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

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

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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<tr>
<td>2-OAADPR</td>
<td>2'-O-acetyl-ADP-riboside</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>4E binding protein 1</td>
</tr>
<tr>
<td>ac</td>
<td>acetyl-group</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-coenzyme A-carboxylase</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AIRg</td>
<td>acute insulin response to glucose</td>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
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<td>ALT</td>
<td>alanine transaminase</td>
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<td>ATP</td>
<td>adenosine monophosphate</td>
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<td>AMPK</td>
<td>5’-AMP-activated protein kinase</td>
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<tr>
<td>An</td>
<td>Annexin</td>
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<td>AST</td>
<td>aspartate transaminase</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BMAL1</td>
<td>aryl hydrocarbon receptor translocator-like protein 1</td>
</tr>
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<td>coronary artery disease</td>
</tr>
<tr>
<td>cADPR</td>
<td>cyclic ADP-ribose</td>
</tr>
<tr>
<td>CLOCK</td>
<td>circadian locomotore output cycles protein kaput</td>
</tr>
<tr>
<td>CPT</td>
<td>carnitine palmitoyltransferase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>CXCL16</td>
<td>chemokine (C-X-C motif) ligand 16</td>
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<td>eNAMPT</td>
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</tr>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
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<td>fatty acid synthase</td>
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<td>forkhead box protein O1</td>
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<td>fasting plasma glucose</td>
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<td>FRD</td>
<td>fructose-rich diet</td>
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<tr>
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<td>glucose-6-phosphatase</td>
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<tr>
<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
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<td>hepatocellular carcinoma</td>
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<td>HIF1α</td>
<td>hypoxia inducible factor 1α</td>
</tr>
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<td>homeostatic model assessment</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>iNAMPT</td>
<td>intracellular NAMPT</td>
</tr>
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<td>ITT</td>
<td>insulin tolerance test</td>
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<tr>
<td>MART</td>
<td>mono (ADP-ribosyl) transferase</td>
</tr>
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<td>MCP-I</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MnSOD</td>
<td>mitochondrial superoxide dismutase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NA</td>
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</tr>
<tr>
<td>NAAD</td>
<td>nicotinic acid adenine dinucleotide</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADS</td>
<td>nicotinamide adenine dinucleotide synthase</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NAM</td>
<td>nicotinamide</td>
</tr>
<tr>
<td>NAMN</td>
<td>nicotinic acid mononucleotide</td>
</tr>
<tr>
<td>NAMPT</td>
<td>nicotinamide phosphoribosyltransferase</td>
</tr>
<tr>
<td>NAPRT</td>
<td>nicotinate phosphoribosyltransferase</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NMN</td>
<td>nicotinamide mononucleotide</td>
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<td>NMNAT</td>
<td>nicotinamide/nicotinic acid mononucleotide adenyllyltransferase</td>
</tr>
<tr>
<td>NR</td>
<td>nicotinamide riboside</td>
</tr>
<tr>
<td>NRK</td>
<td>nicotinamide riboside kinase</td>
</tr>
<tr>
<td>NSPC</td>
<td>neuronal stem and/or progenitor cell</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
</tr>
<tr>
<td>P38 MAPK</td>
<td>mitogen-activated protein kinase P38</td>
</tr>
<tr>
<td>p70S6K</td>
<td>70S ribosomal protein S6 kinase</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBEF</td>
<td>pre-b cell colony-enhancing factor</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor gamma coactivator 1α</td>
</tr>
<tr>
<td>PHH</td>
<td>primary human hepatocytes</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PNPLA3</td>
<td>patatin-like phospholipase domain containing 3</td>
</tr>
<tr>
<td>PRPP</td>
<td>5′-phosphoribosyl-1-pyrophosphate</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SCDI</td>
<td>stearoyl-CoA-desaturase 1</td>
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<tr>
<td>SIRT</td>
<td>sirtuin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>sterol regulatory element-binding protein 1c</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglyceride</td>
</tr>
<tr>
<td>TDO</td>
<td>tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>TGFB</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
<tr>
<td>UCP2</td>
<td>mitochondrial uncoupling protein 2</td>
</tr>
<tr>
<td>VAT</td>
<td>visceral adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WHR</td>
<td>waist–hip ratio</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>γ-GT</td>
<td>γ-glutamyltransferase</td>
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</table>
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). 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 (CD68), 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
Summary

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, hepatocarcinoma 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 hepatzellulären 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.


Zusammenfassung


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: Nikotinamidphosphoribosyltransferase; NMN: Nikotinamidmononukleotid; NMNAT: Nikotinamidmononukleotidadenyllyltransferase; NR: Nikotinamidribosid; PPi: Pyrophosphat; PRPP: 5'-Phosphoribosyl-1-Pyrophosphat; SIRT1: Siruin 1


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


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: 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 reported 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 Ca²⁺ 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 locomotor 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 \textit{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 \textit{NAMPT} in disease development might depend on whether the active site, assembly of the active NAMPT dimer or gene expression of \textit{NAMPT} is affected.

A meta-analysis, which included human studies on the association between eNAMPT and obesity parameters, reported that 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]. \textit{In vitro} studies have shown that expression of \textit{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)\textgreek{a} 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-\textkappa activation [90,91] and production of cytokines, such as TNF\textgreek{a}, IL-6, IL-1\textgreek{b} [91,92], transforming growth factor \textgreek{b} [93] and monocyte chemoattractant protein 1 [94]. Production of inflammatory cytokines and \textit{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 \gamma in 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

<table>
<thead>
<tr>
<th>NAMPT in human studies</th>
<th>NAMPT correlation with</th>
<th>References</th>
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<tr>
<td>↑ levels of NAMPT in serum* and liver† of women with obesity vs normal-weight women</td>
<td>Levels of IL-6*, CRP*, resistin† and TNFα†</td>
<td>Auguet et al. (2013) [96]</td>
</tr>
<tr>
<td>↑ levels of NAMPT in serum* and liver† of women with obesity and NAFLD vs women with obesity without NAFLD</td>
<td>TNFα levels</td>
<td>Genc et al. (2013) [97]</td>
</tr>
<tr>
<td>No change in serum levels of NAMPT in patients with NAFLD vs health individuals</td>
<td>None</td>
<td>Hosseinzadeh-Attar et al. (2013) [98]</td>
</tr>
<tr>
<td>↓ levels of NAMPT in serum after 6-week weight reduction</td>
<td>Carbohydrate intake</td>
<td>Saboori et al. (2013) [99]</td>
</tr>
<tr>
<td>No change in serum levels of NAMPT in healthy women with obesity vs normal-weight women</td>
<td>BMI and levels of IL-6 (boys)</td>
<td>Li et al. (2013) [100]</td>
</tr>
<tr>
<td>↑ serum levels of NAMPT in children with obesity vs normal-weight children</td>
<td>None</td>
<td>Jaleel et al. (2013) [101]</td>
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<tr>
<td>↑ serum levels of NAMPT in children and adolescents with obesity vs normal-weight children and adolescents</td>
<td>None</td>
<td>Akbal et al. (2012) [102]</td>
</tr>
<tr>
<td>↑ serum levels of NAMPT in patients with NAFLD vs individuals without NAFLD</td>
<td>Levels of IL-6*, CRP* and TNFα†</td>
<td>Terra et al. (2012) [103]</td>
</tr>
<tr>
<td>↑ serum levels of NAMPT in women with morbid obesity vs lean women*</td>
<td>Body weight, BMI, WHR, levels of serum triglycerides, LDL cholesterol, insulin and C-peptide, insulin resistance as defined by HOMA and glucosose:insulin ratio</td>
<td>Taskesen et al. (2012) [104]</td>
</tr>
<tr>
<td>↓ serum levels of NAMPT after bariatric surgery†</td>
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<tr>
<td>↑ levels of NAMPT in subcutaneous and visceral adipose tissue† in women with morbid obesity vs lean women</td>
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<tr>
<td>↑ serum levels of NAMPT in adolescents with exogenous pubertal obesity vs healthy adolescents</td>
<td></td>
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<tr>
<td>↑ levels of NAMPT in serum and peripheral blood cells in individuals with obesity vs lean individuals</td>
<td>BMI*(^1), WHR*(^1), % body fat(^2), levels of ALT(^<em>), AST(^</em>), γ-GT(^*) and levels of HIF1α mRNA(^2)</td>
<td>Catalan et al. (2011) [105]</td>
</tr>
<tr>
<td>↑ serum levels of NAMPT in children with obesity vs lean children</td>
<td>Leucocyte counts</td>
<td>Friebe et al. (2011) [14]</td>
</tr>
<tr>
<td>↑ serum levels of NAMPT in adult individuals with obesity vs normal-weight adult individuals</td>
<td>BMI, waist circumference, hip circumference and insulin resistance as defined by HOMA</td>
<td>Reda et al. (2011) [106]</td>
</tr>
<tr>
<td>↑ serum levels of NAMPT in children and adolescents with obesity vs lean children and adolescents</td>
<td>Waist circumference, BMI, whole body insulin sensitivity and levels of IL-6 and thiobarbituric-acid-reactive substances (boys)</td>
<td>Krzystek-Korpacka et al. (2011) [107]</td>
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<td>↑ serum levels of NAMPT in prepubertal children with obesity vs prepubertal lean children</td>
<td>Levels of resistin and IL-6</td>
<td>Martos-Moreno et al. (2011) [108]</td>
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<td>↓ levels of NAMPT in serum and liver in patients with NAFLD vs healthy controls</td>
<td>None</td>
<td>Dahl et al. (2010) [109]</td>
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<td>↓ levels of NAMPT in visceral adipose tissue in patients with NAFLD vs individuals without NAFLD</td>
<td>None</td>
<td>Gaddipati et al. (2010)[110]</td>
</tr>
<tr>
<td>↑ levels of NAMPT in liver in patients with fibrosis vs individuals with NAFLD without fibrosis</td>
<td>Stage of fibrosis</td>
<td>Kukla et al. (2010) [111](^\text{18})</td>
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<td>No change in serum levels of NAMPT in women with obesity vs lean women</td>
<td>None</td>
<td>Ersoy et al. (2010)[112]</td>
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<td>↑ serum levels of NAMPT in individuals with obesity vs healthy, normal-weight individuals</td>
<td>Age, WHR and HbA(_{1c}) levels</td>
<td>Kaminska et al. (2010)[113]</td>
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<td>↓ levels of NAMPT in subcutaneous tissue in individuals with obesity vs normal-weight individuals</td>
<td>None</td>
<td>Barth et al. (2010)[114]</td>
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</table>

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>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>↑ NAMPT levels in human adipocytes</td>
<td>Haider <em>et al.</em> (2006) [79]</td>
</tr>
<tr>
<td>Fructose</td>
<td>↑ NAMPT levels in BAT and WAT of FRD-fed mice&lt;br&gt;↓ NAMPT levels in circulation of FRD-fed mice</td>
<td>Caton <em>et al.</em> (2011) [116]</td>
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<td>Oxidized LDL</td>
<td>↑ NAMPT levels in 3T3-L1 adipocytes</td>
<td>Chen <em>et al.</em> (2013) [81]</td>
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<tr>
<td>Growth hormone</td>
<td>↓ NAMPT levels in 3T3-L1 adipocytes</td>
<td>Kralisch <em>et al.</em> (2005) [82]</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑ NAMPT levels in rheumatoid arthritis&lt;br&gt;↓ NAMPT levels in 3T3-L1 adipocytes</td>
<td>Nowell <em>et al.</em> (2006) [117]&lt;br&gt;Kralisch <em>et al.</em> (2005) [82]</td>
</tr>
<tr>
<td>TNFα</td>
<td>↑ NAMPT levels in adipose tissue&lt;br&gt;↓ NAMPT levels in 3T3-L1 adipocytes</td>
<td>Hector <em>et al.</em> (2007) [92]&lt;br&gt;Kralisch <em>et al.</em> (2005) [82]</td>
</tr>
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<td>Palmitate</td>
<td>↑ NAMPT levels in HepG2 cells</td>
<td>Choi <em>et al.</em> (2011) [118]</td>
</tr>
<tr>
<td>Leucine</td>
<td>↑ NAMPT levels in HFD-fed mice</td>
<td>Li <em>et al.</em> (2012) [119]</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>↑ NAMPT levels in human preadipocytes and adipocytes&lt;br&gt;↑ NAMPT levels in 3T3-L1 adipocytes</td>
<td>Friebe <em>et al.</em> (2011) [84]&lt;sup&gt;85&lt;/sup&gt;&lt;br&gt;Kralisch <em>et al.</em> (2005) [82]</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>↑ NAMPT levels in human preadipocytes&lt;br&gt;↓ NAMPT levels in 3T3-L1 adipocytes</td>
<td>Friebe <em>et al.</em> (2011) [84]&lt;br&gt;Kralisch <em>et al.</em> (2005) [82]</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>↑ NAMPT levels in 3T3-L1 adipocytes&lt;br&gt;↑ NAMPT levels in MCF-7 breast cancer cells</td>
<td>Segawa <em>et al.</em> (2006) [86]&lt;br&gt;Bae <em>et al.</em> (2006) [120]</td>
</tr>
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</table>

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

<table>
<thead>
<tr>
<th>NAMPT in human studies</th>
<th>NAMPT correlation with</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ NAMPT levels in patients with obesity and T2DM vs normal-weight patients with T2DM vs healthy individuals</td>
<td>BMI, WHR, FPG, insulin resistance as defined by HOMA, and levels of triglycerides, total cholesterol, IL-6 and vaspin</td>
<td>El-Mesallamy et al. (2011) [140]</td>
</tr>
<tr>
<td>↑ NAMPT levels in patients with obesity and T2DM vs healthy individuals</td>
<td>T2DM (after controlling for anthropometric variables such as blood pressure, lipid profile and smoking status)</td>
<td>Chen et al. (2006) [141]</td>
</tr>
<tr>
<td>↑ NAMPT levels in patients with long-standing T1DM* and in patients with T2DM‡ vs healthy individuals§</td>
<td>↑ Age, no correlation with HbA1c levels</td>
<td>López-Bermejo et al. (2006) [142]</td>
</tr>
<tr>
<td>↑ NAMPT levels in patients with long-standing T1DM vs patients with T2DM</td>
<td>↑ HbA1c levels</td>
<td></td>
</tr>
<tr>
<td>↑ NAMPT levels in patients with T2DM and minor or severe proteinuria vs healthy individuals</td>
<td>↑ AIRg, fasting insulin levels, insulin sensitivity and 30-min OGTT (insulin secretion)</td>
<td>Yilmaz et al. (2008) [143]</td>
</tr>
<tr>
<td>↑ NAMPT levels in patients with T2DM and severe proteinuria vs patients with T2DM and minor proteinuria</td>
<td>Proteinuria, flow-mediated dilatation</td>
<td></td>
</tr>
<tr>
<td>↑ 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)</td>
<td>CAD</td>
<td>Saddi-Rosa et al. (2013) [73]</td>
</tr>
<tr>
<td>↑ frequency of T allele of NAMPT polymorphism rs9770242 in Brazilian cohort vs North-American, non-Hispanic white cohort</td>
<td></td>
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</tr>
</tbody>
</table>
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

| ↑ NAMPT levels in patients with T2DM vs healthy individuals | T2DM | Motawi *et al.* (2014) [144] |
| ↑ 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 | CVD |

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.
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, prostate, 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 considerably more 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 leukaeemias 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 is 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? Eightly, 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.

1.5 Acknowledgements

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1.6 References


Chapter 1


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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. Consequently 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™, 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-BFGGS 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 ¹⁴C-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 (Tnfa) as markers for inflammation were not changed in HFD mice (Figure 1G).

mRNA expression of the cytokines chemokine (C-X-C motif) ligand 16 (Cxc1l6), monocyte chemoattractant protein 1 (Mcp-1) and transforming growth factor β (Tgff) 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 cpn/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).
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 alpha (Pgc-1alpha) 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, Northwestern 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 1c (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).

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 alpha (Pgc-1alpha) 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, Northwestern 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 1c (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 \textit{Ucp2} while \textit{Pgc1a} and \textit{MnSOD} were unchanged (A) as analysed by qPCR. \textit{Srebp-1c} mRNA expression was up regulated while \textit{Fas}, \textit{Acc} and \textit{Scd1} mRNA expression was declined compared to Ctl mice (B). \textit{G6pc} and \textit{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 \textit{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 \textit{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 \textit{Nampt} levels, while an up regulation of \textit{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 \textit{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 \textit{Nampt} in humans have been reported. In obese women hepatic \textit{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 study found higher \textit{NAMPT} mRNA expression level in fibrotic livers [13]. Opposite, it has also been reported that patients with NAFLD showed lower levels of \textit{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.

3.6 Funding
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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


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, Tgfb, and Mcp-1, 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

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

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Chapter 4

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

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* both authors contributed equally to this work

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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 complex 1 (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, respectively. 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% CO₂.

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% CO₂.

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).
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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 hepatocytes 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 downstream 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\textsubscript{50} (EC\textsubscript{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.

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for providing Hep3B cells.
4.7 References


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


Presentations

**Oral**


**Poster**


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:
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- 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
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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:
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Part Tommaso deGiorgis:
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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


Melanie Penke
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

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- HPLC analysis for NAD concentrations
- Measurement of NAMPT activity and serum NAMPT concentrations
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- Oral glucose tolerance test, insulin tolerance test
- Serum collection
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Author contribution statement, Melanie Penke
The NAD salvage pathway during the progression of non-alcoholic fatty liver disease

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Journal: Biochem Biophys Res Communication
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- 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
Selbstständigkeitserklärung


Leipzig, 20.07.2015

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Melanie Penke
Danksagung


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