The Longer Life Foundation
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FINAL REPORT

Diagnostic and therapeutic applications of a novel plasma metabolite, nicotinamide mononucleotide (NMN), for age-associated metabolic complications in humans

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Abstract

This study aimed to understand the importance of systemic NAD biosynthesis regulated by nicotinamide phosphoribosyltransferase (NAMPT), a key NAD biosynthetic enzyme, in predicting and extending the health span in humans and to explore the potential of nicotinamide mononucleotide (NMN), a product of the NAMPT reaction and a novel metabolite in mouse and human plasma, as a biomarker of aging and a therapeutic agent to prevent and treat age-associated metabolic complications, such as type 2 diabetes, in humans. In our previous studies, we have demonstrated that NAMPT functions as an intra- and extracellular NAD biosynthetic enzyme and plays a critical role in maintaining normal NAD biosynthesis and glucose-stimulated insulin secretion in pancreatic β cells. We also found that plasma NMN levels decrease over age in mice. In this study, therefore, we hypothesized that plasma NMN levels might serve as a novel functional biomarker to predict the risks of age-associated metabolic complications and that NMN administration might be an effective intervention to prevent/treat those age-associated complications, such as type 2 diabetes, in mice and humans. To address this hypothesis, we conducted both mouse and human studies. In the mouse study [SPECIFIC AIM (1)], we examined the importance of NAMPT-mediated NAD biosynthesis in the pathogenesis of high-fat diet (HFD)- and age-induced type 2 diabetes. We successfully demonstrated that both HFD and aging compromise NAMPT-mediated NAD biosynthesis, contributing to the pathogenesis of diet- and age-induced type 2 diabetes. Short-term NMN administration dramatically ameliorated the pathophysiology of type 2 diabetes, partly through SIRT1 activation, providing proof of the concept that promoting NAD biosynthesis by administering NMN can be an effective nutriceutical anti-aging intervention. In the human study [SPECIFIC AIM (2)], we measured plasma NMN levels in 40 obese non-diabetic human subjects and analyzed possible relationships between plasma NMN levels and various metabolic parameters. Whereas this initial assessment failed to show any significant correlations, the study provided critical information to set up the next human study that will look much deeper into the dynamics of NAMPT-mediated systemic NAD biosynthesis in humans. Therefore, these findings clearly provide new insights into the physiological significance of NAMPT-mediated systemic NAD biosynthesis and therapeutic applications of NMN for metabolism and aging in rodents and humans.

Lay Summary

This study aimed to understand the importance of a new metabolic regulatory system termed systemic NAD biosynthesis in predicting and extending the health span in humans and to explore the potential of nicotinamide mononucleotide (NMN), a key chemical in this systemic NAD biosynthesis system, as a biomarker of aging and a therapeutic agent to prevent and treat age-associated metabolic complications, such as type 2 diabetes, in humans. Nicotinamide adenine dinucleotide (NAD) is an important chemical that functions as an essential currency for cellular energy metabolism in all living organisms. In mammals, nicotinamide (a form of vitamin B_3) is a major substrate to synthesize NAD, and NMN is a critical intermediate that is synthesized from nicotinamide. Nicotinamide phosphoribosyltransferase (NAMPT) is the key enzyme that catalyzes the conversion from nicotinamide to NMN. In our previous studies, we demonstrated that NAMPT functions both inside and outside the cells and plays a critical role in maintaining normal insulin secretion in pancreatic β cells. We also found that plasma NMN levels decrease over age in mice. In this study, therefore, we hypothesized that plasma NMN levels might serve as a novel functional biomarker to predict the risks of age-associated metabolic complications, such as type 2 diabetes, and that NMN administration might be an effective intervention to prevent/treat those age-associated complications in mice and humans. To address this hypothesis, we conducted both mouse and human studies. In the mouse study [SPECIFIC AIM (1)], we examined the importance of NAMPT-mediated NAD biosynthesis in the pathophysiology of high-fat diet (HFD)- and age-induced type 2 diabetes. We successfully demonstrated that both HFD and aging compromise NAMPT-mediated NAD biosynthesis, contributing to the pathophysiology of diet- and age-induced type 2 diabetes. Short-term NMN administration dramatically ameliorated metabolic complications caused by type 2 diabetes, providing proof of the concept that promoting NAD biosynthesis by administering NMN can be an effective nutriceutical anti-aging intervention. In the human study [SPECIFIC AIM (2)], we measured plasma NMN levels in 40 obese non-diabetic human subjects and analyzed possible relationships between plasma NMN levels and various metabolic parameters. Whereas this initial assessment failed to show any significant correlations, the study provided critical information to set up the next human study that will look much deeper into the dynamics of NAMPT-mediated systemic NAD biosynthesis in humans. Therefore, these findings clearly provide new insights into the physiological significance of NAMPT-mediated systemic NAD biosynthesis and therapeutic applications of NMN for metabolism and aging in rodents and humans.

1. Specific Aims

In this proposed study, we addressed the following hypothesis by two specific aims:

HYPOTHESIS: *Intra- and extracellular NAMPT (iNAMPT and eNAMPT) regulates systemic NAD biosynthesis in mammals, and the concentration of NMN in plasma is a critical determinant for the regulation of systemic NAD biosynthesis and the maintenance of normal tissue functions. The age-associated decline in plasma NMN levels affects cells that rely on circulating NMN for their normal functions, such as pancreatic β cells and neurons, and causes age-associated declines in their functions. Therefore, plasma NMN levels might serve as a novel biomarker to predict the risks of age-associated metabolic complications, and NMN administration might be an effective intervention to prevent/treat those age-associated complications, such as type 2 diabetes, in mice and humans.*

SPECIFIC AIM (1): To investigate the pathophysiological role of NAMPT-mediated systemic NAD biosynthesis in age-associated metabolic complications, particularly in obesity and type 2 diabetes, we will use a cohort of NAMPT-deficient heterozygous (*Nampt*$^{+/-}$) and control (*Nampt*$^{+/+}$) mice and examine whether *Nampt* haplodeficiency accelerates the development of metabolic complications over age or under a high-fat diet (HFD). We will also examine whether NMN administration can prevent and/or improve those complications in aging and HFD-fed *Nampt*$^{+/+}$ and *Nampt*$^{+/-}$ mice.

SPECIFIC AIM (2): To examine whether plasma NMN levels can be used as a biomarker to assess the risk of age-associated metabolic complications, we will measure eNAMPT and NMN levels in plasma samples from human obese non-diabetic subjects at the age of 30s-60s. These plasma samples will be provided by Dr. Samuel Klein's laboratory, and plasma NMN levels in particular will be measured by a new mass spectrometry-driven methodology in collaboration with Dr. R. Reid Townsend in the Department of Medicine.

2. Results

2.1. SPECIFIC AIM (1) – Mouse Studies

For this Specific Aim, we have successfully conducted all proposed experiments during two budgetary years. Through these experiments, we have made critical findings demonstrating the importance of NAMPT-mediated NAD biosynthesis in the pathogenesis of type 2 diabetes and providing proof of the concept for the application of NMN as an anti-aging nutriceutical intervention.

As partly reported in the last year's progress report, we have demonstrated that both HFD and aging compromise NAMPT-mediated NAD biosynthesis, resulting in significant decreases in NAMPT protein and NAD levels in multiple metabolic tissues. These defects contribute to insulin

resistance in the liver and impaired glucose-stimulated insulin secretion (GSIS) in pancreatic β cells. Strikingly, NMN administration can significantly improve hepatic insulin sensitivity and GSIS. In the HFD-fed diabetic liver, NMN also restores gene expression related to oxidative stress, inflammatory response, and circadian rhythm, partly through the activation of the mammalian NAD-dependent deacetylase SIRT1. These findings strongly suggest that underlying defects in NAMPT-mediated NAD biosynthesis play an important role in the pathogenesis of diet- and age-induced type 2 diabetes and also that promoting NAD biosynthesis by administering NMN, a key NAD intermediate, can be an effective intervention to treat the pathophysiology of type 2 diabetes.

The manuscript reporting all these striking findings is currently under revision for *Cell Metabolism*. The manuscript is attached as an appendix with this final report.

2.2. SPECIFIC AIM (2) – Human Studies

Despite serious troubles with the mass spec machine in the Townsend lab, we managed to conduct experiments proposed in this Specific Aim. The results, which are described below, provided important ideas on how to translate our research to humans. Based on these results, Dr. Samuel Klein and I are currently working together on another human protocol that will allow us to examine more details of NAMPT-mediated systemic NAD biosynthesis in human subjects.

In the first budgetary year, we successfully developed a mass spectrometry-driven methodology that enabled us to detect major NAD precursors and intermediates in human and mouse plasma samples. For example, we were able to detect nicotinamide, NMN, and nicotinamide riboside (NR) in human plasma samples (Figure 1A). Because of the matrix

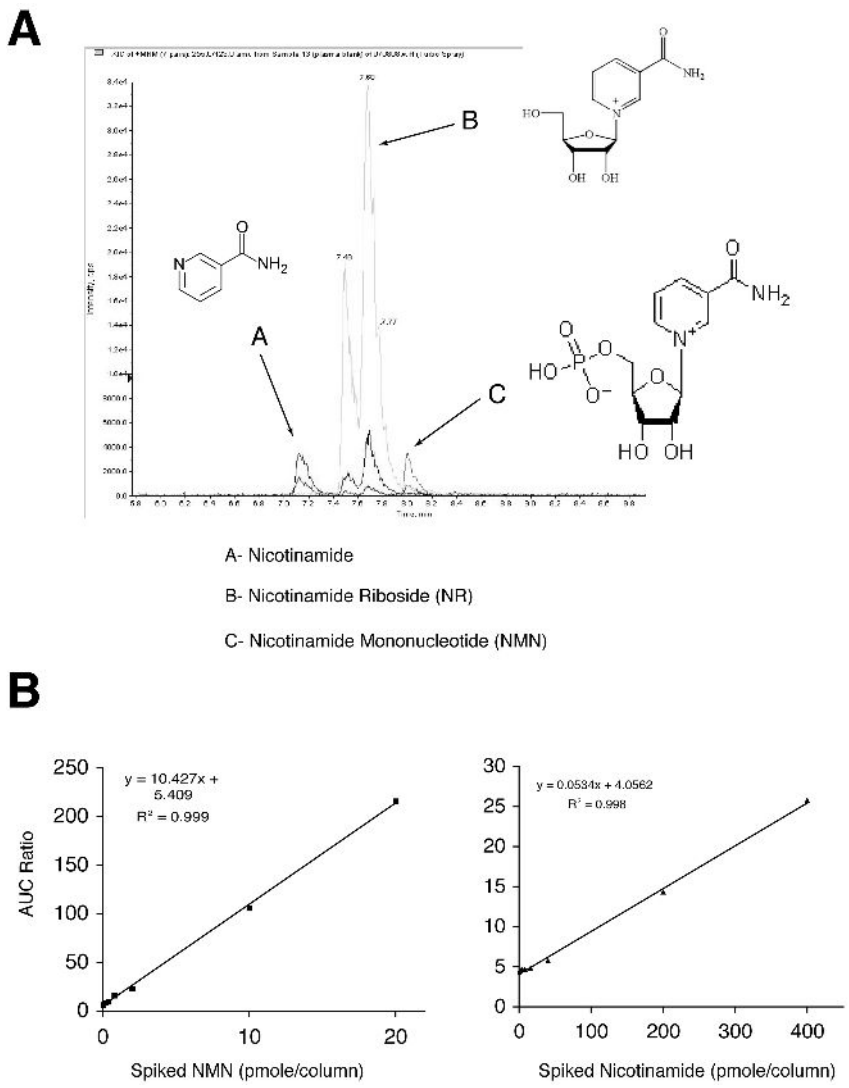


Figure 1. (**A**) Detection of nicotinamide, nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN) in human plasma by mass spectrometry. (**B**) Standard curves of NMN and nicotinamide using human plasma samples spiked with NMN (left) and nicotinamide (right).

effects, the development of a quantitative measurement method for these NAD-related chemicals was quite challenging. Nonetheless, we found appropriate column and buffer conditions to obtain linear standard curves of NMN and nicotinamide using human plasma samples spiked with these compounds (Figure 1B).

Using this mass spec-driven quantitative measurement method, we analyzed human plasma samples from 20 subjects with the highest and another 20 with the lowest disposition index (DI) values, as originally proposed. Specimens and metabolic data were collected from those individuals in 2005-2006 as a part of other studies funded by Dr. Klein's discretionary funds. We did not include a lean control group because 1) it was practically very difficult to recruit lean control people who had a similar range of disposition indices and 2) the main purpose of this specific aim was to study possible correlations between plasma NMN levels and metabolic parameters reflecting pancreatic β cell function. Body composition and metabolic variables in selected 40 obese subjects

Table 1. Body composition and metabolic variables in 40 obese subjects with low and high DI values

matching ID	Age	Sex (female = 1, male = 2)	BMI	% body fat	Fasting glucose (mg/dL)	Fasting insulin (ng/mL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	Systolic BP (mm of Hg)	Diastolic BP (mm of Hg)	DI	AIRg	Si
20 lowest														
009334	55	2	33.6	29.1	90.9	17.3	128	117	46	117	66	74	41	1.79
004432	45	1	32.91	41.9	96.0	14.0	127	113	44	130	86	160	46	3.53
007233	63	2	35.43	32.3	92.5	24.8	133	101	31	149	89	249	198	1.26
900531	43	1	41.9	49.8	98.5	22.0	216	87	54	140	78	262	216	1.21
004532	34	2	40.71	40.2	90.1	19.8	91	99	31	130	77	308	116	2.65
005633	60	1	41.76	47.9	79.4	12.5	153	178	42	147	80	309	498	0.62
902331	32	1	37.71	44.6	92.0	15.3	168	106	45	126	84	309	116	2.67
003732	35	1	37.97	46.3	87.8	13.0	72	94	30	105	64	335	162	2.08
002932	42	1	37.35	48.3	85.0	12.8	132	111	63	109	63	361	391	0.92
009134	54	2	37.47	33.1	86.8	13.5	85	139	36	148	85	427	307	1.39
004332	57	1	33.7	37.7	98.6	19.5	N/A	N/A	N/A	159	79	428	189	2.26
004032	63	1	31.13	42.9	93.4	12.0	91	141	59	116	68	434	193	2.25
902531	33	2	32.8	24.6	84.1	14.8	172	113	30	135	83	442	406	1.09
008434	47	1	35.53	43.8	83.3	8.5	193	164	49	130	87	448	561	0.80
004732	38	2	34.04	29.3	100.8	15.3	159	124	42	142	89	530	373	1.42
900831	55	1	40.18	50.5	86.6	22.5	115	194	59	142	76	576	344	1.68
902431	53	2	35.33	37.9	90.3	13.0	159	132	39	125	72	585	348	1.68
900731	44	1	33.82	41.0	80.0	10.8	99	98	39	144	90	619	237	2.61
902231	50	1	31.09	40.5	88.0	7.0	137	99	64	133	77	625	150	4.18
003832	36	1	33.65	44.9	83.1	11.3	271	122	56	127	82	670	238	2.82
AVERAGE	46.95		35.9	40.3	89.4	15.0	142.2	122.7	45.2	132.5	78.5	407.5	256.3	1.9
SD	10.2		3.3	7.4	6.1	4.7	49.2	29.3	11.3	14.1	8.3	161.5	143.0	0.9
SE	2.29		0.7	1.6	1.4	1.0	11.0	6.6	2.5	3.2	1.9	36.1	32.0	0.2
20 highest														
006533	64	2	36.48	36.6	74.0	16.8	210	187	37	143	82	1042	733	1.42
003232	46	1	37.21	42.4	88.1	17.8	57	128	57	147	92	1106	717	1.54
006433	35	1	38.93	45.0	71.4	19.3	227	99	29	117	72	1151	479	2.40
008134	39	1	39.33	49.4	81.4	18.3	204	65	51	124	78	1174	1132	1.04
005133	32	1	38.46	45.4	79.3	8.5	116	111	39	116	74	1191	451	2.64
003532	53	1	40.2	51.8	87.4	12.5	70	122	61	134	75	1224	507	2.41
003032	47	2	40.42	35.1	103.1	33.8	100	118	43	131	78	1287	1002	1.28
003932	61	1	32.43	43.3	94.5	13.0	78	123	60	110	54	1314	415	3.17
900631	46	1	31.63	37.3	79.1	10.5	120	108	61	122	80	1341	707	1.90
900131	34	1	35.46	37.7	73.5	11.0	97	136	60	121	71	1563	841	1.86
003632	46	1	32.73	40.5	90.3	4.3	47	139	54	128	61	1660	233	7.13
008634	44	1	39.66	50.2	86.9	9.5	107	128	57	122	80	1677	404	4.16
005833	53	2	34	28.2	87.0	9.0	124	162	38	131	90	1777	617	2.88
009034	40	1	33.07	45.0	86.6	14.0	108	110	47	122	73	2204	1615	1.36
003332	48	1	35.72	36.9	77.0	17.0	294	142	40	123	79	2255	1012	2.23
901931	45	1	30.51	43.2	92.3	4.5	75	95	67	105	50	2258	421	5.36
901331	53	1	35.63	47.8	94.1	10.0	63	123	56	107	68	2712	945	2.87
901831	64	1	39.52	45.3	89.3	10.3	103	108	47	137	69	3277	879	3.73
900331	45	1	35.59	35.8	92.0	6.3	49	121	59	106	67	3810	394	9.66
004933	38	1	34.33	41.3	77.1	14.3	44	115	47	120	82	3942	1755	2.25
AVERAGE	46.65		36.1	41.9	85.2	13.0	114.7	122.0	50.5	123.1	73.5	1898.1	762.8	3.06
SD	9.27		3.1	5.9	8.3	6.6	67.9	25.1	10.3	11.5	10.5	900.3	402.2	2.1
SE	2.07		0.7	1.3	1.9	1.5	15.2	5.6	2.3	2.6	2.4	201.3	89.9	0.5
TTEST	0.923		0.874	0.459	0.081	0.287	0.158	0.933	0.135	0.027	0.102	0.00000001	0.0000051	0.039

were already evaluated by Dr. Klein's group (Table 1).

Possible correlations between plasma NMN levels and various metabolic parameters were assessed for those 40 subjects. As shown in Figure 2, we were not able to find any significant correlations between plasma NMN levels and any of the metabolic parameters we assessed. Based on our mouse studies, we particularly expected to see a positive correlation between NMN levels and the acute insulin response to glucose (AIR_g; an index of insulin secretion in response to the glucose bolus) or fasting insulin levels in each DI group. However, as shown in Figures 2A and 2F, we did not observe any trend between NMN levels and either of AIR_g or fasting insulin levels.

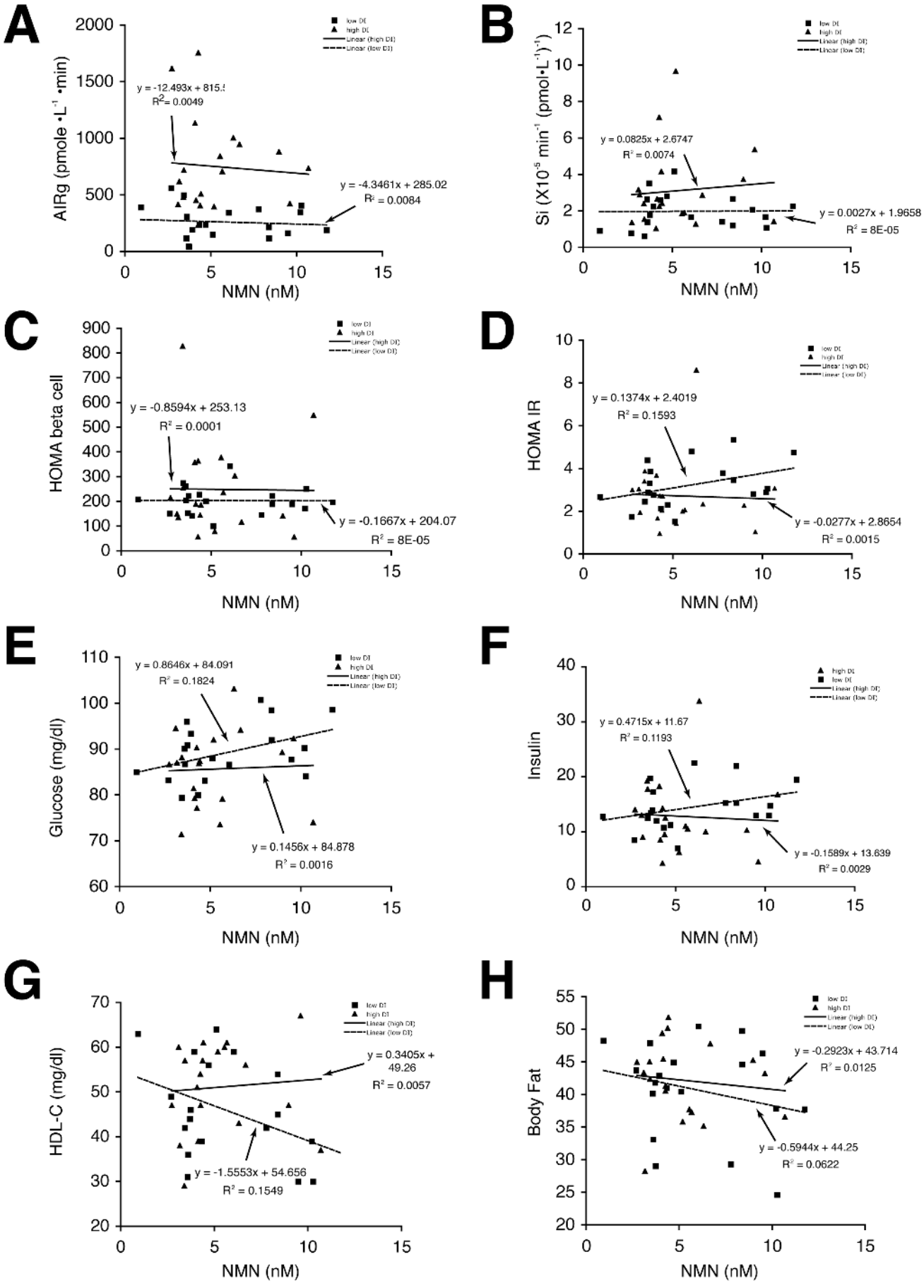
The fact that we did not see any meaningful correlations between plasma NMN levels and metabolic parameters is somewhat surprising. These initial "negative" results need to be evaluated carefully, and there are several important points to discuss:

1) We noticed that NMN levels detected in those plasma samples tended to be lower than those in freshly collected plasma samples, implying that NMN in these samples might have suffered some degradation during storage. Therefore, in our next study that Dr. Klein and I are currently planning, we should measure NMN levels as soon as possible after collecting plasma samples.

2) Given that NAMPT-mediated NAD biosynthesis exhibits circadian oscillation, plasma NMN levels might also show such an oscillation. If this is the case, we should collect plasma samples at the time when plasma NMN or eNAMPT levels reach highest to detect any pathophysiologically relevant differences in plasma NMN levels. Thus, to optimize our assay condition, we are currently planning to examine if plasma eNAMPT and NMN levels exhibit circadian oscillation in human subjects.

3) As described in the attached manuscript, we found that after intraperitoneal administration, NMN was immediately taken up into tissues and converted to NAD within 15 min in mice (see Figure 1C and S1C in the attached manuscript), implicating that plasma NMN levels might be kept low and constant because the equilibrium of NMN distribution is always from plasma to tissues. If this is the case, we would never be able to see significant differences in plasma NMN levels. Instead, NMN and NAD levels in some particular tissues or cell types should reflect the extent of systemic NAD biosynthesis mediated by both iNAMPT and eNAMPT. Interestingly, it has been reported that NAMPT-mediated NAD biosynthesis is critical for neutrophilic granulocyte differentiation in humans (Skokowa *et al.*, Nat. Med. 15: 151-158, 2009). More recently, it has also been reported that serum eNAMPT levels are positively correlated with circulating leukocyte counts (Friebe *et al.*, Diabetologia,

Figure 2 (next page). Relationships between plasma NMN levels and various metabolic parameters in 40 obese subjects with low and high disposition index (DI) values. Closed triangles and rectangles represent values from human subjects with high and low DI values, respectively. Plasma NMN levels were measured with our mass spectrometry-driven quantitative method. AIRg (**A**), S_I (**B**), HOMA beta cell (**C**), HOMA IR (**D**), fasting glucose (**E**), fasting insulin (**F**), HDL cholesterol (**G**), and body fat (**H**) were obtained or calculated from Table 1.


A
AIRg (pmole •L⁻¹ •min)
NMN (nM)
low DI
high DI
Linear (high DI)
Linear (low DI)
y = -12.493x + 815.
R²= 0.0049
y = -4.3461x + 285.02
R² = 0.0084
B
Si (X10⁻⁵ min⁻¹ (pmol•L⁻¹)⁻¹)
NMN (nM)
y = 0.0825x + 2.6747
R² = 0.0074
y = 0.0027x + 1.9658
R² = 8E-05
C
HOMA beta cell
NMN (nM)
y = -0.8594x + 253.13
R² = 0.0001
y = -0.1667x + 204.07
R² = 8E-05
D
HOMA IR
NMN (nM)
y = 0.1374x + 2.4019
R² = 0.1593
y = -0.0277x + 2.8654
R² = 0.0015
E
Glucose (mg/dl)
NMN (nM)
y = 0.8646x + 84.091
R² = 0.1824
y = 0.1456x + 84.878
R² = 0.0016
F
Insulin
NMN (nM)
high DI
low DI
y = 0.4715x + 11.67
R² = 0.1193
y = -0.1589x + 13.639
R² = 0.0029
G
HDL-C (mg/dl)
NMN (nM)
y = 0.3405x + 49.26
R² = 0.0057
y = -1.5553x + 54.656
R² = 0.1549
H
Body Fat
NMN (nM)
y = -0.2923x + 43.714
R² = 0.0125
y = -0.5944x + 44.25
R² = 0.0622

Epub on Feb 6, 2011). We also have a preliminary result showing that peripheral blood mononuclear cells (PBMCs) are sensitive to NMN treatment (data not shown). Taken together, we speculate that NMN and NAD levels in PBMCs might properly reflect the extent of eNAMPT-mediated NMN synthesis in blood circulation. Dr. Klein and I are currently working together on a new human protocol that will allow us to analyze NMN and NAD levels in PBMCs with 3-hr intervals through 24 hrs, as well as those in biopsy samples from white adipose tissue and skeletal muscle, in human subjects.

Although our initial assessments with 40 obese subjects failed to show meaningful correlations, this study provided critical information to set up the next human study. Given that NAMPT-mediated NAD biosynthesis is highly conserved between rodents and humans, it is highly likely that this NAD biosynthesis system also has significant relevance in human physiology. For example, it has been shown that NAMPT expression and NAD levels are enhanced in multiple tissues and cell types in rodents in response to various stimuli including low energy intake (Yang *et al.*, Cell 130: 1095-1107, 2007; Fulco *et al.*, Dev. Cell 14: 661-673, 2008; Cantó *et al.*, Cell Metab. 11: 213-219, 2010). Dr. Klein's and our groups have recently obtained an interesting preliminary result showing that calorically restricted human subjects tend to show increases in NAD levels in WAT and skeletal muscle (Figure 3), implying that NAMPT-mediated NAD biosynthesis likely plays an important role in regulating NAD levels in response to low energy intake in humans. Therefore, it is critical to continue our effort of identifying appropriate assay conditions to assess NAMPT-mediated systemic NAD biosynthesis in human subjects. Indeed, this effort has become much more important, now that we have clearly demonstrated the potential of NMN as an effective anti-aging nutriceutical intervention in mice [see SPECIFIC AIM (1)]. Most recently, we have initiated a collaborative project under the Sponsored Research Agreement with Oriental Yeast Co. in Japan to conduct a long-term NMN administration experiment. Using these preclinical results, we are planning to move forward to a human clinical trial for NMN. This Pilot & Feasibility grant from the Longer Life Foundation has provided significant support to make all these translational efforts possible. I greatly appreciate this generous two-year grant support from the Foundation.

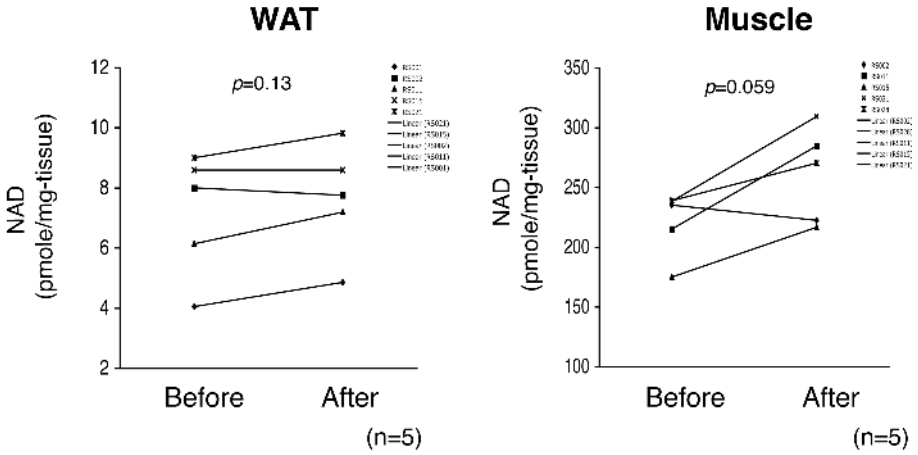


Figure 3. NAD levels in white adipose tissue and skeletal muscle from five human subjects before and after caloric restriction. This measurement was conducted by the Imai lab, which is a part of the human protocol approved for Dr. Klein's Resvida study.

3. Appendix

The manuscript that contains all results for SPECIFIC AIM (1) is attached with this final report. This is currently under revision for *Cell Metabolism*.