W. Kiess, J. Yoshino, S.-I. Imai, et al., Target enzyme mutations are the molecular basis for resistance towards pharmacological inhibition of nicotinamide phosphoribosyltransferase., BMC Cancer. 10 (2010) 677.

[175] T.-Q. Bi, X.-M. Che, X.-H. Liao, D.-J. Zhang, H.-L. Long, H.-J. Li, et al., Overexpression of Nampt in gastric cancer and chemopotentiating effects of the Nampt inhibitor FK866 in combination with fluorouracil., Oncol. Rep. 26 (2011) 1251–7. doi:10.3892/or.2011.1378.

[176] I. Gehrke, E.D.J. Bouchard, S. Beiggi, A.G. Poeppl, J.B. Johnston, S.B. Gibson, et al., On-target effect of FK866, a nicotinamide phosphoribosyl transferase inhibitor, by apoptosis-mediated death in chronic lymphocytic leukemia cells., Clin. Cancer Res. 20 (2014) 4861–72. doi:10.1158/1078-0432.CCR-14-0624.

[177] M. Cea, G. Zoppoli, S. Bruzzone, F. Fruscione, E. Moran, A. Garuti, et al., APO866 activity in hematologic malignancies: a preclinical in vitro study., Blood. 113 (2009) 6035–7; author reply 6037–8. doi:10.1182/blood-2009-03-209213.

[178] D. Soncini, I. Caffa, G. Zoppoli, M. Cea, A. Cagnetta, M. Passalacqua, et al., Nicotinamide phosphoribosyltransferase promotes epithelial-to-mesenchymal transition as a soluble factor independent of its enzymatic activity., J. Biol. Chem. 289 (2014) 34189–204. doi:10.1074/jbc.M114.594721.

[179] B. Tan, D.A. Young, Z.-H. Lu, T. Wang, T.I. Meier, R.L. Shepard, et al., Pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme essential for NAD+ biosynthesis, in human cancer cells: metabolic basis and potential clinical implications., J. Biol. Chem. 288 (2013) 3500–11. doi:10.1074/jbc.M112.394510.

[180] K.S. Tummala, A.L. Gomes, M. Yilmaz, O. Graña, L. Bakiri, I. Ruppen, et al., Inhibition of de novo NAD(+) synthesis by oncogenic URI causes liver tumorigenesis through DNA damage., Cancer Cell. 26 (2014) 826–39. doi:10.1016/j.ccell.2014.10.002.

[181] A. von Heideman, A. Berglund, R. Larsson, P. Nygren, Safety and efficacy of NAD depleting cancer drugs: results of a phase I clinical trial of CHS 828 and overview of published data., Cancer Chemother. Pharmacol. 65 (2010) 1165–72. doi:10.1007/s00280-009-1125-3.

[182] K. Holen, L.B. Saltz, E. Hollywood, K. Burk, A.-R. Hanauske, The pharmacokinetics, toxicities, and biologic effects of FK866, a nicotinamide adenine dinucleotide biosynthesis inhibitor., Invest. New Drugs. 26 (2008) 45–51. doi:10.1007/s10637-007-9083-2.

[183] US National Library of Medicine. ClinicalTrials.gov [online], CHS 828 in Treating Patients With Solid Tumors (Phase 1), 2012.

[184] US National Library of Medicine. ClinicalTrials.gov [online], A Phase I/II Study of GMX1777 in Combination With Temozolomide for the Treatment of Metastatic Melanoma, 2013.

[185] US National Library of Medicine. ClinicalTrials.gov [online], Safety and Efficacy of GMX1777 in the Treatment of Refractory Solid Tumors or Lymphomas, 2013.

[186] US National Library of Medicine. ClinicalTrials.gov [online], A Study of APO866 for the Treatment of Cutaneous T-cell Lymphoma, 2013.

[187] US National Library of Medicine. ClinicalTrials.gov [online], A Phase I/II Study to Assess the Safety and Tolerability of APO866 for the Treatment of Refractory B-CLL, 2013.

[188] US National Library of Medicine. ClinicalTrials.gov [online], A Study to Assess APO866 for the Treatment of Advanced Melanoma, 2013.

[189] M. Chan, M. Gravel, A. Bramoullé, G. Bridon, D. Avizonis, G.C. Shore, et al., Synergy between the NAMPT inhibitor GMX1777(8) and pemetrexed in non-small cell lung cancer cells is mediated by PARP activation and enhanced NAD consumption., Cancer Res. 74 (2014) 5948–54. doi:10.1158/0008-5472.CAN-14-0809.

Chapter 2

Hypothesis, aims and scientific questions

Non-alcoholic fatty liver disease (NAFLD) is an increasing public health problem with limited treatment options and incomplete characterisation of pathogenic factors. Therefore it is crucial to understand the pathological mechanisms to find novel targets for the treatment of NAFLD. It has been already shown that the NAD salvage pathway and NAD-dependent enzymes, e.g. sirtuin 1, are regulators of a majority of processes during the progression of NAFLD such as mitochondrial biogenesis, apoptosis, inflammation or *de novo* lipogenesis. Consequentially the hypothesis of my doctoral thesis is that the NAD salvage pathway is dysregulated during the progression of non-alcoholic fatty liver disease.

The aim of the study described in *Chapter 3* was to investigate the NAD salvage pathway in the early stage of NAFLD in a diet-induced mouse model of hepatic steatosis. The following questions were aimed to be answered:

1. Does hepatic steatosis caused by high-fat diet affect hepatic NAMPT expression and activity as well as NAD levels?
2. Are the NAD-dependent enzymes SIRT1 and SIRT3 dysregulated in hepatic steatosis?
3. How are downstream targets of SIRT1 and SIRT3 and thus central metabolic pathways in NAFLD regulated?

Contrary some other studies, NAMPT mRNA, protein abundance and activity as well as hepatic NAD levels were increased in murine steatotic livers. While SIRT3 protein abundance was unchanged in the present study, SIRT1 protein levels and activity were up regulated which was further demonstrated by altered acetylation and transcription of SIRT1 downstream targets. This study suggests that the hepatic NAD salvage pathway is enhanced during hepatic lipid accumulation indicating that the maintenance of NAD levels could be a useful therapy option to suppress progression from steatosis to NASH.

NASH is a high risk factor for the development of the hepatocellular carcinoma (HCC), a malignant disease with currently only one treatment option. *Chapter 4* of this thesis was focused on whether or not inhibition of NAMPT by its specific inhibitor FK866 could be a potential therapeutic approach in HCC. That implied the following scientific questions:

1. Are hepatocarcinoma cells and primary human hepatocytes differentially affected by FK866?
2. Can NAMPT inhibition induce energy depletion and apoptosis in hepatocarcinoma cells?
3. Which molecular mechanisms underlie the effect of FK866?

It was found that primary human hepatocytes are less sensitive to FK866 than hepatocarcinoma cells. FK866 treatment led to decreased NAD and ATP levels as well as to induction of apoptosis. The effects of FK866 were mediated by the AMPK/mTOR signalling pathway. This study points out that inhibition of NAD biosynthesis by FK866 could be a new approach in the therapy of HCC.

Chapter 3

Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet

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3.1 Abstract

Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme for NAD salvage and the abundance of Nampt has been shown to be altered in non-alcoholic fatty liver disease. It is, however, unknown how hepatic Nampt is regulated in response to accumulation of lipids in the liver of mice fed a high-fat diet (HFD). HFD mice gained more weight, stored more hepatic lipids and had an impaired glucose tolerance compared with control mice. NAD levels as well as Nampt mRNA expression, protein abundance and activity were significantly increased in HFD mice. Enhanced NAD levels were associated with deacetylation of p53 and Nfκb indicating increased activation of Sirt1. Despite impaired glucose tolerance and increased hepatic lipid levels in HFD mice, NAD metabolism was significantly enhanced. Thus, improved NAD metabolism may be a compensatory mechanism protect against negative impact of hepatic lipid accumulation.

3.2 Introduction

More than 30 % of Western adult population is affected by non-alcoholic fatty liver diseases (NAFLD) with a rapidly increasing number in obese and diabetic people. NAFLD covers a wide range of conditions from simple steatosis caused by accumulation of triglycerides to non-alcoholic steatohepatitis (NASH) which is associated with inflammatory processes. NASH is a risk factor for fibrosis and cirrhosis which are associated with a significant liver-related mortality and a median survival of 6 years [1].

The pathological pathways leading to NASH are not yet fully understood. Interestingly, specific sirtuins (Sirts) seem to affect the development of NAFLD by regulating processes like hepatic gluconeogenesis, mitochondrial biogenesis and fatty acid synthesis. Liver-specific Sirt1 knockout mice develop hepatic steatosis and show impaired insulin signaling on a high-fat diet (HFD) [2], while global transgenic mice overexpressing Sirt1 are protected against the negative effects of a HFD such as inflammation, impaired glucose tolerance and hepatic steatosis [3]. Additionally, whole-body Sirt3 knockout mice show enhanced storage of hepatic triglycerides and insulin resistance [4] although liver-specific Sirt3 knockout mice do not display an overt phenotype [5].

Sirtuins are nicotinamide adenine dinucleotide (NAD) dependent in respect to their deacetylase activity. The key enzyme in the mammalian NAD salvage pathway starting from nicotinamide is nicotinamide phosphoribosyltransferase (Nampt) which converts nicotinamide to nicotinamide mononucleotide (NMN), an intermediate in NAD biosynthesis [6]. Additionally, Nampt is secreted from adipocytes [7], hepatocytes [8], and leucocytes [9]. Nampt circulates in the blood where it acts as a NMN biosynthetic enzyme [10] and/or cytokine [11]. Several human studies have investigated how Nampt protein levels in biopsies and blood are affected in individuals with steatosis and NASH, however, results are conflicting [12–14].

It has been shown that rats fed a HFD develop fatty liver and hepatic insulin resistance already after 3 days of HFD feeding [15]. Moreover, another study in mice showed that hepatic insulin

resistance is present already after 3 days on a HFD before insulin resistance develops in muscle and adipose tissue [16]. Thus, hepatic insulin resistance develops very rapidly, but whether NAD metabolism is also affected in the early stages of the development of NAFLD is unknown. We hypothesize a link between the development of NAFLD and the ability to maintain NAD levels. To define the effect of lipid accumulation on hepatic NAD metabolism, we fed mice a HFD for 11 weeks and examined the effect on hepatic NAD metabolism. Despite hepatic lipid accumulation and impaired whole body glucose tolerance in mice fed a HFD, we found an improved hepatic NAD metabolism suggesting that this may be a compensatory mechanism to protect against the negative impact of hepatic lipid accumulation on inflammation and apoptosis.

3.3 Methods and Procedures

Mice and Ethical approval

All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the EU convention for protection of vertebra animals used for scientific purposes (Council of Europe 123, Strasbourg, France, 1985). Twenty-four male mice were obtained from Taconic when they were 10 weeks of age (C57BL/6Ntac, #DIO-B6). At the time of arrival in the laboratory, mice had been on a HFD (Research Diets Industry, D12492) or corresponding chow diet (Ctl) for 4 weeks. Mice were kept in a 12:12-h light:dark cycle and kept on the diet for additional 7 weeks. Water was given ad libitum. Mice were either single- (s) or group-housed (gr). To confirm that housing conditions (i.e., single- vs. group-housing) did not affect NAD metabolism, we measured Nampt protein abundance and NAD levels in a separate cohort of mice. In this cohort we applied 26 carefully weight matched male mice (C57BL/6JBomTac) at 7 weeks of age. Upon arrival, the mice were acclimatized on a standard chow diet for 10 days before they were randomly assigned to the experimental groups and fed either a HFD (D12492) or a corresponding low fat diet (D12450J) for 11 weeks. The mice were either single-housed (5 mice per group per diet) or group-housed (8 mice per group per diet, 4 mice per cage) from the point of arrival to the day of termination. Housing conditions did not affect hepatic Nampt protein and NAD levels (Figure S1A,B). After 11 weeks on their respective diets, mice were anesthetized (Pentobarbital, 100 mg/kg body weight) and livers were carefully dissected, frozen in liquid nitrogen, and stored at -80°C until further analysis. Mice were all sacrificed at the same time point.

Metabolic characterization

Animals were weighed and body composition was assessed by MR scanning (EchoMRI^{TM}, USA). At the age of 14 and 15 weeks an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were performed, respectively. Both tests were performed after a 6-hour fast and blood glucose measurements were done from the tail vein. For the OGTT a bolus of glucose (25 % glucose/saline solution, 2 g glucose/kg lean body mass) was delivered by oral gavage, and tail vein blood glucose

was measured just before and at 20, 40, 60, 90 and 120-minutes after the glucose bolus. The ITT was performed following 8 days of recovery after the OGTT. Insulin (100U/ml) was diluted in gelofusine (B. Braun, Denmark) for a working solution of 0.333U/ml, and administered by intraperitoneal injection (0.75 U/kg lean body mass). Blood glucose in the tail blood was measured before and at 15, 30, 45, 60, 90 and 120-minutes after the injection. The mice were conscious, and placed in their cages during both tests. A week before collecting tissues, 200 µl of blood was drawn from the mandible into EDTA coated tubes, left to clot at room temperature for 30-minutes and spun down at 2000xg at 4 C° for 10-minutes. Supernatants were transferred to clean tubes and stored at -80 C°.

Triacylglycerol measurement of liver tissue

The triacylglycerol (TAG) content of the liver tissue was quantified by ^{1}H-high resolution magic-angle spinning-nuclear magnetic resonance spectroscopy. Five to fifteen milligrams of intact liver tissue was transferred into a 4mm zirconia HR-MAS rotor with a volume of 15 µl. Additionally, 5 µl of a 100 µM solution of trisodium phosphate was added as reference. The NMR spectra were recorded using a 600MHz Bruker Avance III NMR spectrometer (Bruker, Rheinstetten, Germany). All measurements were conducted under MAS at a frequency of 9kHz and a temperature of 25°C. The water signal was suppressed by presaturation (1mW power for 3s). NMR spectra were excited by a $\pi/2$ pulse of 4 µs duration. To allow for complete relaxation, the delay time between successive scans was 30 s. The amount of TAG was determined by comparing the integrals of the trisodium phosphate signal with the glycerol backbone signal at 4.29 ppm which is exclusively found in TAG. All spectra were corrected for baseline and phase distortions using Spinworks (University of Manitoba). Deconvolution of the signals was performed using an in-house written SciPy script. The peaks were fitted to a Voigt-Profile applying a constrained least-squares approach based on the L-BGFGS optimization algorithm [17]. The TAG content is expressed per gram liver tissue.

Protein extraction, Western blot analyses and immunoprecipitation

Approximately ten milligrams liver tissue was lysed in modified RIPA buffer as previously described [18]. Protein concentration was determined using Pierce BCA protein assay (Thermo Scientific) and equal amounts of protein were separated by SDS-PAGE and transferred using a semi-dry transfer apparatus to nitrocellulose membranes. Next, membranes were blocked in 5 % non-fat dry milk in TBS buffer containing 0.1 % Tween 20. Applied antibodies are listed in Supplementary Table S1. Detection of proteins was carried out using Luminata Classico Western HRP Substrate (Merck Millipore) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Gapdh immunoblotting was performed as a loading control. For immunoprecipitation livers were incubated with anti-acetyl lysine antibody conjugated to µMACS Protein A Micro Beads (Miltenyi Biotec) overnight and eluted with 1x SDS sample buffer. After 5 min heating, samples were analyzed by Western blotting with an anti-Srebp1 antibody (H-160, Santa Cruz Biotechnology).

Total RNA extraction and realtime qPCR

Total RNA of liver tissue was extracted by TRIzol® Reagent (Life Technologies) according to manufacturer's protocol. One microgram of total RNA was transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen). Quantitative PCR analyses were performed using the qPCR Master Mix Plus Low ROX (Eurogentec) or Absolute qPCR SYBR Green Low ROX Mix (Thermo Scientific) and the Applied Biosystems 7500 Real Time PCR System. Primer sequences are summarized in Supplementary Table S2.

NAD measurement

NAD was measured by EnzyChrom™ NAD^+/NADH Assay Kit (E2ND-100, Biotrend) according to manufacturer's protocol or reversed-phase HPLC using the Chromaster Purospher STAR RP-18 endcapped 3 µm Hibar RT 150-3 HPLC column (Merck). Ten milligram of frozen liver tissue was sonicated in 100 µl 1 M perchloric acid. After a 10-minute incubation period on ice samples were centrifuged and the supernatant was neutralized with 3 M potassium carbonate. After repeated centrifugation samples were loaded onto the column as previously described [18].

Nampt enzyme activity

For determination of Nampt activity ten milligrams of liver tissue was lysed in 100 µl Nampt enzyme assay buffer (0.1 M sodium phosphate, pH 7.4) and protein concentration was determined using BCA protein assay. Thirty micrograms of protein was added to the reaction buffer and incubated at 37°C for 1h. Afterwards the assay using radiolabeled ^{14}C-nicotinamide was performed as previously described [8].

Serum Nampt measurement

Nampt in mouse serum was measured by the Nampt (Visfatin/PBEF) (mouse/rat) Dual ELISA Kit (AdipoGen Inc., Seoul, South Korea), according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed with the open source language R in version 3.0.2 for triacylglycerol measurements or with GraphPad Prism® software (5.03) for all other statistical comparisons. Significance levels were calculated by unpaired, two-tailed t-tests or by repeated two-way ANOVA with subsequent Bonferroni post-test where appropriate. For TAG measurement of liver tissue a two-tailed Kruskal-Wallis test was conducted to determine significant differences between Ctl and HFD groups, because data were not normally distributed. Data represent mean ± SEM. $p<0.05$ was considered significant. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to Ctl mice.

3.4 Results

Mice fed a high-fat diet have impaired glucose tolerance and increased hepatic lipid content while markers for fibrosis and inflammation are not altered

To determine how a HFD affects hepatic NAD metabolism, a cohort of mice were put on a HFD (60 % of total energy from fat) or Ctl diet for 11 weeks at 6 weeks of age. After 11 weeks on HFD, mice were significantly heavier (39.0±4.2 vs. 29.9±2.5 g) (Figure 1A) and had significantly increased amounts of body fat (13.3±1.1 vs. 3.3±0.6 g) (Figure 1B) compared to Ctl mice. Lean body mass was significantly higher in Ctl vs. HFD mice (25.5±0.5 vs. 23.0±0.3 g) (Figure 1C). HFD mice showed a significantly impaired oral glucose tolerance ($p<0.0001$). Significant differences in fasting blood glucose as well as at 20, 40, 60 and 90 min were found between the Ctl and HFD groups (Figure 1D). In the insulin tolerance test a main effect of diet ($p=0.0018$) and a borderline significant interaction effect were observed ($p=0.055$; observed power,$P=0.78$) (Figure 1E). Hepatic TAG content was 2.1-fold increased compared to Ctl mice (93.2 ± 12.6 vs. 44.1 ± 3.8 mg/g tissue) (Figure 1F). To further evaluate the state of NAFLD we measured a marker for macrophage infiltration, CD68, which was not different between both groups. *α-smooth muscle actin* (*α-Sma*) was decreased in HFD mice while other markers for fibrosis (*fibrinogen, collagen I*) were not different. *Interleukin-6* (*Il-6*) and *tumor necrosis factor alpha* (*Tnfα*) as markers for inflammation were not changed in HFD mice (Figure 1G). mRNA expression of the cytokines *chemokine (C-X-C motif) ligand 16* (*Cxcl16*), *monocyte chemoattractant protein 1* (*Mcp-I*) and *transforming growth factor β* (*Tgfβ*) were not different in the livers of mice fed a HFD compared to Ctl. mRNA expression of the co receptor of the T cell receptor *CD8* and the fatty acid translocase *CD36* were not changed in the livers of the HFD mice (Supplementary Fig. S2A). *Carnitine palmitoyltransferase (Cpt)-Iα* and-*II* are two mitochondrial enzymes which are important for β oxidation of long-chain fatty acids. Both stayed stable in mice fed a HFD (Supplementary Fig. S2B). Furthermore, we detected less endoplasmic reticulum stress in livers of HFD mice indicated by lower levels of phosphorylated eIF2α compared to Ctl mice (Supplementary Fig. S2C).

High-fat diet up regulates hepatic Nampt mRNA expression, protein levels, activity, and NAD levels

Nampt levels have been shown to be either up or down regulated in different studies on insulin resistance and glucose intolerance [19–21]. Interestingly, both mRNA and protein levels of Nampt were increased in HFD mice compared with Ctl mice by 1.9-fold and 1.7-fold, respectively (Figure 2A,B). This was in line with a significant 1.5-fold increase in Nampt enzyme activity (41.4±4.2 vs 65.8±5.8 cpm/µg protein x h) in the HFD fed mice (Figure 2C). Because Nampt is the key regulator of the mammalian NAD salvage pathway we measured hepatic NAD levels and found that NAD was significantly increased by 1.6-fold in the HFD mice compared to Ctl mice (4.2±0.3 vs. 6.7±0.5 nmol/ mg protein) (Figure 2D). Since hepatocytes are able to secrete Nampt [8], we measured

Nampt in mouse serum samples from the mice. However, no differences between HFD and Ctl group were observed (19.8±4.9 vs. 14.6±1.3 ng/ml) (Figure 2E).

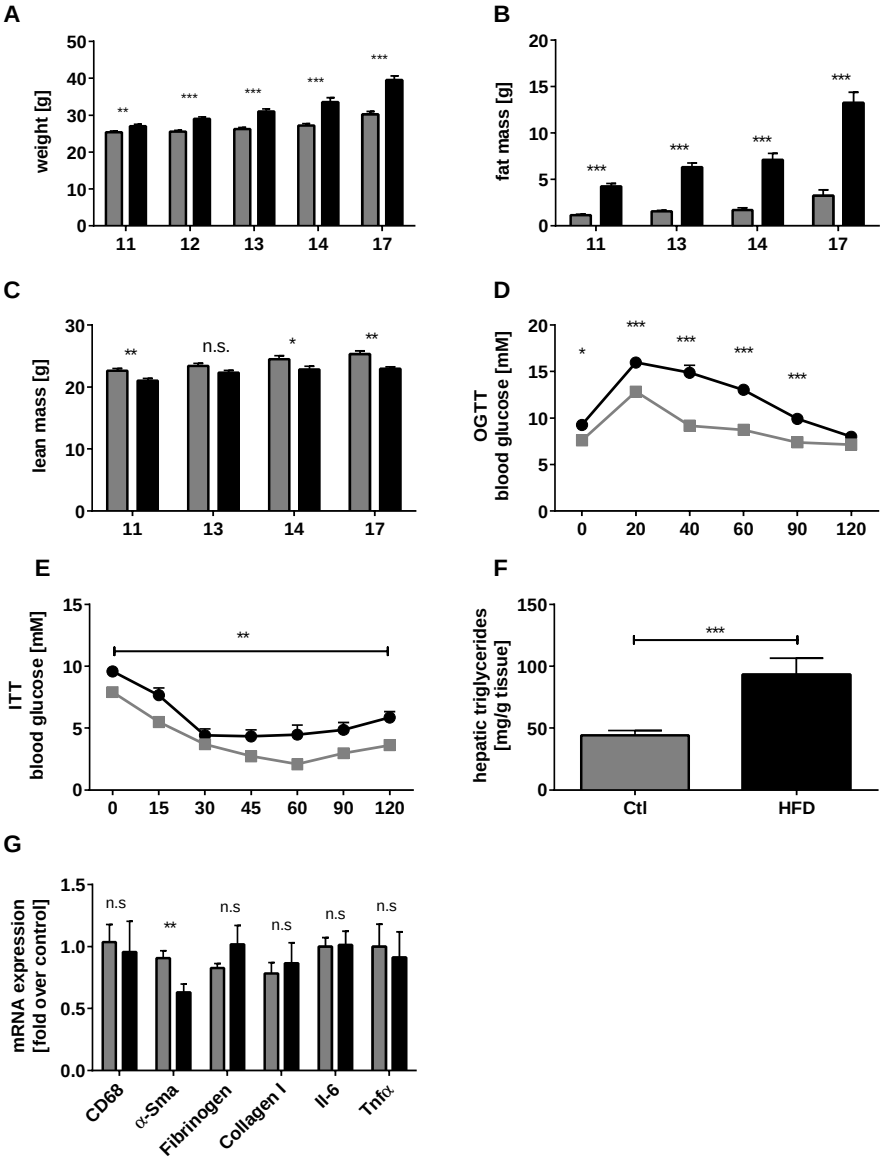

Fig. 1. Mice fed a high fat diet have impaired glucose tolerance and increased hepatic lipid content while markers for fibrosis and inflammation are not altered. Mice fed a high fat diet (HFD) for 11 weeks gained weight (A) and fat body mass (B) while lean body mass (C) was decreased compared to controls (Ctl). The HFD mice showed an impaired oral glucose tolerance (D) and insulin tolerance (E) compared to Ctl. Livers of HFD mice showed higher levels of triglycerides (F) but no indication for macrophage infiltration as measured by *CD68* expression. *α-Sma* was significantly down regulated in livers of mice fed a HFD while *fibrinogen* and *collagen I* expression as markers for fibrosis stayed stable. mRNA expressions of markers for inflammation (*TNFα* and, *Il-6*) were unchanged in both groups (G). Data are shown as means ± SEM. n.s., not significant, *p < 0.05, **p < 0.01 and ***p < 0.001 compared to Ctl mice.

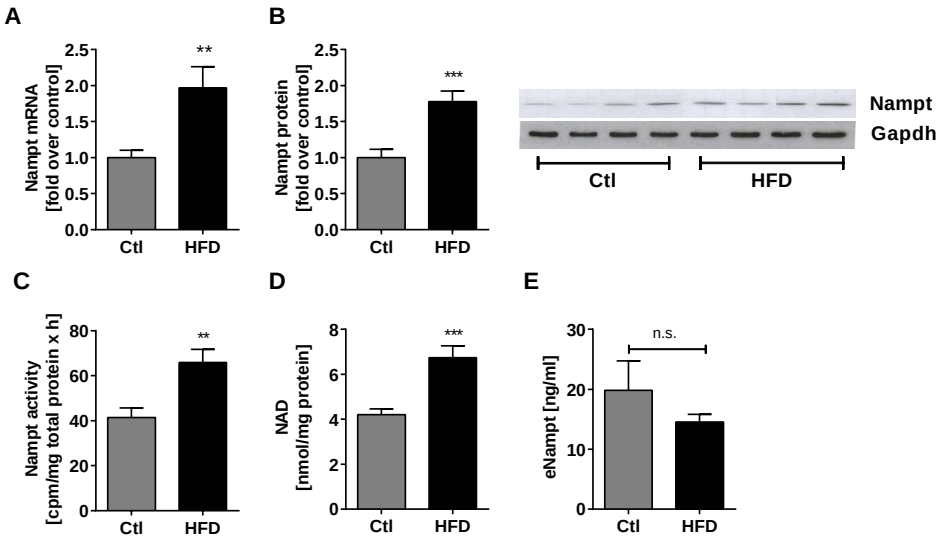

Fig. 2. HFD up regulates hepatic Nampt expression and activity as well as NAD levels. After a HFD for 11 weeks murine livers showed an increased Nampt mRNA (A) and protein amount (B) measured by qPCR or western blot analyses, respectively, compared to Ctl mice. Nampt activity (C) and intracellular NAD levels (D) measured by HPLC were enhanced. Serum Nampt levels were unchanged between both groups (E). Data are shown as means ± SEM (n = 12). One representative Western Blot is shown. **p < 0.01 and ***p < 0.001 compared to Ctl mice.

Acetylation of Nfκb and p53 is decreased while Sirt1 protein is increased in HFD mice

Since NAD is a required substrate for deacetylases and since total hepatic acetylation of the HFD mice was down regulated (Supplementary Fig. S3A), we measured the acetylation state of Nfκb (ac-Nfκb) and p53 (ac-p53), which are involved in the regulation of inflammation and apoptosis, respectively [22]. Total NfκB protein was up regulated in livers of HFD mice compared to Ctl mice while the acetylation of NfκB stayed stable resulting in 2.1-fold less acetylated NfκB (Figure 3A). We found that total and phosphorylated p53 protein was not changed (Supplementary Fig. S2D), while acetylated p53 was significantly reduced by 1.8-fold (Figure 3A), implicating that p53 was less active in murine livers of HFD mice compared to Ctl mice. In support of this finding, Bax, a downstream target of p53, was decreased by 1.6-fold (Figure 3A).

As NAD levels were up regulated resulting in lower levels of acetylated p53 and NfκB, we further investigated protein and mRNA levels of Sirt1 and Sirt3 which are proteins known to be regulated during NAFLD development [2,4]. No differences in *Sirt1* and *Sirt3* mRNA levels were detected between groups (Figure 3B). However, Sirt1 protein was significantly increased by 1.4-fold in the HFD group compared to Ctl while Sirt3 protein amount stayed stable (Figure 3C).

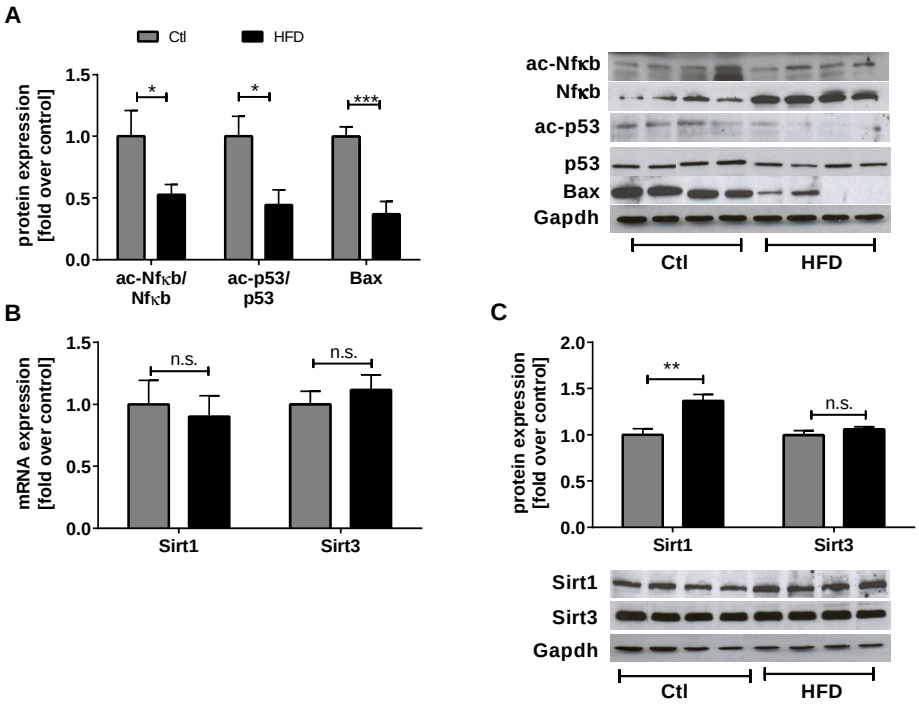

Fig.3. Acetylation state of Nfκb and p53 are down regulated in HFD mice which is associated with increased Sirt1 protein expression. The acetylation status of Nfκb (ac-Nfκb), acetylated p53 (ac-p53) and total Bax protein were decreased in livers of mice fed a HFD compared to Ctl mice as shown by densitometric analysis of Western blots (A). Sirt1 and Sirt3 mRNA (B) and protein levels (C) in liver tissues were measured by qPCR andWestern blot analyses, respectively. Hepatic Sirt1 protein was up regulated in mice fed a HFD for 11 weeks compared to Ctl mice. Data are shown as means ± SEM (n = 12). One representativeWestern blot is shown. Acetylated protein is normalized to the corresponding total protein. Gapdh is used as loading control. n.s., not significant, *p<0.05, **p<0.01 and ***p<0.001 compared to Ctl mice.

Expression of sirtuin downstream targets is altered in mice fed a HFD

The expression of further sirtuin targets that are associated with the pathogenesis of NAFLD was analyzed. *Mitochondrial uncoupling protein 2 (Ucp2)* was significantly reduced by 1.4-fold in HFD mice compared to Ctl mice while *peroxisome proliferator-activated receptor gamma coactivator 1 α* (*Pgc-1α)* and *mitochondrial superoxide dismutase (MnSOD)*, markers of the mitochondrial biogenesis, were unchanged (Figure 4A). Sirt3 deacetylates MnSOD at K122 resulting in increased MnSOD activity [23]. Using an antibody targeting acetylated MnSOD K122 (kindly provided by Prof. David Gius, North Westwestern University) we measured the acetylation state of MnSOD and found no differences on total protein and acetylation level (Supplementary Fig. S3B). Sirt1 is a transcriptional regulator of *sterol regulatory element-binding protein 1 (Srebp-1c)* [24], a key regulator of *de novo* lipogenesis in the liver. *Srebp-1c* was enhanced by 1.9-fold while its downstream targets *fatty acid synthase (Fas), stearoyl-CoA-desaturase 1* (*Scd1*) and *acetyl-coA-carboxylase* (*Acc*) were significantly declined (Figure 4B). Acetylation of Srebp-1 was not significantly different in both groups (Supplementary Fig. S3C). Glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pepck), key enzymes of gluconeogenesis, were both downregulated by 1.8-fold and 2.2-fold, respectively, in the livers of the HFD mice compared to control chow (Figure 4C).

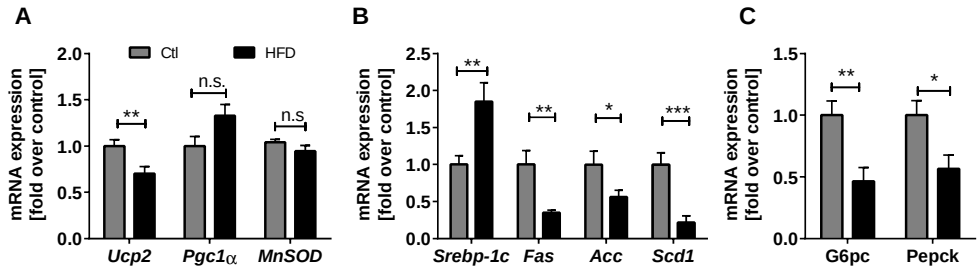

Fig. 4. Sirt1 downstream targets were altered in mice fed a HFD. HFD mice showed a reduced expression of *Ucp2* while *Pgc1α* and *MnSOD* were unchanged (A) as analysed by qPCR. *Srebp-1c* mRNA expression was up regulated while *Fas, Acc* and *Scd1* mRNA expression was declined compared to Ctl mice (B). *G6pc* and *Pepck* were both decreased in mice fed a HFD (C). n.s., not significant, *p < 0.05, **p < 0.01 and ***p < 0.001 compared to Ctl mice.

3.5 Discussion

The impact of hepatic lipid accumulation on NAD metabolism in the liver is incompletely known. To this end we evaluated the effect of a HFD intervention in mice on the hepatic NAD salvage pathway. The HFD mouse model employed in this study represents the human phenotype of hepatosteatosis with increased hepatic TAG accumulation, but without any indications of macrophage infiltration or fibrosis. Our data are also in accordance with data from other studies using HFD mice [25]. Mice on HFD had impaired glucose tolerance, higher fasting blood glucose levels as well as a borderline significant reduction in insulin tolerance compared to mice on normal chow. Collectively, this indicates that HFD-fed mice had decreased peripheral insulin sensitivity.

Interestingly, we found an up regulation in the capacity for NAD salvage in the livers of the HFD-fed mice, indicated by up regulated expression and activity of the NAD salvage enzyme Nampt and increased NAD levels. The enhanced NAD salvage we observed could be a prerequisite for the up regulation of mitochondrial metabolism as an adaptive mechanism in response to chronic fat overload as described in other studies [26], since an increase in β-oxidation and tricarboxylic acid cycle flux would require increased levels of NAD. In general, it has been shown that mice fed a HFD exhibit decreased hepatic Nampt levels, while an up regulation of Nampt ameliorated the negative impact of HFD in the liver and augmented glucose tolerance [27,28]. In contrast, and in accordance with our study, another study showed an up regulation of *Nampt* mRNA in a rat model of NAFLD [29]. Reasons for the discrepant results of animal studies could be the composition and duration of the HFD as well as mouse strain-dependent differences [30]. Conflicting data about the role of hepatic Nampt in humans have been reported. In obese women hepatic *NAMPT* mRNA levels in the liver were shown to be higher compared to lean women and were even further increased in obese women with NAFLD, which is in line with our data [14]. A different human studyfound higher *NAMPT* mRNA expression level in fibrotic livers [13]. Opposite, it has also been reported that patients with NAFLD showed lower levels of *NAMPT* mRNA expression in the liver [12]. The reason for the discrepancies between these studies is unknown but it could be due to variations in the progression of NAFLD.

As a result of increased NAD levels an enhanced Sirt1 deacetylase activity in the livers of the HFD-fed mice was detected as shown by less acetylation of Nfκb on lysine 310 [22] and p53 on lysine 382 [31]. By deacetylating p53 at lysine 382 Sirt1 is able to repress the activity of p53, a master regulator of apoptosis and cell cycle progression [31]. In liver biopsies from subjects with NAFLD Sirt1 was shown to be decreased leading to increased acetylation of p53 compared to Ctl patients [32]. The lower activity of p53 in the present study was confirmed by the observed down regulation of the p53 downstream target Bax in HFD murine livers [33]. Nfκb is a critical factor in the innate immunity response and is directly affected by Sirt1 which deacetylates the ReIA/p65 component of Nfκb leading to a down regulation of its activity and degradation [22]. A global over expression of Sirt1 in mice subjected to HFD has been shown to improve hepatic inflammation by decreasing activity of Nfκb [3].

The increased Sirt1 activity in HFD-fed mice was confirmed by detecting a down regulation of *Ucp2*. This mitochondrial inner membrane protein negatively regulates reactive oxygen species (ROS) production and has been demonstrated by others to be up regulated in NAFLD [34]. Sirt1 is known to regulate *Ucp2* by binding to its promoter resulting in down regulation of *Ucp2* mRNA expression [35]. Our data indicate that a higher activity of Sirt1 led to a down regulation of *Ucp2* in HFD mouse livers suggesting that oxidative stress was not present in our murine model at the analysed time point. The absence of oxidative stress was supported by the fact that MnSod, a key ROS scavenging enzyme [23], was not different on mRNA level as well as on total and acetylated protein level in our murine model.

Srebp-1c can be inhibited by Sirt1 and is a key regulator of *de novo* lipogenesis, which plays a crucial role in the pathogenesis of NAFLD. Srebp-1c is mainly activated by saturated fatty acids and elevated insulin levels [36,37]. In line with these studies, we found higher *Srebp-1c* mRNA expression in livers of HFD-fed mice. Despite increased TAG accumulation in the liver of HFD-fed mice it might however be reasonably assumed that Srebp-1c was less active due to deacetylation by Sirt1 [38], emphasized by lower mRNA levels of its downstream targets *Fas, Acc* and *Scd1*. However, in our study acetylation of Srebp-1 was not significantly altered in mice fed a HFD compared to Ctl. Transcriptional activity of Srebp-1c is also regulated by phosphorylation due to activation of AMPK which suppresses Srebp-1c cleavage and nuclear translocation in insulin resistant LDL receptor deficient mice and therefore represses *de novo* lipogenesis [39]. Interestingly we could also find a decreased expression of the two key enzymes of gluconeogenesis G6pc and Pepck which might be due to higher activity of Sirt1 leading to an increased phosphorylation [40] and decreased acetylation of FoxO1 [41] and thus inhibiting its transcription of G6pc and Pepck.

Besides elevated Sirt1 activity, we found increased hepatic Sirt1 protein expression in HFD-fed mice. There are several contradictory studies on Sirt1 expression in the livers of NAFLD animal models. Rats on a HFD for 3 months were reported to develop hepatic steatosis accompanied by decreased Sirt1 protein levels [42], while in other studies Sirt1 over expression was found to be protective against the negative impact of a HFD [2,3]. In contrast, it has been shown that liver-specific Sirt1 knockout mice stored less fat in white adipose tissue and liver under HFD conditions [43]. In

addition, liver-specific Sirt1 knockout mice were more glucose tolerant and had lower levels of blood glucose and insulin than wild type controls [43]. Taken together, the association between Sirt1 protein and activity levels, hepatic lipid accumulation and resulting metabolic disturbances is not yet clarified and may be associated with disease progression and inflammatory conditions.

In summary, we detected an up regulation of the NAD salvage pathway and concomitantly increased Sirt1 deacetylase activity in the livers of mice fed a HFD diet for 11 weeks. One could suppose that up regulation of Nampt activity and NAD levels are an early compensatory mechanism to protect the liver against the negative consequences of lipid accumulation. The negative impact of an 11-week HFD was being counteracted via higher Nampt and Sirt1 activities. It is possible that a more prolonged HFD exposure would lead to impaired regulation of hepatic Nampt and NAD levels, thus contributing to the development of steatohepatitis.

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MP, SS, MD, BJ, TG, SGV, JT, WK and AG conceived experiments, MP, PL, AM and SR carried out experiments, MP, MD PL, AM, JT and AG analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

3.8 References

[1] G. Tarantino, C. Finelli, What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome?, World J. Gastroenterol. 19 (2013) 3375–84. doi:10.3748/wjg.v19.i22.3375.

[2] A. Purushotham, T.T. Schug, Q. Xu, S. Surapureddi, X. Guo, X. Li, Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation., Cell Metab. 9 (2009) 327–38. doi:10.1016/j.cmet.2009.02.006.

[3] P.T. Pfluger, D. Herranz, S. Velasco-Miguel, M. Serrano, M.H. Tschöp, Sirt1 protects against high-fat diet-induced metabolic damage., Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 9793–8. doi:10.1073/pnas.0802917105.

[4] M.D. Hirschey, T. Shimazu, E. Goetzman, E. Jing, B. Schwer, D.B. Lombard, et al., SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation, Nature. 464 (2010) 121–125. doi:10.1038/nature08778.

[5] P.J. Fernandez-Marcos, E.H. Jeninga, C. Canto, T. Harach, V.C.J. de Boer, P. Andreux, et al., Muscle or liver-specific Sirt3 deficiency induces hyperacetylation of mitochondrial proteins without affecting global metabolic homeostasis., Sci. Rep. 2 (2012) 425. doi:10.1038/srep00425.

[6] J.R. Revollo, A.A. Grimm, S. Imai, The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals., Curr. Opin. Gastroenterol. 23 (2007) 164–70. doi:10.1097/MOG.0b013e32801b3c8f.

[7] M. Tanaka, M. Nozaki, A. Fukuhara, K. Segawa, N. Aoki, M. Matsuda, et al., Visfatin is released from 3T3-L1 adipocytes via a non-classical pathway, Biochem. Biophys. Res. Commun. 359 (2007) 194–201. doi:10.1016/j.bbrc.2007.05.096.

[8] A. Garten, S. Petzold, A. Barnikol-Oettler, A. Körner, W.E. Thasler, J. Kratzsch, et al., Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes, Biochem. Biophys. Res. Commun. 391 (2010) 376–381. doi:10.1016/j.bbrc.2009.11.066.

[9] D. Friebe, M. Neef, J. Kratzsch, S. Erbs, K. Dittrich, a. Garten, et al., Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans, Diabetologia. 54 (2011) 1200–1211. doi:10.1007/s00125-010-2042-z.

[10] J.R. Revollo, A. Korner, K.F. Mills, A. Satoh, T. Wang, A. Garten, et al., Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme, Cell Metab. 6 (2007) 363–375. doi:10.1016/j.cmet.2007.09.003.

[11] B. Samal, Y. Sun, G. Stearns, C. Xie, S. Suggs, I. McNiece, Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor., Mol. Cell. Biol. 14 (1994) 1431–7. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=358498&tool=pmcentrez&rendertype=abstract (accessed April 01, 2015).

[12] T.B. Dahl, J.W. Haukeland, A. Yndestad, T. Ranheim, I.P. Gladhaug, J.K. Damås, et al., Intracellular nicotinamide phosphoribosyltransferase protects against hepatocyte apoptosis and is down-regulated in nonalcoholic fatty liver disease, J. Clin. Endocrinol. Metab. 95 (2010) 3039–3047. doi:10.1210/jc.2009-2148.

[13] M. Kukla, M. Ciupińska-Kajor, M. Kajor, M. Wyleżoł, K. Żwirska-Korczala, M. Hartleb, et al., Liver visfatin expression in morbidly obese patients with nonalcoholic fatty liver disease undergoing bariatric surgery., Pol. J. Pathol. 61 (2010) 147–153. http://www.ncbi.nlm.nih.gov/pubmed/21225497.

[14] T. Auguet, X. Terra, J.A. Porras, J.M. Orellana-Gavaldà, S. Martinez, C. Aguilar, et al., Plasma visfatin levels and gene expression in morbidly obese women with associated fatty liver disease, Clin. Biochem. 46 (2013) 202–208. doi:10.1016/j.clinbiochem.2012.11.006.

[15] V.T. Samuel, Z.-X. Liu, X. Qu, B.D. Elder, S. Bilz, D. Befroy, et al., Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease., J. Biol. Chem. 279 (2004) 32345–53. doi:10.1074/jbc.M313478200.

[16] N. Turner, G.M. Kowalski, S.J. Leslie, S. Risis, C. Yang, R.S. Lee-Young, et al., Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding., Diabetologia. 56 (2013) 1638–48. doi:10.1007/s00125-013-2913-1.

[17] C. Zhu, R.H. Byrd, P. Lu, J. Nocedal, Algorithm 778: L-BFGS-B: Fortran subroutines for large-scale bound-constrained optimization, ACM Trans. Math. Softw. 23 (1997) 550–560. doi:10.1145/279232.279236.

[18] S. Schuster, M. Penke, T. Gorski, S. Petzold-Quinque, G. Damm, R. Gebhardt, et al., Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes, PLoS One. 9 (2014). doi:10.1371/journal.pone.0091045.

[19] D. Friebe, M. Neef, J. Kratzsch, S. Erbs, K. Dittrich, A. Garten, et al., Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans, Diabetologia. 54 (2011) 1200–1211. doi:10.1007/s00125-010-2042-z.

[20] Z. Goktas, S. Owens, M. Boylan, D. Syn, C.-L. Shen, D.B. Reed, et al., Associations between tissue visfatin/nicotinamide, phosphoribosyltransferase (Nampt), retinol binding protein-4, and vaspin concentrations and insulin resistance in morbidly obese subjects., Mediators Inflamm. 2013 (2013) 861496. doi:10.1155/2013/861496.

[21] M. Olszanecka-Glinianowicz, A. Owczarek, M. Bożentowicz-Wikarek, A. Brzozowska, M. Mossakowska, T. Zdrojewski, et al., Relationship between circulating visfatin/NAMPT, nutritional status and insulin resistance in an elderly population - results from the PolSenior substudy., Metabolism. 63 (2014) 1409–18. doi:10.1016/j.metabol.2014.07.013.

[22] F. Yeung, J.E. Hoberg, C.S. Ramsey, M.D. Keller, D.R. Jones, R.A. Frye, et al., Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase., EMBO J. 23 (2004) 2369–80. doi:10.1038/sj.emboj.7600244.

[23] R. Tao, M.C. Coleman, J.D. Pennington, O. Ozden, S.-H. Park, H. Jiang, et al., Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress, Mol. Cell. 40 (2010) 893–904. doi:10.1016/j.molcel.2010.12.013.

[24] A. Defour, K. Dessalle, A. Castro Perez, T. Poyot, J. Castells, Y.S. Gallot, et al., Sirtuin 1 regulates SREBP-1c expression in a LXR-dependent manner in skeletal muscle., PLoS One. 7 (2012) e43490. doi:10.1371/journal.pone.0043490.

[25] Y. Takahashi, Y. Soejima, T. Fukusato, Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis., World J. Gastroenterol. 18 (2012) 2300–8. doi:10.3748/wjg.v18.i19.2300.

[26] C. Koliaki, M. Roden, Hepatic energy metabolism in human diabetes mellitus, obesity and non-alcoholic fatty liver disease., Mol. Cell. Endocrinol. 379 (2013) 35–42. doi:10.1016/j.mce.2013.06.002.

[27] J. Yoshino, K.F. Mills, M.J. Yoon, S. Imai, Nicotinamide Mononucleotide, a Key NAD+ Intermediate, Treats the Pathophysiology of Diet- and Age-Induced Diabetes in Mice, Cell Metab. 14 (2011) 528–536. doi:10.1016/j.cmet.2011.08.014.

[28] R. Tao, D. Wei, H. Gao, Y. Liu, R. a. Depinho, X.C. Dong, Hepatic FoxOs regulate lipid metabolism via modulation of expression of the nicotinamide phosphoribosyltransferase gene, J. Biol. Chem. 286 (2011) 14681–14690. doi:10.1074/jbc.M110.201061.

[29] W.-C. Chang, H. Jia, W. Aw, K. Saito, S. Hasegawa, H. Kato, Beneficial effects of soluble dietary Jerusalem artichoke (Helianthus tuberosus) in the prevention of the onset of type 2 diabetes and non-alcoholic fatty liver disease in high-fructose diet-fed rats., Br. J. Nutr. 112 (2014) 709–17. doi:10.1017/S0007114514001421.

[30] J.T. Heiker, A. Kunath, J. Kosacka, G. Flehmig, A. Knigge, M. Kern, et al., Identification of genetic loci associated with different responses to high-fat diet-induced obesity in C57BL/6N and C57BL/6J substrains., Physiol. Genomics. 46 (2014) 377–84. doi:10.1152/physiolgenomics.00014.2014.

[31] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, et al., hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase., Cell. 107 (2001) 149–59. http://www.ncbi.nlm.nih.gov/pubmed/11672523 (accessed March 28, 2015).

[32] R.E. Castro, D.M.S. Ferreira, M.B. Afonso, P.M. Borralho, M. V Machado, H. Cortez-Pinto, et al., miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease., J. Hepatol. 58 (2013) 119–25. doi:10.1016/j.jhep.2012.08.008.

[33] L.M. Rozan, W.S. El-Deiry, p53 downstream target genes and tumor suppression: a classical view in evolution., Cell Death Differ. 14 (2007) 3–9. doi:10.1038/sj.cdd.4402058.

[34] M. Kohjima, M. Enjoji, N. Higuchi, M. Kato, K. Kotoh, T. Yoshimoto, et al., Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease., Int. J. Mol. Med. 20 (2007) 351–8. http://www.ncbi.nlm.nih.gov/pubmed/17671740 (accessed April 01, 2015).

[35] L. Bordone, M.C. Motta, F. Picard, A. Robinson, U.S. Jhala, J. Apfeld, et al., Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells., PLoS Biol. 4 (2006) e31. doi:10.1371/journal.pbio.0040031.

[36] M. Aragno, C.E. Tomasinelli, I. Vercellinatto, M.G. Catalano, M. Collino, R. Fantozzi, et al., SREBP-1c in nonalcoholic fatty liver disease induced by Western-type high-fat diet plus fructose in rats., Free Radic. Biol. Med. 47 (2009) 1067–74. doi:10.1016/j.freeradbiomed.2009.07.016.

[37] J.T. Haas, J. Miao, D. Chanda, Y. Wang, E. Zhao, M.E. Haas, et al., Hepatic insulin signaling is required for obesity-dependent expression of SREBP-1c mRNA but not for feeding-dependent expression., Cell Metab. 15 (2012) 873–84. doi:10.1016/j.cmet.2012.05.002.

[38] B. Ponugoti, D.-H. Kim, Z. Xiao, Z. Smith, J. Miao, M. Zang, et al., SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism., J. Biol. Chem. 285 (2010) 33959–70. doi:10.1074/jbc.M110.122978.

[39] Y. Li, S. Xu, M.M. Mihaylova, B. Zheng, X. Hou, B. Jiang, et al., AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice., Cell Metab. 13 (2011) 376–88. doi:10.1016/j.cmet.2011.03.009.

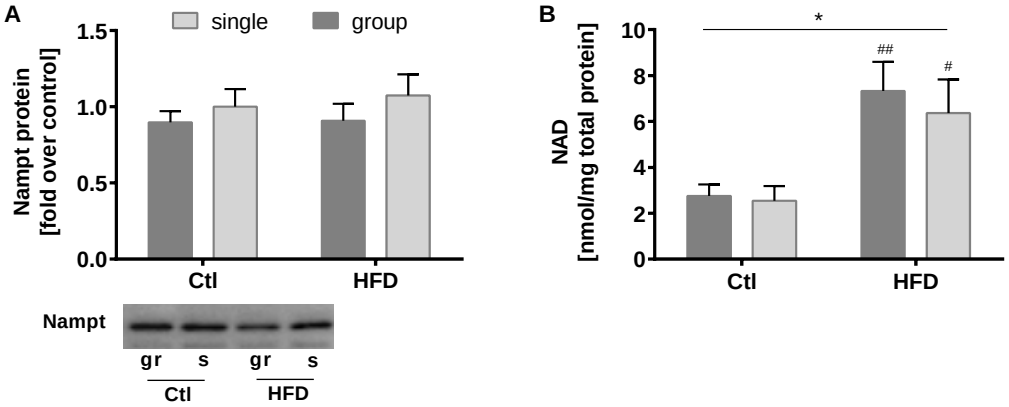
[40] R.-H. Wang, H.-S. Kim, C. Xiao, X. Xu, O. Gavrilova, C.-X. Deng, Hepatic Sirt1 deficiency in mice impairs mTorc2/Akt signaling and results in hyperglycemia, oxidative damage, and insulin resistance., J. Clin. Invest. 121 (2011) 4477–90. doi:10.1172/JCI46243.

[41] M.C. Motta, N. Divecha, M. Lemieux, C. Kamel, D. Chen, W. Gu, et al., Mammalian SIRT1 represses forkhead transcription factors., Cell. 116 (2004) 551–63. http://www.ncbi.nlm.nih.gov/pubmed/14980222 (accessed January 21, 2015).

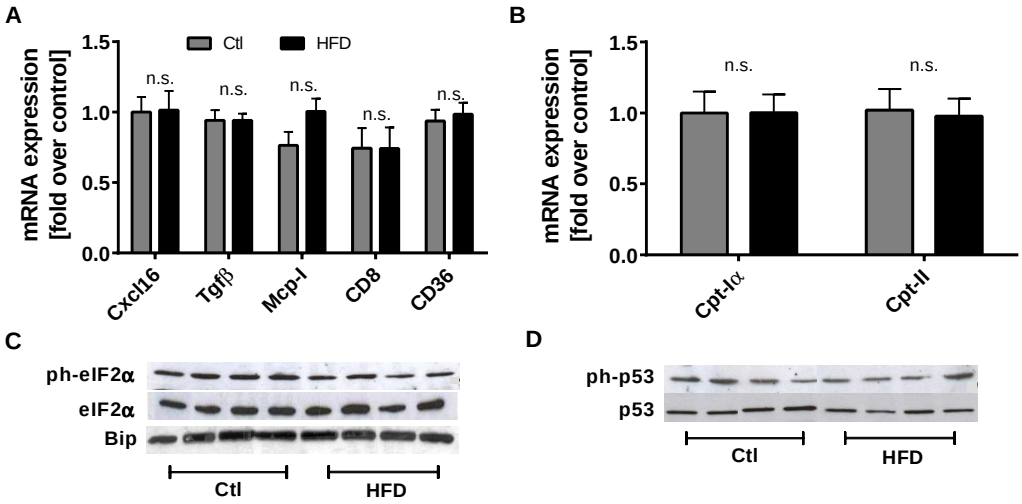
[42] X.-Q. Deng, L.-L. Chen, N.-X. Li, The expression of SIRT1 in nonalcoholic fatty liver disease induced by high-fat diet in rats., Liver Int. 27 (2007) 708–15. doi:10.1111/j.1478-3231.2007.01497.x.

[43] D. Chen, J. Bruno, E. Easlon, S.-J. Lin, H.-L. Cheng, F.W. Alt, et al., Tissue-specific regulation of SIRT1 by calorie restriction., Genes Dev. 22 (2008) 1753–7. doi:10.1101/gad.1650608.

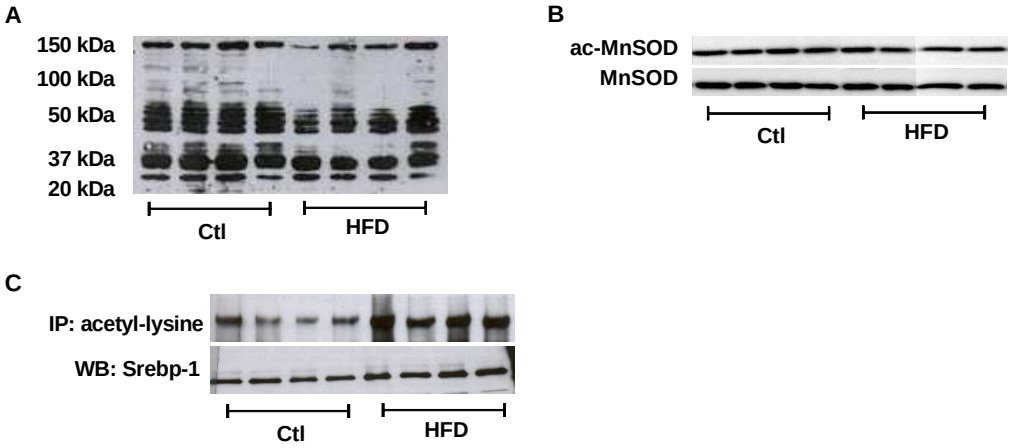
3.9 Appendix: Supplementary Data



Supplementary Fig.1 Hepatic Nampt protein level (A) and NAD levels (B) were not significantly different between single- and group-housed mice. One representative Western blot is shown (n=5). *p<0.05 Ctl-group vs. HFD-single mice. $^{\#}$p<0.05, $^{\#\#}$p<0.01 Ctl mice vs. equivalent housed HFD mice. gr: group-housed; s: single-housed.



Supplementary Fig. 2 A) The cytokines *Cxcl16*, *Tgfβ* and *Mcp-I*, as well as the the T-cell co-receptor *CD8* and the fatty acid translocase *CD36* were unchanged. B) *Cpt-Iα* and *Cpt-II* mRNA as enzymes for the β oxidation of long chain fatty acids were not different between both groups. C) ph-eIf2α was decreased in HFD mice to (Ctl) while Bip stayed stable as markers for ER stress. D) phospho p53 (ph-p53) was not different between both groups. One representative Western Blot is shown (n=12).



Supplementary Fig.3 Total acetylated protein was down regulated in liver tissue of mice fed a high fat diet (A) while specific acetylation of MnSOD (B) was not altered in mice fed a HFD compared to Ctl. Acetylated Srebp-1 (C) was immunoprecipitated with acetyl lysine antibody and blotted for Srebp-1 but no differences were detected. One representative Western blot is shown (n=12).

Supplementary Table 1 Antibodies used for Western blot analyses

antibody	**dilution**	**manufacturer**
Visfatin Antibody (#NP_005737.1)	1:1000	Bethyl Laboratories
Acetyl-NF-κB p65 (Lys310) (#3045)	1:1000	Cell Signaling
Acetyl-p53 (Lys379) Antibody (#2570)	1:1000	Cell Signaling
Bax Antibody (#2772)	1:1000	Cell Signaling
Bip Antibody (#3183)	1:1000	Cell Signaling
eIF2α Antibody (#9722)	1:1000	Cell Signaling
NF-κB p65 (#8242)	1:1000	Cell Signaling
Phospho-eIF2α (Ser51) Antibody (#9721)	1:1000	Cell Signaling
Phospho-p53 (Ser15) Antibody (#9284)	1:1000	Cell Signaling
SirT1 Antibody (Mouse Specific) (#2028)	1:1000	Cell Signaling
SirT3 (D22A3) Rabbit mAb (#5490)	1:1000	Cell Signaling
Acetylated-Lysine Antibody (#9441)	1:1000	Cell Signaling
Anti-p53, C-Terminal antibody (#SAB4503021)	1:500	Sigma Aldrich
Anti-Glyceraldehyde-3-Phosphate Dehydrogenase Antibody, clone 6C5 (#MAB374)	1:100000	Millipore
Anti-MnSOD (#06-984)	1:4000	Millipore
MnSOD K122	1:4000	Friendly provided by David Gius

Supplementary Table 2 Sequences of primer and probes used for Taqmen or SYBR Green analyses

Target	**Forward Primer**	**Reverse Primer**	**Probe**
α-smooth muscle actin	CTG ACA GAG GCA CCA CTG AA	CAT CTC CAG AGT CCA GCA CA	
Carnitine palmitoyltransferase Iα	CTT CAA TAC TTC CCG CAT CC	CTG CTG TCC TTG ACG TGT TG	
Carnitine palmitoyltransferase II	GCT CCG AGG CAT TTG TC	CAT CGC TGC TTC TTT GGT	
CD8	CAA AGA AAA TGG ACG CCG AAC TTG G	TGG CAC GAC AGA ACT GAA GTA CAT C	
CD36	AACTCTGCAGGTTTGC AGCTCT	AGCTTCCACTCTT GCTGTAGG	
CD68	CTT AAA GAG GGC TTG GGG CA	ACT CGG GCT CTG ATG TAG GT	
Collagen I	TGG CCT TGG AGG AAA CTT TG	CTT GGA AAC CTT GTG GAC CAG	
Cyclophillin	ATG TGG TTT TCG GCA AAG TT	TGA CAT CCT TCA GTG GCT TG	
Fatty acid synthase	CCC TTG ATG AAG AGG GAT CA	GAA CAA GGC GTT AGG GTT GA	
Fibronectin	TGC AGT GAC CAA CAT TGA TCG C	AAA AGC TCC CGG ATT CCAT CC	
Glucose 6-phosphatase	CCT GTG AGA CCG GAC CAG	AAA GAT AGC AAG AGT AGA AGT GAC CAT	
Interleukin-6	ACC AGA GGA AAT TTT CAA TAG GC	TGA TGC ACT TGC AGA AAA CA	
mitochondrial uncoupling protein 2	CAG CTA CTG TCA GTT CCG CC	CCC GAT CCC CTC GAT TTT CC	
monocyte chemotactic protein 1	AGGAGCCATACCTGT AAATGCC	ATGCCGTGGATGA ACTGAGG	
Nicotinamide phosphoribosyltransferase	GAT GGT CTG GAA TAC AAG TTA CAT GAC T	ATG AGC AGA TGC CCC TAT GC	AGG AGT CTC TTC GCA AGA GAC TGC T
Peroxisome proliferator-activated receptor gamma coactivator 1α	ATG TGT CGC CTT CTT GCT CT	ATC TAC TGC CTG GGG ACC TT	
Phosphoenolpyruvate carboxykinase	GTG CAT GAA AGG CCG CACCA	GAT CCG CAT GCT GGC CAC C	
Sirtuin 1	CGG CTA CCG AGG TCC ATA TAC	CCG CAA GGC GAG CAT AGA TA	
Sirtuin 3	AGG TGG AGG AAG CAG TGA GA	GCT TGG GGT TGT GAA AGA AA	
Sterol regulatory element-binding protein-1	GGA GCC ATG GAT TGC ACA TTT G	CAA ATA GGC CAG GGA AGT CAC	

Superoxide dismutases	GTG TCT GTG GGA GTC CAA GG	AGC GGA ATA AGG CCT GTT GT	
Transforming growth factor β	AAG TTG GCA TGG TAG CCC TT	GCC CTG GAT ACCAAC TAT TGC	
Tumor necrosis factor α	ATG GGC TTT CCG AAT TCA C	GAG GCA ACC TGA CCA CTC TC	

Chapter 4

FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signalling in hepatocarcinoma cells

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4.1 Abstract

Background: Nicotinamide phosphoribosyltransferase (NAMPT) is the key enzyme of the NAD salvage pathway starting from nicotinamide. Cancer cells have an increased demand for NAD due to their high proliferation and DNA repair rate. Consequently, NAMPT is considered as a putative target for anti-cancer therapies. There is evidence that AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) become dysregulated during the development of hepatocellular carcinoma (HCC). Here, we investigated the effects of NAMPT inhibition by its specific inhibitor FK866 on the viability of hepatocarcinoma cells and analysed the effects of FK866 on the nutrient sensor AMPK and mTOR complex1 (mTORC1) signalling.

Results: FK866 markedly decreased NAMPT activity and NAD content in hepatocarcinoma cells (Huh7 cells, Hep3B cells) and led to delayed ATP reduction which was associated with increased cell death. These effects could be abrogated by administration of nicotinamide mononucleotide (NMN), the enzyme product of NAMPT. Our results demonstrated a dysregulation of the AMPK/mTOR pathway in hepatocarcinoma cells compared to non-cancerous hepatocytes with a higher expression of mTOR and a lower AMPK activation in hepatocarcinoma cells. We found that NAMPT inhibition by FK866 significantly activated AMPKα and inhibited the activation of mTOR and its downstream targets p70S6 kinase and 4E-BP1 in hepatocarcinoma cells. Non-cancerous hepatocytes were less sensitive to FK866 and did not show changes in AMPK/mTOR signalling after FK866 treatment.

Conclusion: Taken together, these findings reveal an important role of the NAMPT-mediated NAD salvage pathway in the energy homeostasis of hepatocarcinoma cells and suggest NAMPT inhibition as a potential treatment option for HCC.

4.2 Introduction

The co-factor nicotinamide adenine dinucleotide (NAD) plays a crucial role in multiple cellular processes and is substrate for a variety of enzymes and regulatory proteins [1]. In humans a main portion of NAD is generated via the nicotinamide (NAM) salvage pathway, in which nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step in the biosynthesis of NAD yielding nicotinamide mononucleotide (NMN) [2,3]. As NAD is rapidly consumed in cells (1h) and converted to NAM [4], NAMPT is essential for the replenishment of the intracellular NAD pool. The development of many cancers is associated with increased NAMPT expression [5]. Cancer cells have a high rate of NAD turnover due to their increased energy demand and a high activity of NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs) and sirtuins, required for DNA repair, genome stability and proliferation [1,6]. Therefore, cancer cells are more susceptible to NAMPT inhibition than normal cells [7,8]. In previous studies, we found that NAMPT is released from hepatocytes [9] as well as differentially expressed and more enzymatically active in hepatocarcinoma cells compared to non-cancerous human hepatocytes [10].

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths [11]. The only available proven systemic therapy for HCC is the multi-targeting kinase inhibitor sorafenib [12]. An effective second-line agent for patients with sorafenib failure or intolerance has yet to be identified. This has led to an intensive search for molecular pathways and novel compounds for the treatment and prevention of HCC. Targeting NAMPT activity and intracellular NAD content represents a novel therapeutic concept for HCC. The specific NAMPT inhibitor FK866 is a competitive inhibitor that was selected by an anticancer screening system differentiating acute cytotoxicity from growth inhibition [13,14]. FK866 has been evaluated in a broad variety of tumors, including solid tumors and leukemia [5,15,16] *in vitro* and in nude mouse xenografts [17–19], where FK866 was able to reduce or attenuate tumor growth.

In HCC tissue, AMP-activated protein kinase (AMPK), a major regulator of cellular energy homeostasis that coordinates multiple metabolic pathways, has been shown to be dysregulated compared to normal tissue [20,21]. AMPK activity opposes tumor development and negatively regulates the Warburg effect (aerobic glycolysis) leading to suppression of tumor growth *in vivo* [20–22]. AMPK translates changes in glucose availability and fluctuation of energy to mammalian target of rapamycin (mTOR) and thereby acts as a master energy sensor to modulate cellular activities in response to energy stress [23,24]. mTOR, a serine/threonine protein kinase, has been observed to be increased in multiple human cancers, including HCC, where it is associated with less differentiated tumors, earlier tumor recurrence, and worse survival outcomes [25,26]. Inhibition of mTOR has proven efficacious in clinical trials [26,27]. Recently, there is great scientific interest in finding molecular pathways and novel compounds that target AMPK/mTOR signalling as a new treatment option for HCC.

Little is known about the interaction of NAMPT and AMPK/mTOR signalling during the development of HCC. In this study, we investigated the effects of the NAMPT inhibitor FK866 on hepatocarcinoma cells and non-cancerous human hepatocytes. We asked whether or not FK866-induced energy stress might activate AMPK and modify the mTOR signalling pathway and whether the observed effects could be rescued by the NAMPT enzyme product NMN.

4.3 Material and Methods

Material

Cell culture media, supplements and antibiotics were obtained from PAA (Cölbe, Germany) or Invitrogen (Karlsruhe, Germany). FK866, nicotinamide mononucleotide (NMN) and camptothecin were purchased from Sigma-Aldrich (Munich, Germany). Etoposide was purchased from Merck Millipore (Darmstadt, Germany).

Hepatocarcinoma cell lines

Huh7 cells (p53-mutated) and Hep3B cells (p53-deficient) were maintained in DMEM medium with high glucose or MEM medium, repectively. Media were supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine, 100 IU penicillin and 100 µg/mL streptomycin. All cells were grown at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2.

Primary human hepatocytes

Tissue samples from patients undergoing liver surgery at the University Medical Center Regensburg were used. Primary human hepatocytes (PHH) were isolated and cultivated as described recently [28]. Briefly, non-neoplastic tissue samples from liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. PHHs were isolated using a modified two-step EGTA/collagenase perfusion procedure and plated on collagen coated dishes. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research, Regensburg, Germany), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. All experiments involving human tissues and cells have been carried out in accordance to *The Code of Ethics of the World Medical Association* (Declaration of Helsinki). Cells were seeded in Williams' Medium E containing 2 mM glutamine, 10^{-7}mol/L dexamethasone, 100 IU penicillin, 100 µg/mL streptomycin and 10 %FBS. All cells were grown at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2.

Cell treatments

FK866 was dissolved in DMSO to create a stock solution of 10 mM. NMN was dissolved in the appropriate medium for a stock solution of 100 mM. After 16h serum starvation, cells were treated with the indicated concentration of FK866 alone or in combination with NMN [500 µM] for 24, 48 and 72 h.

Cell viability and apoptosis

Cell viability analysis was conducted using the cell proliferation reagent WST-1 (Roche, Grenzach-Wyhlen, Germany) according to manufacturer´s instructions. To examine the effects of FK866 on cell death, the number of dead cells was measured by FACS analysis at different time points (48 h, 72 h) using the AnnexinV-FITC Apoptosis Detection Kit (BD Pharmingen™, Franklin Lakes, USA). Adherent and floating cells were analysed according to manufacturer´s protocol. Samples were analysed using a Beckton-Dickinson FACS LSRII. As positive control, apoptosis was induced via camptothecin [2 µM] and etoposide [85 µM] for 24 h. Annexin$^+$ (An^+)and double-stained An^+/propidium iodide (PI^+) cells were considered as dead cells.

ATP measurement

ATP levels were measured with the luminescent-based CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA) according to the manufacturer's protocol.

Western Blot

Protein extraction and Western Blot analysis were performed as described previously [10]. Primary antibodies used for immunoblotting included anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-tubulin, anti-phospho p70S6 kinase (Thr389), anti-p70S6 kinase, anti-phospho-4E-BP1 (Ser65), anti-4E-BP1, anti-acetylated lysine (Cell Signaling, Beverly, MA, USA) and anti-GAPDH (MerckMillipore, Schwalbach, Germany). Appropriate secondary antibodies were purchased from DAKO (Hamburg, Germany). Immunoblotting for GAPDH or tubulin was performed to verify equivalent amounts of loaded protein. Densitometric analysis was performed using ImageJ 1.41 Software (NIH, USA).

NAMPT enzymatic activity

NAMPT activity was measured by the conversion of ^{14}C- labelled nicotinamide to ^{14}C-NMN using a method previously described [10,29]. Radioactivity of ^{14}C-NMN was quantified in a liquid scintillation counter in counts per minute (cpm) (Wallac 1409 DSA, PerkinElmer). NAMPT activity (cpm) was normalized to total protein concentration as measured by the BCA protein assay.

NAD measurement

Concentrations of NAD from whole-cell extracts were quantified by HPLC analysis using a SUPELCOSIL™ LC-18-T HPLC column (Sigma Aldrich) at a flow rate of 0,8ml/min with 100 % buffer A (potassium phosphate buffer pH 6.0) from 0–2min, a linear gradient to 85 % Buffer A/15 % Buffer B (100 % methanol) from 2-5min, 85 % Buffer A/15 % Buffer B from 5-10min, a linear gradient to 100 % Buffer A from 10–12min and 100 % Buffer A from 12–15min. NAD was eluted as a sharp peak at 8min and quantitated based on the peak area compared to a standard curve and normalized to total protein concentration as measured by the BCA protein assay.

4.4 Results

FK866-induced NAMPT inhibition significantly decreased NAD levels in human hepatocarcinoma cells which could be ameliorated by NMN administration

We stimulated hepatocarcinoma cells with FK866 [10nM] and found significantly reduced NAMPT activity (-74.9±8.1 % in Huh7 cells, -38.1±3.7 % in Hep3B cells) (Fig.1A) which caused a sharp decline of NAD levels (Huh7 cells 3.3± 0.3 µmol/g protein [con] vs. 0.3± 0.2 µmol/g protein [10nM FK866]; Hep3B cells 2.2± 0.7 µmol/g protein [con] vs. 0.2± 0.08 µmol/g protein [10nM FK866]) (Fig.1B). Co-treatment with NMN restored intracellular NAD levels in all tested cell lines (Fig.1B).

To investigate the sensitivity of non-cancerous human hepatocytes towards FK866, we used the same treatment conditions as for hepatocarcinoma cells and found that non-cancerous hepatocytes showed no significant reduction in NAMPT activity and NAD levels at 10nM FK866 after 48 h (Supplement Fig.1A,B). Emerging evidence suggests that the cellular acetylation state is associated with the energy state of a cell [30]. We could show that FK866-induced NAD depletion led to a decreased activity of NAD-dependent lysine deacetylases as measured by an increased global acetylation of lysine residues (+1.9-fold, $p<0.001$) (Fig.1C). The administration of NMN abrogated the FK866-induced hyperacetylation of lysine residues ($p<0.001$) (Fig.1C).

NAMPT inhibition by FK866 reduced cell viability, induced energy stress and led to delayed cell death in human hepatocarcinoma cells

We could detect a decreased cell viability in hepatocarcinoma cells (-49.4±4.6 % in Huh7 cells, -20.6±2.8 % in Hep3B cells) (Fig.2A) after 24 h of FK866 treatment. We wanted to investigate whether FK866-induced NAD depletion would result in a reduction of ATP generation and therefore would induce cellular energy stress in hepatocarcinoma cells. Time course studies revealed that ATP levels were lowered in Huh7 cells (-49.6±9.5 %, $p<0.01$) and Hep3B cells (-61.1±6.8 %, $p<0.001$) after 48 h of treatment with 10nM FK866 (Fig.2B). The ATP levels further declined after 72 h in Huh7 cells (-90.2±2.5 %, $p<0.001$) and Hep3B cells (-91.1±1.5 %, $p<0.001$) (Fig.2C). The co-administration of NMN could ameliorate ATP levels in Huh7 and Hep3B cells after 48 and 72 h (Fig.2B,C). After 72 h, subsequent to the drop of NAD levels, the effects of FK866 on cell death became evident when measuring An^+/PI^+-stained cells. Hep3B cells, a p53-deficient cell line, already displayed an increase in An^+/PI^+ cells after 48 h of FK866 treatment (+1.8-fold, $p<0.01$) (Supplement Fig.2A) indicating that FK866-induced cell death did not depend on p53 function. Huh7 cells treated with FK866 [10nM] for 72 h showed a 1.5-fold increase in An^+/PI^+ cells compared to control cells ($p<0.05$) (Fig.2D) whereas the number of An^+/PI^+ Hep3B cells increased further (+3.0-fold, $p<0.01$). Co-stimulation with NMN ameliorated the induction of cell death in Huh7 cells ($p=0.09$) and completely rescued FK866-induced cell death in Hep3B cells ($p<0.01$) (Fig.2D).

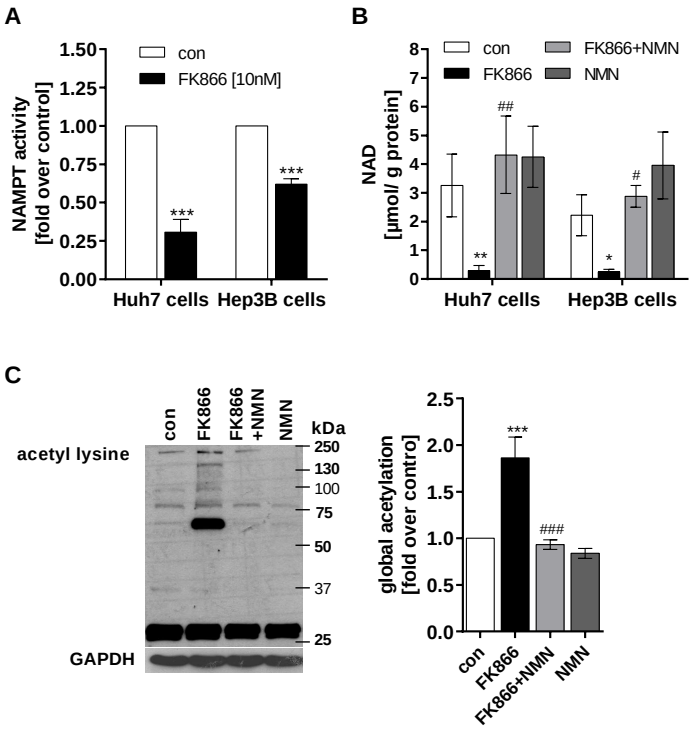

Fig.1 FK866 reduced NAMPT activity and NAD content and increased global acetylation of lysine residues. A) NAMPT activity and B) NAD content were measured after 24 h and were normalized to total protein amount in each sample (n=3). C) Western Blot analysis of acetylated lysine residues in lysates of Hep3B cells treated with FK866 [10nM], a combination of FK866+NMN or NMN alone for 48 h. GAPDH was used as loading control. Densitometric analysis of each lane was performed in four independent Western Blots (n=4). Cells stimulated with serum-free medium were used as control [con] and were set 1. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p<0.05, **p<0.01, ***p<0.001 compared to serum-free medium control; #p<0.05, ##p<0.01, ###p<0.001 compared to FK866 [10nM]).

Dysregulation of the AMPK/mTOR signalling pathway in hepatocarcinoma cells compared to non-cancerous hepatocytes

Growing evidence suggests that mTOR and AMPK dysregulation play an important role in hepatocellular carcinogenesis [20,30]. Therefore, we compared the protein amount of mTOR and its downstream target p70S6 kinase and also AMPKα activation in non-cancerous primary human hepatocytes and hepatocarcinoma cells. An increased protein level of total mTOR and p70S6 kinase was found in hepatocarcinoma cells compared to non-cancerous hepatocytes (Fig.3A). In contrast, AMPK activation was enhanced in non-cancerous primary human hepatocytes (PHH) compared to Huh7 and Hep3B cells despite equal AMPKα total protein amount (Fig.3A). This suggests that mTOR signalling and AMPK activation are involved in metabolic adaptation of hepatocarcinoma cells and might be interesting targets for prevention of cancer cell growth.

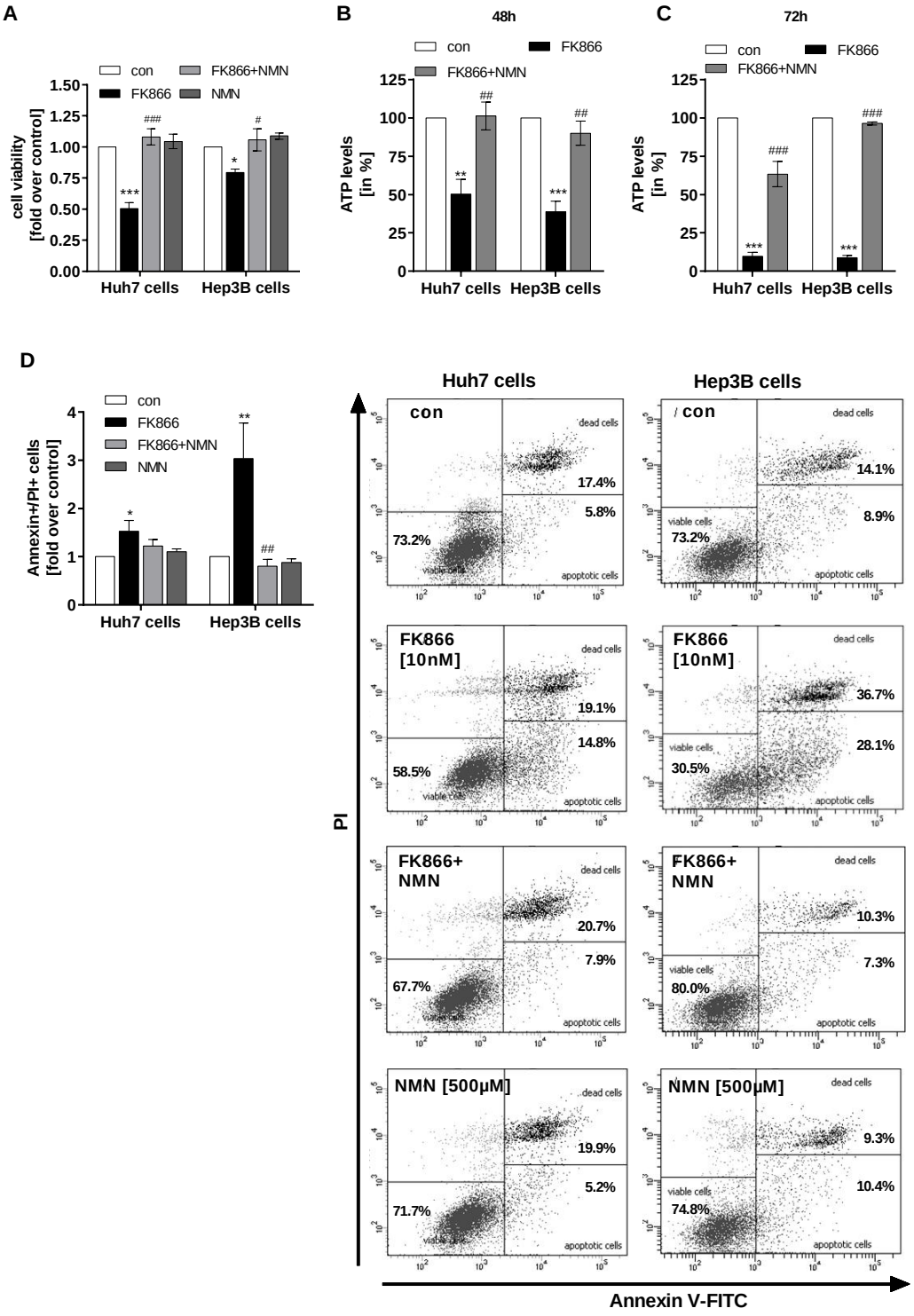

Fig.2 FK866-induced NAMPT inhibition reduced cell viability and ATP content and induced delayed cell death in hepatocarcinoma cells. A) Cell viability of Huh7 and Hep3B cells after 24 h was measured using WST1-reagent (n=4). ATP content after B) 48 h and C) 72 h treatment with 10nM FK866 (n=3). Cells stimulated with serum-free medium were used as control [con] and were set 100 %. D) AnnexinV-FITC/PI assay of Huh7 and Hep3B cells treated with FK866, FK866+NMN or NMN alone for 72 h (n=3). Cells stimulated with serum-free medium were used as control [con] and were set 1. Representative dot plots of the AnnexinV-FITC/PI staining in Huh7 and Hep3B cells are shown including the percentage of viable, An^+ and An^+/PI^+ cells. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p<0.05, **p<0.01, ***p<0.001 compared to serum-free medium control; #p<0.05, ##p<0.01, ###p<0.001 compared to FK866 [10nM]).

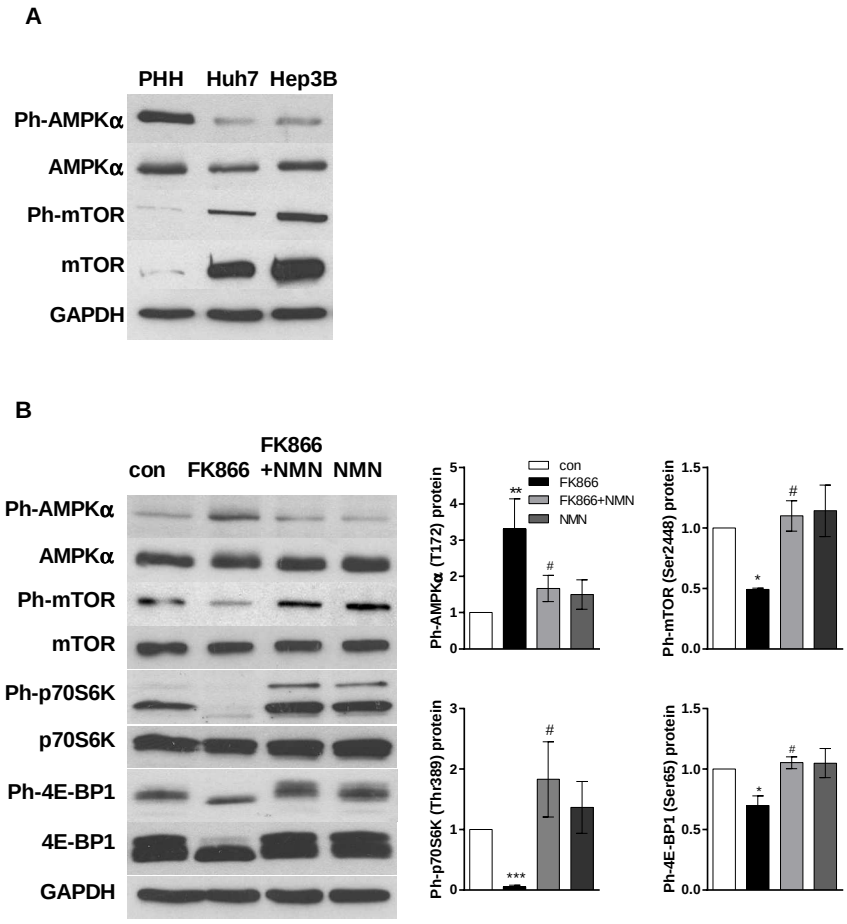

Fig.3 Expression of mTOR and AMPK in hepatocarcinoma cells and non-cancerous human hepatocyes and its regulation by FK866. A) Western blot analysis of AMPK and mTOR expression in lysates of non-cancerous, primary human hepatocytes (PHH), Huh7 and Hep3B cells (n=3). B) Western blot analysis of the AMPK/mTORC1 signalling pathway in lysates of Huh7 cells treated with FK866 [10nM], a combination of FK866 [10nM]+NMN [500 µM] or NMN [500 µM] alone for 48 h (n=3). GAPDH was used as loading control. One representative blot out of 3 independent experiments is shown. Background-corrected densitometric values were normalized to control (serum-free medium). Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p<0.05, **p<0.01, ***p<0.001 compared to serum-free medium control; #p<0.05, ##p<0.01, ###p<0.001 compared to FK866 [10nM].

FK866-induced energy stress activated AMPKα and led to inhibition of mTOR complex1 signalling in hepatocarcinoma cells

To test the efficacy of FK866-induced NAD depletion to activate AMPK and inhibit the mTOR signalling pathway, we measured the phosphorylation state of different members of the AMPK/mTOR complex1 cascade. FK866 treatment increased the phosphorylation of AMPKα at Thr172 (+3.3-fold, p<0.01) in hepatocarcinoma cells (Fig.3B). This was associated with a significant down regulation of phosphorylated mTOR (Ser2448) by -50.7±0.1 % (p<0.05) and the phosphorylation of its down-stream target p70S6 kinase (by -94.7±2.4 %, p<0.001) and 4E-BP1 (by -30.0±.0.1 %, p<0.05) indicating reduced protein synthesis and cell growth (Fig.3B). Co-treatment with NMN [500 µM] completely reversed the FK866-induced effects on AMPK activation and mTOR complex1 signalling inhibition suggesting that the NMN biosynthetic activity of NAMPT is relevant in mediating the effects of FK866. NMN alone had no impact on AMPK activation and mTORC1 signalling in

hepatocarcinoma cells (Fig.3B). Non-cancerous human hepatocytes treated with equal amounts of FK866 for 48 h did not show significant changes in AMPK activation and mTOR phosphorylation (Supplement Fig.1C) verifying their lower sensitivity to FK866.

4.5 Discussion

During malignant transformation the cellular metabolism undergoes multiple molecular and metabolic adaptations to support cell growth and survival. NAD is a key determinant in cancer cell biology as it is essential for redox reactions and key component of signalling pathways that regulate transcription, DNA repair, apoptosis and metabolism [1]. In mammals, NAMPT is a main regulator of the intracellular NAD pool [2,3]. Here, we investigated whether or not the NAMPT inhibitor, FK866, would affect intracellular NAD and ATP concentrations in hepatocarcinoma cells and consequently would be able to regulate the activity of the metabolic sensors AMPK and mTOR. Our study showed that FK866 rapidly reduced NAD levels in hepatocarcinoma cells and led to delayed ATP depletion which could be ameliorated by administration of NMN. Break down of ATP levels was associated with increased cell death. In contrast to another study [31], we demonstrated that FK866 reduced NAMPT activity, depleted NAD and ATP content and induced cell death in p53-deficient Hep3B cells suggesting that FK866-mediated cell death does not depend on functional p53. Our results are in line with a study performed in chronic lymphocytic leukemia cells [15]. In our study, especially Hep3B cells showed a high sensitivity to FK866 and an increased number of dead cells occurred already after 48 h of FK866 treatment. Interestingly, non-cancerous human hepatocytes subjected to the same FK866 treatment as hepatocarcinoma cells did not display reduced NAMPT activity and NAD content even at a FK866 concentration 10-fold of the EC_{50} (EC_{50} 8.2 nM) indicating a lower sensitivity of non-cancerous cells to FK866. This has also been described for normal blood cells [8,31]. Therefore, FK866 represents an interesting compound in cancer cell therapy as it progressively exhausts NAD content in cells with a high NAD turnover that mainly rely on nicotinamide and the NAMPT-mediated NAD salvage pathway as source of NAD. Cancer cells have a significantly higher NAD turnover than normal cells to sustain their rapid proliferation, relative genomic instability, permanently ongoing DNA repair, increased aerobic glycolysis and increased activity of NAD-dependent deacetylases [1,13,14]. This is in line with results of our previous study showing that the expression of SIRT1, a NAD-dependent deacetylase, was significantly higher in hepatocarcinoma cells than in non-cancerous hepatocytes [10].

In this study we could demonstrate that NAMPT inhibition by FK866 led to a sharp decline of intracellular ATP levels and therefore induced energy stress. As a key physiological energy sensor, AMPK is a major regulator of cellular energy homeostasis that coordinates multiple metabolic pathways to balance energy supply [24]. Several studies have shown that AMPK activators exhibit inhibitory effects on cancer cell growth [32,33]. AMPK is known to phosphorylate and activate tuberous sclerosis complex (TSC)2, a negative regulator of mTOR [34]. Therefore, the AMPK/mTOR pathway serves as a signalling nexus for regulating cellular metabolism, energy homeostasis, and cell

growth, and dysregulation of each pathway may contribute to the development of HCC [20,26]. Since the discovery that the mTOR pathway is hyperactivated in many cancers including HCC [25,26,30,35], there is a great interest in finding molecular pathways and novel compounds that target AMPK/mTOR signalling as novel treatment option for HCC. We could show that components of the mTORC1 cascade were significantly higher expressed in hepatocarcinoma cells than in non-cancerous hepatocytes. Additionally, our data revealed that the activation of AMPK was significantly decreased in hepatocarcinoma cells. Reduced AMPK activity has also been detected in primary human breast cancer [36] and lymphoma [21] cells. Thus, a dysregulated AMPK activity may represent an important regulatory step during tumor initiation and progression, allowing cancer cells to gain a metabolic growth advantage by enhancing aerobic glycolysis (Warburg effect) [21]. We made the intriguing discovery that FK866 acts as an AMPK activator in cancer cells potentially through its ability to induce cellular energy stress. Activation of AMPK was associated with a down regulation of the mTORC1 pathway. All FK866 induced effects could be completely reversed by NMN suggesting that these effects were mediated by NAD. mTORC1 inhibition led to decreased activation of its two downstream targets, 70S ribosomal protein S6 kinase (p70S6K) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). p70S6K and 4E-BP1 are major regulators of protein translation and cellular growth [35]. This contradicts a study performed in neuronal cells where FK866 or a NAMPT knock down was shown to reduce AMPK activation [37]. However, this can be explained by the use of non-cancerous neuronal cells compared to cancer cells in our study.

In summary, our study showed the importance of the NAMPT-mediated NAD salvage pathway for energy homeostasis in hepatocarcinoma cells. Furthermore, FK866-induced NAMPT inhibition led to activation of AMPK and inhibition of mTOR signalling suggesting a putative use of FK866 alone or as a chemotherapeutic sensitizing drug to reduce cancer cell growth. In every case of potential therapeutic use, administration of NMN as antidote may be useful to modulate or counteract FK866 toxicity. Only early stages of HCC are curable with today's treatment protocols, therefore new therapeutic strategies are urgently needed and NAMPT inhibition represents a potential novel treatment approach.

4.6 Acknowledgements

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4.7 References

[1] A. Chiarugi, C. Dölle, R. Felici, M. Ziegler, The NAD metabolome--a key determinant of cancer cell biology., Nat. Rev. Cancer. 12 (2012) 741–52. doi:10.1038/nrc3340.

[2] J.R. Revollo, A.A. Grimm, S. Imai, The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals., Curr. Opin. Gastroenterol. 23 (2007) 164–70. doi:10.1097/MOG.0b013e32801b3c8f.

[3] C. Dölle, R.H. Skoge, M.R. Vanlinden, M. Ziegler, NAD biosynthesis in humans--enzymes, metabolites and therapeutic aspects., Curr. Top. Med. Chem. 13 (2013) 2907–17.

[4] M. Rechsteiner, D. Hillyard, B.M. Olivera, Magnitude and significance of NAD turnover in human cell line D98/AH2., Nature. 259 (1976) 695–6. http://www.ncbi.nlm.nih.gov/pubmed/175293 (accessed June 12, 2015).

[5] T.-Q. Bi, X.-M. Che, X.-H. Liao, D.-J. Zhang, H.-L. Long, H.-J. Li, et al., Overexpression of Nampt in gastric cancer and chemopotentiating effects of the Nampt inhibitor FK866 in combination with fluorouracil., Oncol. Rep. 26 (2011) 1251–7. doi:10.3892/or.2011.1378.

[6] T. Bi, X. Che, Nampt/PBEF/visfatin and cancer., Cancer Biol. Ther. 10 (2010) 119–25. doi:10.4161/cbt.10.2.12581.

[7] B.K. Thakur, T. Dittrich, P. Chandra, A. Becker, W. Kuehnau, J.-H. Klusmann, et al., Involvement of p53 in the cytotoxic activity of the NAMPT inhibitor FK866 in myeloid leukemic cells., Int. J. Cancer. 132 (2013) 766–74. doi:10.1002/ijc.27726.

[8] M. Cea, A. Cagnetta, M. Fulciniti, Y.-T. Tai, T. Hideshima, D. Chauhan, et al., Targeting NAD+ salvage pathway induces autophagy in multiple myeloma cells via mTORC1 and extracellular signal-regulated kinase (ERK1/2) inhibition., Blood. 120 (2012) 3519–29. doi:10.1182/blood-2012-03-416776.

[9] A. Garten, S. Petzold, A. Barnikol-Oettler, A. Körner, W.E. Thasler, J. Kratzsch, et al., Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes., Biochem. Biophys. Res. Commun. 391 (2010) 376–81. doi:10.1016/j.bbrc.2009.11.066.

[10] S. Schuster, M. Penke, T. Gorski, S. Petzold-Quinque, G. Damm, R. Gebhardt, et al., Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes, PLoS One. 9 (2014). doi:10.1371/journal.pone.0091045.

[11] World Cancer Report, (n.d.). http://www.iarc.fr/en/publications/books/wcr/ (accessed June 12, 2015).

[12] J.M. Llovet, S. Ricci, V. Mazzaferro, P. Hilgard, E. Gane, J.-F. Blanc, et al., Sorafenib in advanced hepatocellular carcinoma., N. Engl. J. Med. 359 (2008) 378–90. doi:10.1056/NEJMoa0708857.

[13] M. Hasmann, I. Schemainda, FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis., Cancer Res. 63 (2003) 7436–42.

[14] J.A. Khan, X. Tao, L. Tong, Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents., Nat. Struct. Mol. Biol. 13 (2006) 582–8. doi:10.1038/nsmb1105.

[15] I. Gehrke, E.D.J. Bouchard, S. Beiggi, A.G. Poeppl, J.B. Johnston, S.B. Gibson, et al., On-target effect of FK866, a nicotinamide phosphoribosyl transferase inhibitor, by apoptosis-mediated death in chronic lymphocytic leukemia cells., Clin. Cancer Res. 20 (2014) 4861–72. doi:10.1158/1078-0432.CCR-14-0624.

[16] A. Nahimana, A. Attinger, D. Aubry, P. Greaney, C. Ireson, A. V Thougaard, et al., The NAD biosynthesis inhibitor APO866 has potent antitumor activity against hematologic malignancies., Blood. 113 (2009) 3276–86. doi:10.1182/blood-2008-08-173369.

[17] B. Tan, D.A. Young, Z.-H. Lu, T. Wang, T.I. Meier, R.L. Shepard, et al., Pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme essential for NAD+ biosynthesis, in human cancer cells: metabolic basis and potential clinical implications., J. Biol. Chem. 288 (2013) 3500–11. doi:10.1074/jbc.M112.394510.

[18] M.K. Christensen, K.D. Erichsen, U.H. Olesen, J. Tj??rnelund, P. Fristrup, A. Thougaard, et al., Nicotinamide phosphoribosyltransferase inhibitors, design, preparation, and structure-activity relationship, J. Med. Chem. 56 (2013) 9071–9088.

[19] M. Muruganandham, A.A. Alfieri, C. Matei, Y. Chen, G. Sukenick, I. Schemainda, et al., Metabolic signatures associated with a NAD synthesis inhibitor-induced tumor apoptosis identified by 1H-decoupled-31P magnetic resonance spectroscopy., Clin. Cancer Res. 11 (2005) 3503–13. doi:10.1158/1078-0432.CCR-04-1399.

[20] J. Cheng, T. Huang, Y. Li, Y. Guo, Y. Zhu, Q. Wang, et al., AMP-activated protein kinase suppresses the in vitro and in vivo proliferation of hepatocellular carcinoma., PLoS One. 9 (2014) e93256. doi:10.1371/journal.pone.0093256.

[21] B. Faubert, G. Boily, S. Izreig, T. Griss, B. Samborska, Z. Dong, et al., AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo., Cell Metab. 17 (2013) 113–24. doi:10.1016/j.cmet.2012.12.001.

[22] L. Zheng, W. Yang, F. Wu, C. Wang, L. Yu, L. Tang, et al., Prognostic significance of AMPK activation and therapeutic effects of metformin in hepatocellular carcinoma., Clin. Cancer Res. 19 (2013) 5372–80. doi:10.1158/1078-0432.CCR-13-0203.

[23] D.R. Bolster, S.J. Crozier, S.R. Kimball, L.S. Jefferson, AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling., J. Biol. Chem. 277 (2002) 23977–80. doi:10.1074/jbc.C200171200.

[24] D.G. Hardie, The AMP-activated protein kinase pathway--new players upstream and downstream., J. Cell Sci. 117 (2004) 5479–87. doi:10.1242/jcs.01540.

[25] L. Zhou, Y. Huang, J. Li, Z. Wang, The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma., Med. Oncol. 27 (2010) 255–61. doi:10.1007/s12032-009-9201-4.

[26] R.E. Ashworth, J. Wu, Mammalian target of rapamycin inhibition in hepatocellular carcinoma., World J. Hepatol. 6 (2014) 776–82. doi:10.4254/wjh.v6.i11.776.

[27] F. Chiarini, C. Evangelisti, J.A. McCubrey, A.M. Martelli, Current treatment strategies for inhibiting mTOR in cancer., Trends Pharmacol. Sci. 36 (2015) 124–35. doi:10.1016/j.tips.2014.11.004.

[28] T.S. Weiss, S. Pahernik, I. Scheruebl, K.-W. Jauch, W.E. Thasler, Cellular damage to human hepatocytes through repeated application of 5-aminolevulinic acid., J. Hepatol. 38 (2003) 476–82. http://www.ncbi.nlm.nih.gov/pubmed/12663240 (accessed June 12, 2015).

[29] G.C. Elliott, J. Ajioka, C.Y. Okada, A rapid procedure for assaying nicotinamide phosphoribosyltransferase., Anal. Biochem. 107 (1980) 199–205. http://www.ncbi.nlm.nih.gov/pubmed/6449162 (accessed June 12, 2015).

[30] A. Villanueva, D.Y. Chiang, P. Newell, J. Peix, S. Thung, C. Alsinet, et al., Pivotal role of mTOR signaling in hepatocellular carcinoma., Gastroenterology. 135 (2008) 1972–83, 1983.e1–11. doi:10.1053/j.gastro.2008.08.008.

[31] B.K. Thakur, T. Dittrich, P. Chandra, A. Becker, W. Kuehnau, J.-H. Klusmann, et al., Involvement of p53 in the cytotoxic activity of the NAMPT inhibitor FK866 in myeloid leukemic cells., Int. J. Cancer. 132 (2013) 766–74. doi:10.1002/ijc.27726.

[32] J. Sinnett-Smith, K. Kisfalvi, R. Kui, E. Rozengurt, Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: dependence on glucose concentration and role of AMPK., Biochem. Biophys. Res. Commun. 430 (2013) 352–7. doi:10.1016/j.bbrc.2012.11.010.

[33] J. Woodard, L.C. Platanias, AMP-activated kinase (AMPK)-generated signals in malignant melanoma cell growth and survival., Biochem. Biophys. Res. Commun. 398 (2010) 135–9. doi:10.1016/j.bbrc.2010.06.052.

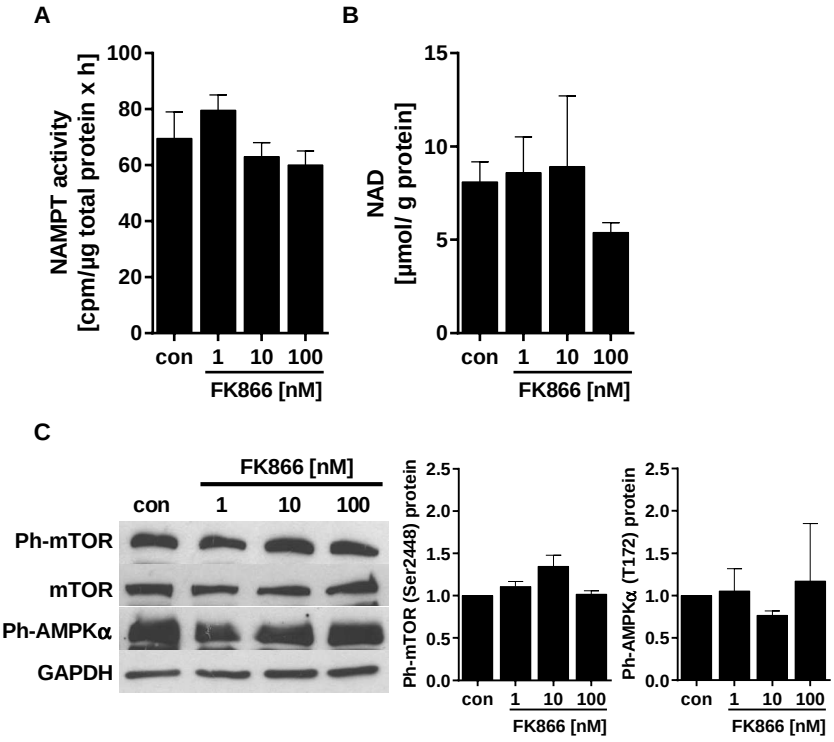
[34] K. Inoki, T. Zhu, K.-L. Guan, TSC2 mediates cellular energy response to control cell growth and survival., Cell. 115 (2003) 577–90. http://www.ncbi.nlm.nih.gov/pubmed/14651849 (accessed June 12, 2015).

[35] D.M. Sabatini, mTOR and cancer: insights into a complex relationship., Nat. Rev. Cancer. 6 (2006) 729–34. doi:10.1038/nrc1974.

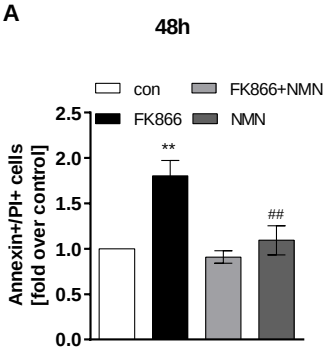
[36] S.M. Hadad, L. Baker, P.R. Quinlan, K.E. Robertson, S.E. Bray, G. Thomson, et al., Histological evaluation of AMPK signalling in primary breast cancer., BMC Cancer. 9 (2009) 307. doi:10.1186/1471-2407-9-307.

[37] P. Wang, T.-Y. Xu, Y.-F. Guan, W.-W. Tian, B. Viollet, Y.-C. Rui, et al., Nicotinamide phosphoribosyltransferase protects against ischemic stroke through SIRT1-dependent adenosine monophosphate-activated kinase pathway., Ann. Neurol. 69 (2011) 360–74. doi:10.1002/ana.22236.

4.8 Appendix: Supplementary Data



Supplementary Fig.1 Effects of FK866 on NAMPT activity, NAD content and AMPKα/mTOR activity in non-cancerous human hepatocytes. Non-cancerous primary human hepatocytes were stimulated with different doses of FK866 [1, 10, 100 nM] in serum-free medium for 48 h. A) NAMPT activity and B) NAD content were measured and normalized to total protein amount in each sample. C) Western blot analysis of mTOR expression and AMPKα activation were performed using specific antibodies against phospho-mTOR (Ser2448), total mTOR and phospho-AMPKα (Thr172). Background-corrected densitometric values were normalized to control (serum-free medium). Data are represented as mean± SEM of two independent experiments (n=2) and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test.



Supplementary Fig.2 FK866 induced cell death in Hep3B cells after 48 h. A) AnnexinV-FITC/PI assay of Hep3B cells treated with FK866 [10 nM], FK866 [10 nM] + NMN [500 µM] or NMN alone for 48 h (n=3). An^+ and An^+/PI^+- stained cells were considered as dead cells and are depicted in the graph. Cells stimulated with serum-free medium were used as control [con] and were set 1. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test. **p<0.01 compared to serum-free medium control; ##p<0.01 compared to FK866 [10 nM].

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Publications and Presentations

Publications

A. Garten., S. Schuster, M. Penke, T. Gorski, T. deGiorgis., W.Kiess, Physiological and pathophysiological roles of NAMPT and NAD metabolism., Nature Review Endocrinology, accepted

M. Penke, P.S. Larsen, S. Schuster, M. Dall, B.A.H. Jensen, T. Gorski, et al., Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet., Mol. Cell. Endocrinol. 412 (2015) 65–72. doi:10.1016/j.mce.2015.05.028.

W. Kiess, T. Gorski, M. Penke, S. Klamt, T.M. Kapellen, Diabetes mellitus in children and adolescents - a global epidemic which has become neglected., J. Pediatr. Endocrinol. Metab. 28 (2015) 247–50. doi:10.1515/jpem-2015-0900.

S. Schuster*, M. Penke*, T. Gorski, R. Gebhardt, T.S. Weiss, W. Kiess, et al., FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signalling in hepatocarcinoma cells, Biochem. Biophys. Res. Commun. (2015). doi:10.1016/j.bbrc.2015.01.111. *These authors are contributed equally to this work.

W. Kiess, M. Penke, E. Sergeyev, M. Neef, M. Adler, R. Gausche, et al., Childhood obesity at the crossroads., J. Pediatr. Endocrinol. Metab. 28 (2015) 481–4. doi:10.1515/jpem-2015-0168.

S. Schuster, M. Penke, T. Gorski, S. Petzold-Quinque, G. Damm, R. Gebhardt, et al., Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes, PLoS One. 9 (2014). doi:10.1371/journal.pone.0091045.

Presentations

Oral

M. Penke, P, Larsen, S. Schuster, T. Gorski, A. Meusel, S. Richter, S.G. Vienberg, J.T. Treebak, W. Kiess, A. Garten, Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet., German Society of Endocrinology (2015).

M. Penke, J.T. Treebak, S. Schuster, T. Gorski, W. Kiess, A. Garten, Hepatic NAD metabolism is dysregulated by an excessive supply of lipids., 12th International Congress on Obesity (2014).

Poster

M. Penke, P. Larsen, S. Schuster, T. Gorski, A. Meusel, S. Richter, SG. Vienberg, J.T. Treebak, W. Kiess, A. Garten, Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet., German Diabetes Association (2015).

M. Penke, P. Larsen, S. Schuster, T. Gorski, A. Meusel, S. Richter, S. Vienberg, J.T. Treebak, W. Kiess, A. Garten, Hepatic NAD salvage pathway is enhanced in on a high-fat diet., Leipzig Research Festival for LIFE Sciences (2014).

M. Penke, J.T. Treebak, S. Schuster, T. Gorski, W. Kiess, A. Garten, Hepatic NAD metabolism is dysregulated by an excessive supply of lipids., XVIII Lipid Meeting Leipzig (2013).

M. Penke, S. Schuster, T. Gorski, W. Kiess A. Garten, Oleate protects against palmitate-induced apoptosis and normalises NAMPT and NAD levels in HepG2 cells., NAD Metabolism & Signaling (2013).

M. Penke, S. Schuster, A. Garten, T. Gorski, W. Kiess, Autophagy-induced- apoptosis of palmitate is mediated by NAD depletion., Leipzig Research Festival for LIFE Sciences (2012).

Author contribution statement, Melanie Penke
The NAD salvage pathway during the progression of non-alcoholic fatty liver disease

Author contribution statement

Title:	Physiological and pathophysiological roles of NAMPT and NAD metabolism
Journal:	Nature Reviews Endocrinology
Authors:	Antje Garten, Susanne Schuster, Melanie Penke, Theresa Gorski, Tommaso deGiorgis, Wieland Kiess

Part Antje Garten:

- Work conception
- Wrote and revised the manuscript and managed it composition
- Figures and Tables

Part Susanne Schuster:

- Wrote the chapters “Mechanism of action” and “NAMPT in cancer”
- Figure design & drawings
- Revised the manuscript

Part Melanie Penke:

- Wrote the chapters “NAMPT and obesity” and “NAMPT and non-alcoholic fatty liver disease”
- Table “Reported associations between levels of NAMPT and obesity or NAFLD”
- Revised the manuscript

Part Theresa Gorski:

- Wrote the Chapter “NAMPT and type II diabetes”
- Figure and table design
- Revised the manuscript

Part Tommaso deGiorgis:

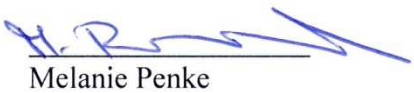
- Wrote the Chapter “NAMPT and type II diabetes”
- Table “Reported associations between levels of NAMPT and diabetes mellitus”
- Revised the manuscript

Part Wieland Kiess:

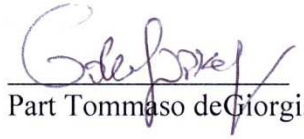
- Work conception
- Revised the manuscript

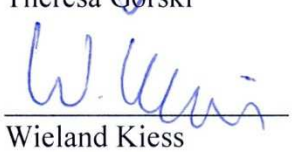

Antje Garten


Susanne Schuster


Melanie Penke


Theresa Gorski


Part Tommaso deGiorgis


Wieland Kiess

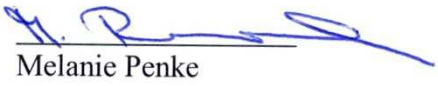
Author contribution statement, Melanie Penke
The NAD salvage pathway during the progression of non-alcoholic fatty liver disease

Author contribution statement

Title: Chapter 2: Hypothesis, aims and scientific questions
Authors: Melanie Penke

Bestätigung über die alleinige Urheberschaft von Kapitel 2

Ich, Melanie Penke, bestätige hiermit die alleinige Urheberschaft des monografisch hinzugefügten Kapitels 2 meiner Dissertation. Dieses Kapitel wurde selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in diesem Kapitel verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht.



Melanie Penke

Author contribution statement, Melanie Penke
The NAD salvage pathway during the progression of non-alcoholic fatty liver disease

Author contribution statement

Title:	Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet
Journal:	Molecular and Cellular Endocrinology
Authors:	Melanie Penke, Per S. Larsen, Susanne Schuster, Morten Dall, Benjamin A. H. Jensen, Theresa Gorski, Andrej Meusel, Sandy Richter, Sara G. Vienberg, Jonas T. Treebak, Wieland Kiess, Antje Garten

Part Melanie Penke:

- Designed, planned and performed in vivo experiments
- Western blot analysis, immunoprecipitation and qPCR
- HPLC analysis for NAD concentrations
- Measurement of NAMPT activity and serum NAMPT concentrations
- Analyzed, evaluated and interpreted the data
- Wrote the publication

Part Per S. Larsen:

- Metabolic characterisation of mice
- Body weight, lean and fat body mass
- Oral glucose tolerance test, insulin tolerance test
- Serum collection
- Analyzed, evaluated and interpreted the data
- Revised the manuscript

Part Susanne Schuster:

- Experimental design
- Analyzed, evaluated and interpreted the data
- Revised the manuscript

Part Morten Dall:

- Measurement of NAMPT protein abundance and NAD levels in the control mouse cohort
- Analyzed, evaluated and interpreted these data

Part Benjamin A. H. Jensen:

- Designed, planned and performed control mouse study
- Revised the manuscript

Part Theresa Gorski:

- Experimental design
- Analyzed, evaluated and interpreted the data
- Revised the manuscript

Part Andrej Meusel:

- Hepatic triacylglycerol measurement by NMR
- Analyzed, evaluated and interpreted these data
- Wrote method part “Triacylglycerol measurement of liver tissue”

Part Sandy Richter:

- Sample preparation, Western blot and qPCR analysis
- Revised the manuscript

Part Sara G. Vienberg:

- Designed, planned and performed mouse study
- Evaluated and interpreted the data
- Revised the manuscript

Part Jonas T. Treebak:

- Planned and designed experiments
- Evaluated and interpreted the data
- Wrote method parts “Mice and Ethical approval” and “Metabolic characterization”
- Revised the manuscript

Part Wieland Kiess:

- Evaluated and interpreted the data
- Revised the manuscript

Part Antje Garten:

- Project idea, study design and work schedule
- Analyzed, evaluated and interpreted the data
- Supported the writing of the manuscript
- Revised the manuscript

Melanie Penke

Per S. Larsen

Susanne Schuster

Morten Dall

Benjamin A. H. Jensen

Theresa Gorski

Andrey Meusel

Sandy Richter

Sara G. Vienberg

Jonas T. Treebak

Wieland Kiess

Antje Garten

Author contribution statement, Melanie Penke
The NAD salvage pathway during the progression of non-alcoholic fatty liver disease

Author contribution statement

Title:	FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signalling in hepatocarcinoma cells
Journal:	Biochem Biophys Res Communication
Authors:	Susanne Schuster*, Melanie Penke*, Theresa Gorski, Rolf Gebhardt, Thomas S. Weiss, Wieland Kiess, Antje Garten *Both authors contributed equally to this work.

Part Susanne Schuster:

- Project idea; designed, planned and performed experiments
- *In vitro* studies on Huh7 cells, HepG2 cells and primary human hepatocytes
- Western Blot analysis and gene expression analysis in Huh7 cells, HepG2 and primary human hepatocytes
- NAMPT activity measurements
- Analyzed, evaluated and interpreted the data
- Wrote the publication

Part Melanie Penke:

- Project idea; designed, planned and performed experiments
- *In vitro* studies on Hep3B cells, HepG2 cells and primary human hepatocytes
- Establishment of HPLC method for NAD measurement
- NAD measurements
- Western Blot analysis and gene expression analysis in Hep3B cells
- Analyzed, evaluated and interpreted the data
- Wrote and revised the manuscript

Part Theresa Gorski:

- Experimental design
- Analyzed, evaluated and interpreted the data
- Revised the manuscript

Part Rolf Gebhardt:

- Provided primary human hepatocytes
- Evaluated and interpreted the data
- Revised the manuscript

Part Thomas S. Weiss:

- Isolation and preparation of primary human hepatocytes
- Revised the manuscript

Part Wieland Kiess:

- Work schedule and study funding
- Evaluated and interpreted the data
- Revised the manuscript

Part Antje Garten:

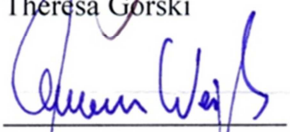
- Project idea, study design and work schedule
- Analyzed, evaluated and interpreted the data
- Revised the manuscript

Susanne Schuster

Melanie Penke

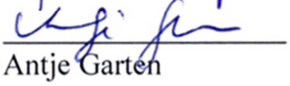
Theresa Gorski

Rolf Gebhardt



Thomas S. Weiss

Wieland Kiess



Antje Garten

Selbstständigkeitserklärung

Hiermit erkläre ich, Dipl.-trop. Melanie Penke, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für die Arbeit erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

Leipzig, 20.07.2015

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Melanie Penke

Danksagung

Eine wissenschaftliche Arbeit ist nie das Werk einer einzelnen Person. Darum ist es jetzt an der Zeit, mich bei allen Menschen zu bedanken, die mir die Erstellung meiner Dissertation ermöglicht haben.

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Zum Schluss möchte ich mich bei den Menschen bedanken, für die die vergangenen Jahre nicht minder aufregend waren: meinen Eltern, meiner Familie, meinem Freund und meinen Freunden. Ihr seid ein wichtiges Fundament meiner Doktorarbeit, habt mich während der ganzen Zeit unterstützt und mir immer wieder bewusst gemacht, was das Wichtigste im Leben ist.