

The differential impact of three different NAD⁺ boosters on circulatory NAD and microbial metabolism in humans

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2. Research Project Protocol

- Research Project Title: "Effect of Oral NAD⁺ Precursors Administration on Blood NAD⁺ Concentration in Healthy Adults"

3. Statistical Analysis Plan

Supplementary Table 1. Changes in the concentrations of NAD⁺, NADP⁺, NADH, NADPH and excretion metabolites MeNam and MeXPY level in whole blood after a 14 days chronic administration of Nam, NR, NMN or placebo. Concentrations are expressed in $\mu\text{mol/L}$ and the adjusted estimates were obtained with a linear mixed model.

Whole Blood	NAD ⁺		NADP ⁺		NADH		NADPH		MeNam		MeXPY	
	Conc.	CI 95%	Conc.	CI 95%	Conc.	CI 95%	Conc.	CI 95%	Conc.	CI 95%	Conc.	CI 95%
NR	46.62	(39.643; 53.592)	4.57	(-0.774; 9.921)	-0.10	(-0.232; 0.035)	-1.77	(-2.981; -0.567)	1.03	(0.554; 1.506)	15.35	(11.958; 18.738)
NMN	40.30	(32.811; 47.786)	6.08	(0.315; 11.851)	-0.03	(-0.171; 0.115)	-2.03	(-3.357; -0.712)	0.56	(0.051; 1.074)	7.64	(4.015; 11.264)
Nam	0.86	(-5.900; 7.628)	0.52	(-4.781; 5.825)	-0.17	(-0.295; -0.037)	-0.92	(-2.128; 0.28)	1.75	(1.29; 2.214)	11.90	(8.616; 15.192)
Placebo	-2.77	(-9.81; 4.275)	2.54	(-2.826; 7.916)	-0.22	(-0.356; -0.085)	-0.71	(-1.929; 0.504)	0.10	(-0.377; 0.586)	0.02	(-3.398; 3.432)
NR vs placebo	49.39	(39.463; 59.307)	2.03	(-5.565; 9.621)	0.12	(-0.069; 0.313)	-1.06	(-2.779; 0.722)	0.93	(0.249; 1.602)	15.33	(10.512; 20.15)
p values	< 0.001		0.595		0.207		0.221		0.008		< 0.001	
NMN vs placebo	43.07	(32.726; 53.407)	3.54	(-4.374; 11.45)	0.19	(-0.005; 0.390)	-1.32	(-3.128; 0.484)	0.46	(-0.251; 1.166)	7.62	(2.621; 12.624)
p values	< 0.001		0.374		0.056		0.148		0.201		0.003	
Nam vs placebo	3.63	(-6.163; 13.427)	-2.02	(-9.595; 5.549)	0.05	(-0.133; 0.242)	-0.21	(-1.926; 1.503)	1.65	(0.976; 2.319)	11.89	(7.13; 16.643)
p values	0.461		0.595		0.563		0.806		< 0.001		< 0.001	
NR vs Nam	45.75	(36.041; 55.466)	4.05	(-3.562; 11.664)	0.07	(-0.119; 0.253)	-0.85	(-2.565; 0.866)	-0.72	(-1.387; -0.058)	3.44	(-1.285; 8.173)
p values	< 0.001		0.291		0.471		0.326		0.034		0.15	
NMN vs Nam	39.43	(29.357; 49.512)	5.56	(-2.403; 13.526)	0.14	(-0.054; 0.331)	-1.11	(-2.942; 0.722)	-1.19	(-1.876; -0.504)	-4.26	(-9.148; 0.62)
p values	< 0.001		0.167		0.156		0.230		0.001		0.086	
NR vs NMN	6.32	(-3.919; 16.557)	-1.51	(-9.306; 6.286)	-0.07	(-0.266; 0.124)	0.26	(-1.517; 2.038)	0.47	(-0.233; 1.168)	7.71	(2.744; 12.672)
p values	0.221		0.700		0.469		0.770		0.187		0.003	

Supplementary Table 2. LLOQ in $\mu\text{mol/L}$ of NAD^+ metabolome in targeted quantitative assays for human whole blood, plasma and urine.

NAD Metabolite types	NAD species	LLOQ in $\mu\text{mol/L}$		
		Human Whole Blood	Human EDTA-Plasma	Human Urine
Active NAD	NAD ⁺	0.2	n.a.	0.005
	NADH	0.2	n.a.	n.a.
	NADP ⁺	0.2	n.a.	n.a.
	NAPDH	0.2	n.a.	n.a.
Salvage Pathway	NAM	0.2	0.02	0.05
	NR	0.02	0.01	0.005
	NMN	0.02	0.01	0.005
Preiss-Handler Pathway	NA	0.02	0.02	0.05
	NAR	n.a.	n.a.	0.005
	NAMN	n.a.	n.a.	n.a.
	NAAD	0.02	n.a.	n.a.
Excretion - metylation and oxydation	1-MeNAM	0.02	0.01	1
	Me2PY (MeXPY in WB)	0.02	0.03	1
	Me4PY	n.a.	0.01	1
	NUR	n.a.	0.01	0.005
	Nam-N-oxide	n.a.	n.a.	0.05
		n.a. = not implemented in the method		

Supplementary Table 3. Changes of urine Nam, NR, NMN, metabolites NAR, MeNam and MeXPY as well as urinary metabolites nicotinuric acid (NUR) and nicotinamide N-oxide (NamOx) in chronic conditions. Data correspond to adjusted estimate and the 95% confidence interval. Interferential concentrations are obtained after comparison between urine V4 versus V3 (T=0). Concentrations are expressed in $\mu\text{mol}/\text{mmol}$ creatinine or nmol/mmol creatinine (*).

Urine	Nam	NR(*)	NMN(*)	MeNam	MeXPY	NAR(*)	NUR(*)	NamOx
NR vs placebo	0.47 (-2.214, 3.144)	1.42 (-2.089, 4.939)	0.41 (-0.043, 0.854)	59.68 (40.796, 78.564)	170.81 (132.436, 209.194)	176.84 (133.718, 219.958)	122.94 (65.444, 180.429)	2.07 (0.152, 3.997)
<i>p</i> values	0.729	0.420	0.075	< 0.001	< 0.001	< 0.001	< 0.001	0.035
NMN vs placebo	-0.04 (-2.814, 2.732)	1.55 (-2.183, 5.282)	0.10 (-0.362, 0.553)	38.04 (18.45, 57.636)	90.45 (51.258, 129.649)	111.52 (67.025, 156.013)	46.47 (-13.667, 106.606)	0.85 (-1.143, 2.848)
<i>p</i> values	0.976	0.409	0.677	< 0.001	< 0.001	< 0.001	0.127	0.396
Nam vs placebo	0.96 (-1.728, 3.656)	0.77 (-2.822, 4.352)	0.28 (-0.158, 0.725)	43.28 (24.39, 62.169)	132.64 (94.433, 170.849)	-0.66 (-43.726, 42.402)	-3.56 (-60.095, 52.985)	4.74 (2.818, 6.662)
<i>p</i> values	0.476	0.671	0.203	< 0.001	< 0.001	0.976	0.900	< 0.001
NR vs Nam	-0.50 (-3.158, 2.16)	0.66 (-2.842, 4.161)	0.12 (-0.326, 0.57)	16.40 (-2.397, 35.197)	38.17 (-0.791, 77.138)	177.50 (134.204, 220.795)	126.49 (68.578, 184.405)	-2.67 (-4.581, -0.75)
<i>p</i> values	0.708	0.707	0.588	0.086	0.055	< 0.001	< 0.001	0.007
NMN vs Nam	-1.01 (-3.762, 1.751)	0.78 (-2.83, 4.398)	-0.19 (-0.653, 0.277)	-5.24 (-24.718, 14.245)	-42.19 (-81.819, -2.557)	112.18 (67.192, 157.17)	50.02 (-10.367, 110.417)	-3.89 (-5.872, -1.902)
<i>p</i> values	0.468	0.665	0.421	0.592	0.037	< 0.001	0.103	< 0.001
NR vs NMN	0.51 (-2.241, 3.254)	-0.12 (-3.748, 3.498)	0.31 (-0.162, 0.782)	21.64 (2.207, 41.066)	80.36 (40.895, 119.828)	65.32 (20.441, 110.197)	76.47 (17.233, 135.7)	1.22 (-0.758, 3.202)
<i>p</i> values	0.713	0.945	0.193	0.03	< 0.001	0.005	0.012	0.221

Supplementary Table 4. Differences of iAUC and Cmax of whole blood NAD⁺, Nam, NR, NMN and NADP⁺ in acute conditions at day 1. Data corresponds to adjusted estimate and the 95% confidence interval. AUC and Cmax are expressed in $\mu\text{mol/L.h}$, and $\mu\text{mol/L}$, respectively.

	NAD ⁺		Nam		NR		NMN		NADP ⁺	
	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax
Nam vs placebo	8.48 (4.44, 12.52)	2.76 (1.26, 4.26)	105.17 (87.39, 122.94)	48.07 (40.91, 55.24)	0.73 (0.38, 1.08)	0.34 (0.16, 0.52)	0.11 (0.06, 0.17)	0.04 (0.01, 0.06)	10.39 (3.44, 17.35)	4.60 (1.02, 8.19)
p-value	0.001	0.003	<0.001	<0.001	0.001	0.002	0.001	0.01	0.015	0.036
NR vs placebo	0.49 (-3.61, 4.58)	-0.26 (-1.78, 1.26)	-11.98 (-30.03, 6.07)	-5.59 (12.87, 1.68)	-0.03 (-0.38, 0.32)	-0.01 (-0.19, 0.17)	0.03 (-0.002, 0.09)	0.02 (-0.01, 0.04)	4.43 (-2.59, 11.44)	2.41 (-1.2, 6.03)
p-value	0.842	0.775	0.272	0.204	0.888	0.92	0.317	0.257	0.296	0.269
NMN vs placebo	-2.24 (-6.5, 2.02)	-1.11 (-2.69, 0.47)	-12.04 (-30.72, 6.65)	-5.80 (-13.34, 1.73)	-0.01 (-0.38, 0.36)	-0.01 (-0.2, 0.18)	0.04 (-0.02, 0.1)	0.03 (0.01, 0.06)	3.50 (-3.79, 10.79)	3.82 (0.06, 7.57)
p-value	0.383	0.245	0.286	0.203	0.958	0.961	0.243	0.025	0.425	0.095
NR vs Nam	-7.99 (-12.01, -3.97)	-3.02 (-4.51, -1.53)	-117.15 (-134.89, -99.4)	-53.66 (-60.81, -46.51)	-0.76 (-1.11, -0.41)	-0.35 (-0.54, -0.17)	-0.08 (-0.14, -0.02)	-0.02 (-0.04, 0)	-5.97 (-12.95, 1.01)	-2.19 (-5.78, 1.41)
p-value	0.002	0.001	<0.001	<.001	0.001	0.002	0.021	0.134	0.158	0.313
NMN vs Nam	-10.72 (-14.88, -6.55)	-3.87 (-5.42, -2.33)	-117.20 (-135.55, -98.55)	-53.88 (-61.27, -46.48)	-0.74 (-1.1, -0.38)	-0.35 (-0.54, -0.16)	-0.07 (-0.13, -0.01)	0.00 (-0.03, 0.02)	-6.89 (-14.17, 0.38)	-0.78 (-4.53, 2.96)
p-value	<0.001	<0.001	<0.001	<.001	0.001	0.003	0.042	0.815	0.119	0.728
NR vs NMN	2.73 (-1.5, 6.96)	0.85 (-0.72, 2.42)	0.06 (-18.54, 18.65)	0.21 (-7.28, 7.70)	-0.02 (-0.38, 0.35)	-0.01 (-0.2, 0.18)	-0.01 (-0.07, 0.05)	-0.02 (-0.04, 0.01)	0.93 (-6.3, 8.15)	-1.40 (-5.13, 2.33)
p-value	0.286	0.37	0.996	0.962	0.933	0.962	0.835	0.232	0.831	0.532

Supplementary Table 5. Differences of iAUC and Cmax of plasma Nam, NR, NMN and MeNam and MeXPY in acute conditions at visit 3 (day 1 of the 2-weeks treatment). Data corresponds to adjusted estimate and the 95% confidence interval. AUC and Cmax are expressed in $\mu\text{mol/L.h}$, and $\mu\text{mol/L}$, respectively.

Plasma	Nam		NR		NMN		MeNam		MeXPY	
	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax
NR vs placebo	0.79 (-14.28, 15.85)	0.35 (-5.22, 5.92)	0.01 (-0.01, 0.04)	0.01 (-0.01, 0.03)	n.a.	n.a.	-0.13 (-2.00, 1.74)	0.07 (-0.66, 0.80)	10.33 (3.37, 17.30)	6.08 (2.87, 9.29)
p values	0.931	0.917	0.435	0.400	n.a.	n.a.	0.907	0.872	0.016	0.003
NMN vs placebo	1.00 (-14.43, 16.44)	0.27 (-5.43, 5.97)	0.04 (0.01, 0.07)	0.03 (0.01, 0.04)	n.a.	n.a.	0.17 (-1.54, 1.88)	0.05 (-0.62, 0.72)	5.64 (-1.54, 12.82)	3.23 (-0.08, 6.54)
p values	0.914	0.938	0.044	0.020	n.a.	n.a.	0.865	0.898	0.194	0.108
Nam vs placebo	127.76 (112.99, 142.54)	55.10 (49.63, 60.56)	0.01 (-0.02, 0.04)	0.01 (0, 0.03)	n.a.	n.a.	16.64 (14.98, 18.3)	5.74 (5.08, 6.39)	77.44 (70.6, 84.27)	30.75 (27.6, 33.91)
p values	< 0.001	< 0.001	0.494	0.230	n.a.	n.a.	< 0.001	< 0.001	< 0.001	< 0.001
NR vs Nam	-126.98 (-142.23, -111.72)	-54.75 (-60.39, -49.11)	0.00 (-0.03, 0.03)	0.00 (-0.02, 0.01)	n.a.	n.a.	-16.77 (-18.54, -15.00)	-5.66 (-6.36, -4.97)	-67.1 (-74.19, -60.02)	-24.67 (-27.94, -21.41)
p values	< 0.001	< 0.001	0.914	0.735	n.a.	n.a.	< 0.001	< 0.001	< 0.001	< 0.001
NMN vs Nam	-126.76 (-142.36, -111.16)	-54.83 (-60.59, -49.06)	0.02 (0, 0.06)	0.01 (0.00, 0.03)	n.a.	n.a.	-16.47 (-18.16, -14.78)	-5.68 (-6.35, -5.02)	-71.8 (-79.01, -64.58)	-27.52 (-30.85, -24.19)
p values	< 0.001	< 0.001	0.170	0.226	n.a.	n.a.	< 0.001	< 0.001	< 0.001	< 0.001
NR vs NMN	-0.22 (-15.94, 15.51)	0.08 (-5.74, 5.89)	-0.02 (-0.05, 0.01)	-0.02 (-0.03, 0.00)	n.a.	n.a.	-0.30 (-2.12, 1.52)	0.02 (-0.70, 0.74)	4.69 (-2.63, 12.02)	2.85 (-0.53, 6.23)
p values	0.932	0.982	0.208	0.129	n.a.	n.a.	0.779	0.964	0.288	0.164

Supplementary Table 6. Differences of iAUC and Cmax of whole blood NAD⁺, Nam, NR, NMN and NADP⁺ in acute conditions at day 14. Data corresponds to adjusted estimate and the 95% confidence interval. iAUC and Cmax are expressed in $\mu\text{mol/L.h}$, and $\mu\text{mol/L}$, respectively.

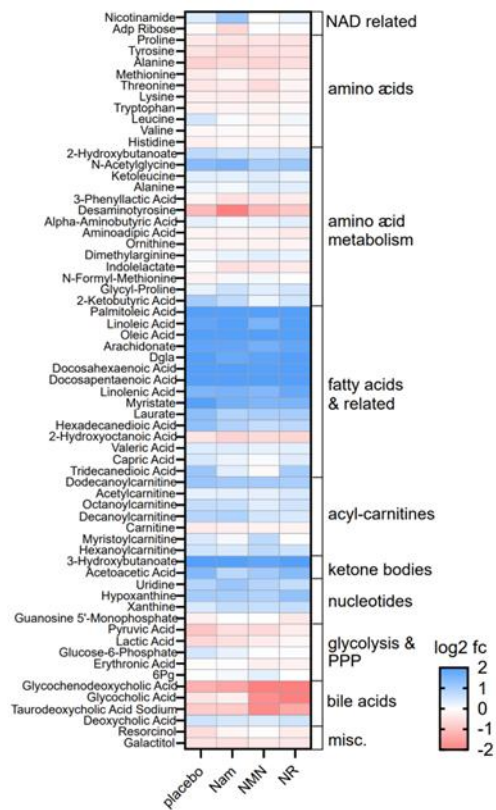
	NAD⁺		Nam		NR		NMN		NADP⁺	
	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax
Nam vs placebo	10.12 (4.78, 15.46)	3.56 (1.57, 5.55)	110.29 (95.96, 124.62)	49.16 (42.76, 55.56)	0.29 (0.21, 0.37)	0.12 (0.09, 0.16)	0.09 (0.04, 0.13)	0.04 (0.01, 0.06)	1.72 (-6.29, 9.72)	0.44 (-3.08, 3.96)
p-value	0.002	0.004	<0.001	<0.1	<0.001	<0.001	0.004	0.006	0.721	0.834
NR vs placebo	7.89 (2.47, 13.31)	4.51 (2.5, 6.53)	1.61 (-12.95, 16.17)	1.06 (-5.44, 7.56)	0.01 (-0.07, 0.1)	0.01 (-0.03, 0.05)	0.01 (-0.04, 0.06)	0.00 (-0.02, 0.03)	-3.41 (-11.49, 4.67)	-1.18 (-4.73, 2.37)
p-value	0.018	<0.001	0.854	0.787	0.766	0.729	0.666	0.764	0.483	0.581
NMN vs placebo	3.17 (-2.45, 8.8)	2.04 (-0.06, 4.13)	1.00 (-14.07, 16.08)	-0.02 (-6.75, 6.71)	0.02 (-0.07, 0.1)	0.01 (-0.03, 0.05)	0.04 (-0.01, 0.09)	0.02 (0.0, 0.04)	-0.74 (-9.14, 7.65)	1.55 (-2.13, 5.24)
p-value	0.349	0.109	0.912	0.996	0.766	0.73	0.205	0.151	0.883	0.484
NR vs Nam	-2.23 (-7.55, 3.1)	0.95 (-1.03, 2.93)	-108.68 (-122.98, -94.38)	-48.10 (-54.49, -41.72)	-0.27 (-0.35, -0.19)	-0.12 (-0.15, -0.08)	-0.07 (-0.12, -0.03)	-0.03 (-0.05, -0.01)	-5.13 (-13.15, 2.9)	-1.62 (-5.16, 1.91)
p-value	0.487	0.425	<0.001	<0.001	<0.001	<0.001	0.011	0.013	0.290	0.445
NMN vs Nam	-6.94 (-12.46, -1.43)	-1.52 (-3.58, 0.53)	-109.29 (-124.06, -94.52)	-49.18 (-55.78, -42.58)	-0.27 (-0.35, -0.19)	-0.12 (-0.15, -0.08)	-0.05 (-0.10, 0.00)	0.03 (-0.04, 0.1)	-2.46 (-10.81, 5.89)	1.11 (-2.57, 4.79)
p-value	0.04	0.219	<0.001	<0.001	<0.001	<0.001	0.108	0.509	0.624	0.616
NR vs NMN	4.72 (-0.88, 10.32)	2.48 (0.39, 4.56)	0.61 (-14.35, 15.56)	1.08 (-5.61, 7.76)	0.00 (-0.09, 0.08)	0.00 (-0.04, 0.04)	-0.03 (-0.07, 0.02)	-0.02 (-0.04, 0.01)	-2.67 (-11.01, 5.67)	-2.73 (-6.39, 0.93)
p-value	0.164	0.052	0.946	0.789	0.94	0.993	0.387	0.245	0.595	0.217

Supplementary Table 7. Differences of iAUC and Cmax of plasma Nam, NR, NMN and MeNam and MeXPY in acute conditions at visit 4 (day 14 of the 2-weeks treatment). Data corresponds to adjusted estimate and the 95% confidence interval. AUC and Cmax are expressed in $\mu\text{mol/L.h}$, and $\mu\text{mol/L}$, respectively.

Plasma	Nam		NR		NMN		MeNam		MeXPY	
	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax	iAUC	Cmax
NR vs placebo	3.27 (-11.46, 18.00)	2.98 (-3.39, 9.35)	0.01 (-0.03, 0.04)	0.00 (-0.02, 0.02)	n.a.	n.a.	0.34 (-3.17, 3.86)	0.97 (-0.38, 2.32)	7.90 (-1.85, 17.66)	3.47 (-0.58, 7.52)
p values	0.711	0.437	0.765	0.829	n.a.	n.a.	0.868	0.230	0.181	0.157
NMN vs placebo	1.28 (-14.04, 16.61)	0.48 (-6.17, 7.13)	0.03 (-0.01, 0.07)	0.02 (-0.01, 0.04)	n.a.	n.a.	1.07 (-2.64, 4.79)	0.96 (-0.47, 2.40)	5.43 (-4.74, 15.59)	3.10 (-1.12, 7.33)
p values	0.889	0.904	0.171	0.249	n.a.	n.a.	0.624	0.262	0.376	0.224
Nam vs placebo	131.04 (116.43, 145.66)	54.51 (48.16, 60.87)	0.01 (-0.03, 0.04)	0.01 (-0.01, 0.03)	n.a.	n.a.	18.62 (15.09, 22.16)	7.37 (6.00, 8.73)	56.99 (47.27, 66.70)	23.87 (19.83, 27.91)
p values	< 0.001	< 0.001	0.706	0.625	n.a.	n.a.	< 0.001	< 0.001	< 0.001	< 0.001
NR vs Nam	-127.77 (-142.89, -112.65)	-51.53 (-58.1, -44.96)	0.00 (-0.04, 0.04)	0.00 (-0.03, 0.02)	n.a.	n.a.	-18.28 (-21.85, -14.71)	-6.73 (-8.100, -5.37)	-49.08 (-59.14, -39.03)	-20.40 (-24.58, -16.21)
p values	< 0.001	< 0.001	0.935	0.789	n.a.	n.a.	< 0.001	< 0.001	< 0.001	< 0.001
NMN vs Nam	-129.76 (-145.48, -114.04)	-54.03 (-60.88, -47.18)	0.02 (-0.02, 0.06)	0.01 (-0.01, 0.03)	n.a.	n.a.	-17.55 (-21.37, -13.73)	-6.38 (-7.84, -4.92)	-51.56 (-61.97, -41.15)	-20.77 (-25.11, -16.43)
p values	< 0.001	< 0.001	0.336	0.517	n.a.	n.a.	< 0.001	< 0.001	< 0.001	< 0.001
NR vs NMN	1.99 (-13.66, 17.64)	2.50 (-4.32, 9.32)	-0.02 (-0.06, 0.01)	-0.01 (-0.04, 0.01)	n.a.	n.a.	-0.73 (-4.46, 3.01)	-0.35 (-1.78, 1.07)	2.48 (-7.96, 12.91)	0.37 (-3.97, 4.71)
p values	0.832	0.542	0.287	0.355	n.a.	n.a.	0.74	0.675	0.693	0.887

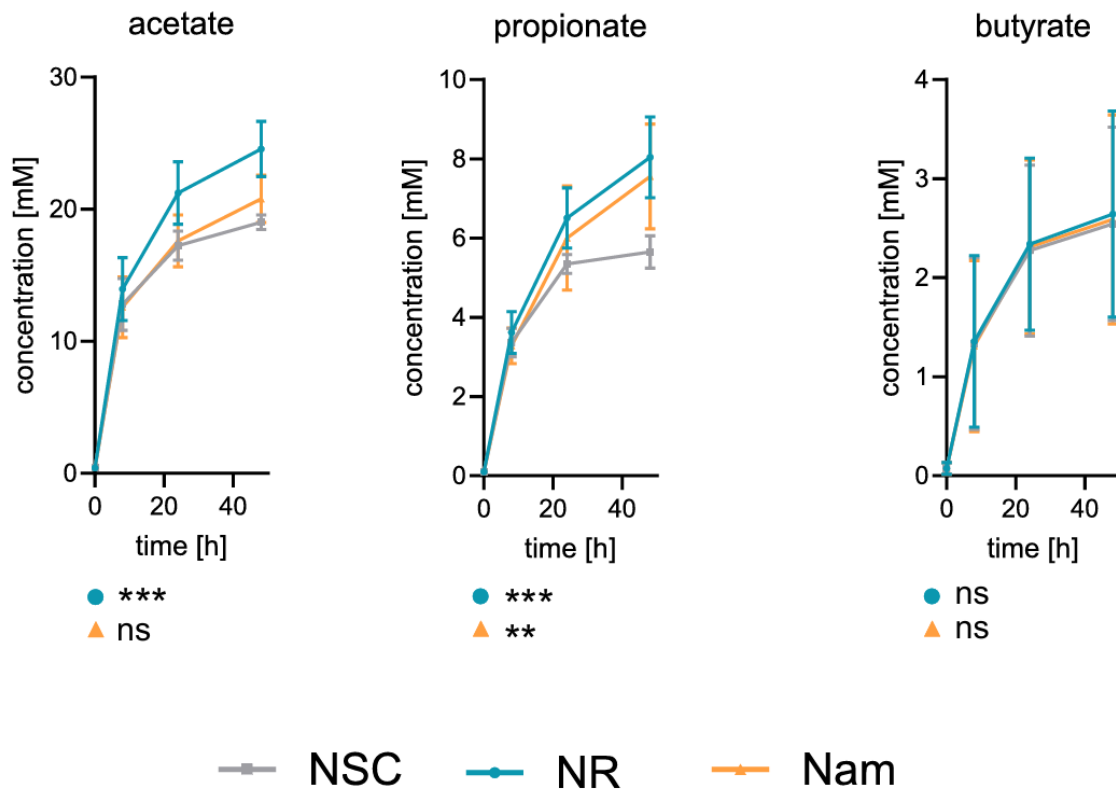
Supplementary Fig. 1.

Acute metabolic changes at visit 3 over 4 hours analyzed by untargeted whole blood metabolomics. The acute log₂ fold-changes of semi-polar and polar metabolites from different classes after receiving a standardized breakfast and either Nam, NMN, NR or placebo supplementation are indicated in the heatmap.



Supplementary Fig. 2.

Impact of NR (blue) and Nam (orange) on propionate, acetate and butyrate by the healthy adult's gut microbiota, compared to a non-substrate control (NSC, grey). Statistical difference related to the treatment effects of NR versus NSC are indicated with * ($0.05 < p < 0.01$), ** ($0.01 < p < 0.001$), *** ($p < 0.001$), ns: not significant.



Research Project Protocol

Research Project Title:	“Effect of Oral NAD ⁺ Precursors Administration on Blood NAD ⁺ Concentration in Healthy Adults”
Research Project N°:	2201NR
Research legislation:	Ordinance on human research with the exception of Clinical trials (HRO) ^[1]
Type of Research Project:	Research project involving human subjects
Risk Categorization:	The current research project protocol and its associated amendments will be of minimal risk (category A, Art 6, letter a, ORH)
Centre:	Nestlé Research - Clinical Innovation Lab – Lausanne Switzerland
Research project period:	April 2022 to June 2023
Project Leader:	Dr Pamela Sun
Sponsor	Société des Produits Nestlé S.A. Avenue Nestlé 55 CH-1800 Vevey Switzerland

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Nestlé Clinical Innovation Lab
Research Project no: 2201NR

PROTOCOL SIGNATURE FORM

Research Project Title: "Effect of Oral NAD⁺ Precursors Administration on Blood NAD⁺ Concentration in Healthy Adults"

The Research Project team has approved the protocol version **[Protocol Final version N° 2 (dated 23.MAY.2022)]** and confirms hereby to conduct the project according to the protocol, the Swiss legal requirements ^[1, 2], current version of the World Medical Association Declaration of Helsinki ^[3] and the principles and procedures for integrity in scientific research involving human beings ^[4].

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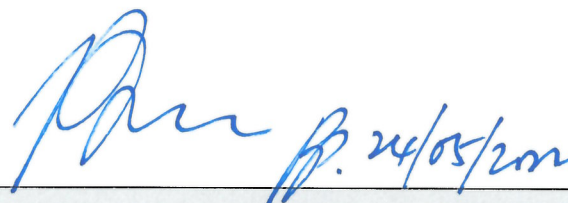
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GLOSSARY OF ABBREVIATIONS

AE	<i>Adverse Event</i>
AUC	<i>Area Under the Curve</i>
BASEC	<i>Business Administration System for Ethical Committees</i>
BMI	<i>Body Mass Index</i>
CER-VD	<i>La Commission cantonale d'éthique de la recherche sur l'être humain</i>
CHF	<i>Confoederatio Helvetica Franc</i>
CIL	<i>Clinical Innovation Lab</i>
Cmax	<i>Maximum Concentration</i>
EC	<i>Ethics Committee</i>
eCRF	<i>Electronic Case Report Form</i>
EDC	<i>Electronic Data Capture</i>
ePRO	<i>Electronic Patient Reported Outcomes</i>
FAS	<i>Full Analysis Set</i>
FOPH	<i>Federal Office of Public Health</i>
g	<i>gram</i>
GCP	<i>Good Clinical Practice</i>
GDPR	<i>General Data Protection Regulation</i>
HILIC	<i>Hydrophilic Interaction Chromatography</i>
HRA	<i>Human Research Act</i>
HRO	<i>Ordinance on Human</i>
ICH	<i>International Conference on Harmonisation</i>
IP	<i>Investigational Product</i>
IRB	<i>Institutional Review Board</i>
ITT	<i>Intention-to-treat</i>
LC-MS/MS	<i>Liquid Chromatography-tandem Mass Spectrometry</i>
LSAF	<i>Life Science Analytics Framework</i>
mg	<i>milligram</i>
μM	<i>micromolar</i>
Me2py	<i>N-methyl-2-pyridone-5-carboxamide</i>
Me4py	<i>N-methyl-4-pyridone-5-carboxamide</i>
MeNAM	<i>N-methyl-nicotinamide</i>
MMRM	<i>Mixed Model for Repeated Measurement</i>
NA	<i>Nicotinic Acid</i>
NAAD	<i>Nicotinic Acid Adenine Dinucleotide</i>
NAADP	<i>Nicotinic Acid Adenine Dinucleotide Phosphate</i>
NAD	<i>Nicotinamide Adenine Dinucleotide</i>
NADH	<i>Reduced form of Nicotinamide adenine dinucleotide</i>
NADP	<i>Nicotinamide Adenine Dinucleotide Phosphate</i>
NAM	<i>Nicotinamide</i>
NAR	<i>Nicotinic Acid Riboside</i>
NCBR	<i>Nestlé Center for Biological Resources</i>
NMN	<i>Nicotinamide Mono Nucleotide</i>
NR	<i>Nicotinamide Riboside</i>
OTC	<i>Over-The-Counter</i>
PBMC	<i>Peripheral Blood Monocyte</i>
PK	<i>Pharmacokinetics</i>
PP	<i>Per Protocol</i>

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<i>SAF</i>	<i>Safety Analysis Set</i>
<i>SD</i>	<i>Standard Deviation</i>
<i>SE</i>	<i>Serious Event</i>
<i>Tmax</i>	<i>Time to maximum concentration</i>
<i>TMF</i>	<i>Trial Master File</i>

1. BACKGROUND AND PROJECT RATIONALE

Nicotinamide adenine dinucleotide (NAD) is a coenzyme playing a central role in human metabolic pathways. Found in all living cells, NAD is involved in redox reactions, carrying electrons from one reaction to another through its two forms: an oxidized (NAD⁺) and reduced (NADH) form. NAD⁺ is also used in other cellular processes, most notably as a substrate of enzymes in post-translational proteins modifications. Given the pivotal role of NAD⁺ in human physiology, studying its metabolism has become essential to monitor genetic and/ or drug- induced perturbations related to metabolic status and diseases such as ageing, cancer, obesity or cardiometabolic diseases (5,6).

NAD metabolism, transport, and function are complex and dynamic (Figure 1). NAD can be metabolized into several molecules, including nicotinamide adenine dinucleotide phosphate (NADP) and nicotinic acid adenine dinucleotide phosphate (NAADP). The metabolites play key roles in energy transduction and cell signaling. Products of NAD liver metabolism, such as nicotinamide (NAM) and n-methyl-nicotinamide (MeNAM), have also emerged as key regulators of energy metabolism, epigenetics, and disease pathophysiology, highlighting the involvement of NAD metabolites in several metabolic pathways apart from the traditionally described oxidation-reduction reactions (7).

Cellular levels of NAD metabolites and the NAD redox ratio (NAD⁺/NADH), appear to decrease during chronological aging (10), and in several other pathological conditions such as adult-onset mitochondrial myopathy (13), schizophrenia (11), and bipolar disorder (12).

Moreover, dysregulation in NAD metabolism has emerged as a contributing factor in the pathogenesis of several disease states therefore therapeutic strategies to increase NAD⁺ levels have been proposed to address NAD related dysregulation (8).

A recognized approach to increase NAD⁺ level is through oral supplementation of its precursors promoting NAD⁺ synthesis *in vivo*. NAD⁺ precursors exist in multiple forms, including nicotinic acid (NA), nicotinamide (NAM), nicotinamide riboside (NR), and nicotinamide mono nucleotide (NMN). However, it is unclear how the various precursors compare in their ability to increase NAD⁺ levels. Moreover, a subset of recent studies indicates that the effects of NAD⁺ supplementation in humans might be less pronounced than what was observed in preclinical studies (5,8,9).

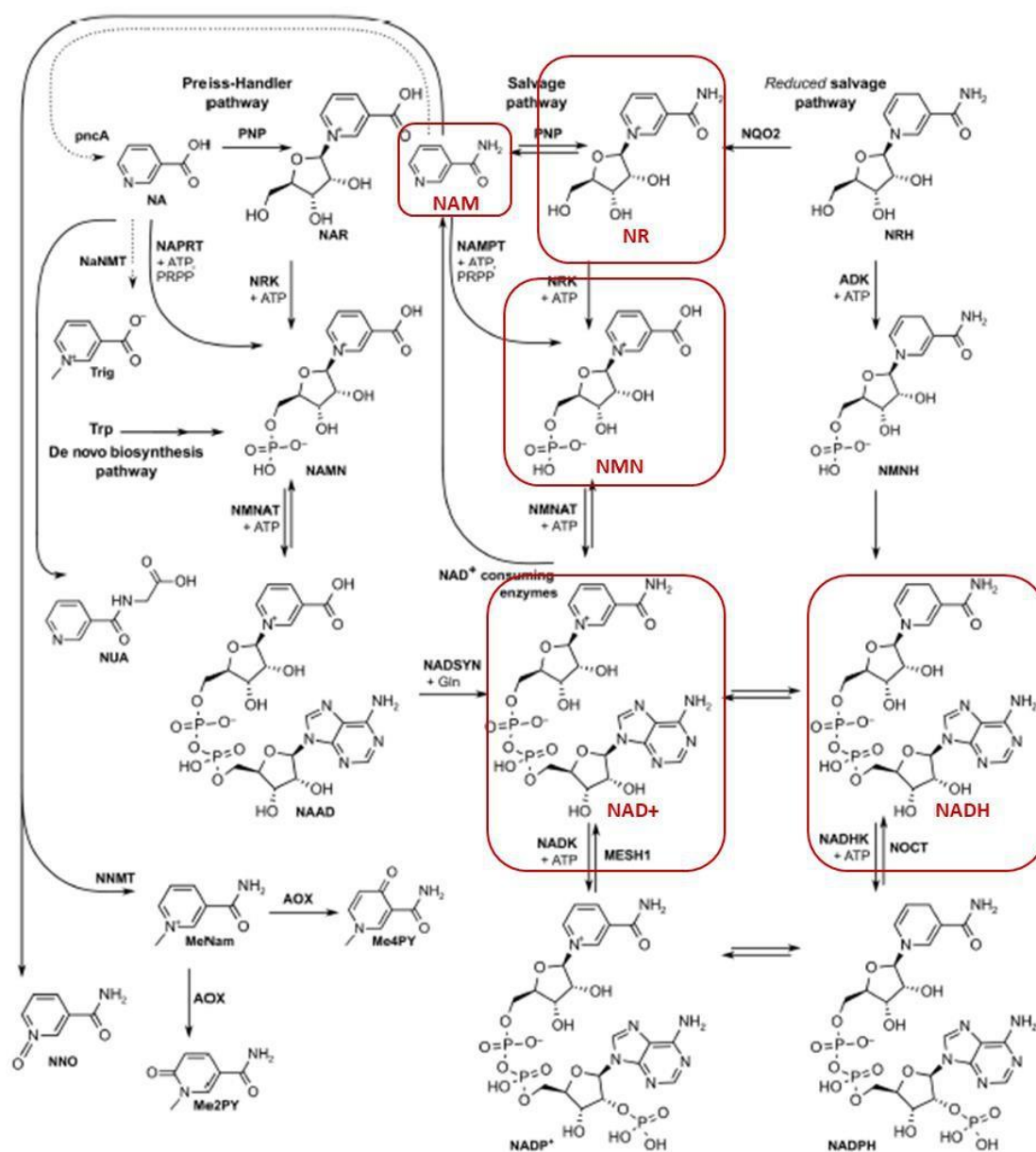


Figure 1. The NAD⁺ metabolome of biosynthetic pathways of NAD(P)(H) (5,9). Metabolites of interest: NA: nicotinic acid; NAR: nicotinic acid riboside; NAM: nicotinamide; NR: nicotinamide riboside; NMN: nicotinamide mononucleotide; NMNH: nicotinamide mononucleotide, reduced form; NAAD: nicotinic acid adenine dinucleotide; NAD⁺: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide, reduced form; NADP⁺: nicotinamide adenine dinucleotide monophosphate; NADPH: nicotinamide adenine dinucleotide monophosphate, reduced form; MeNam: N-methyl-nicotinamide; Me2PY: N-methyl-2-pyridone-5-carboxamide; Me4PY: N-methyl-4-pyridone-3-carboxamide.

1.1 Human pharmacokinetics summary of NAD⁺ precursors

A review of the human pharmacokinetic data on the effect of NAM, NR and NMN is presented in Table 1 and Appendix C. Safety and tolerability of these supplements is reviewed below in section 1.4.

- ✓ NAM: It is anticipated that an acute oral dose of 500 mg of NAM will rapidly increase whole blood levels of NAM, MeNAM and NMN within 1 hour. The anticipated acute or chronic effect on other NAD⁺ metabolites in whole blood such as NAD⁺, NADH or NR has not been elucidated in previous studies.
- ✓ NR: Chronic daily dosing of 1000 mg of NR should increase whole blood NAD⁺ levels by over 50% after 2 weeks intake, but it is not anticipated to show an acute effect on NAD⁺ metabolome.
- ✓ NMN: Chronic dosing of 1000 mg of NMN is anticipated to increase whole blood NAD⁺ level. However, the magnitude of the increase at this dose over 2 weeks chronic dosing is unclear based on current published studies.

The purpose of this study is to compare multiple NAD⁺ precursors towards an optimal approach for increasing whole blood NAD⁺ concentration via oral supplementation.

Table 1. Summary of NAD⁺ metabolites detected in blood after single and repeated dosing of NA, NAM, NR and NMN. Intervention regimen is differentiated to acute (A) or chronic (C); Dose g/d refers to highest dose given in gram per day; Duration refers to exposure time (w, weeks; m, months; sd, single dose); 'n' means number of participants per group. NAD⁺ metabolome species measured are highlighted in color, with green Y indicating a statistically significant increase, red N no statistically significant increase, and when left blank no measurement was performed.

NAD ⁺ precursor		NA			NAM				NR						NMN		
Regimen		C	A	A	C	A	A	C	C	C	C	A	C	C	A	C	C
Dose g/d		1.0	0.2	0.3	1.0	1.0	0.5	1.0	1.0	1.0	1.0	0.5	0.25	0.25			
Duration		4m	sd	sd	1d	sd	sd	9d	2w	3w	6w	sd	10w	3m			
N		8	6	10	13	12	12	8	35	12	30	10	13	10			
Matrix	Whole blood	X	X					X	X	X				X			
	PBMC					X					X		X				
	Erythrocytes						X										
	Plasma			X					X			X	X				
	Serum				X												
Active redox	NAD ⁺	Y	N			N		Y	Y	Y	Y		Y	Y			
	NADH						Y										
	NADP ⁺	Y									N	N					
	NADPH						Y										
Precursors	NAM	Y	Y	Y	Y	N			Y	N	N						N
	NMN	Y	N		Y	N		N		Y	N						Y

NAD ⁺ precursor		NA	NAM			NR						NMN		
	NR	N						N		N	N			Y
	NAAD					N				Y	Y			
	MeNAM			Y	Y	Y			Y	Y		Y		
Metabolites	Me2py					Y				Y		Y	Y	
	Me4py									Y		Y	Y	
Reference		13	16	17	15	19	20	21	22	23	24	26	27	28

1.2 Challenges in NAD⁺ metabolite quantification

A key challenge of NAD⁺ metabolism research relates to significantly different circulating levels of NAD⁺ metabolites reported in the literature. This discrepancy can be attributed to the lack of standardization in sample collection, method of nucleotide extraction, detection assay selection, sample storage, and to the different blood compartments investigated (whole blood, plasma, serum, PBMCs; see Table 2). Therefore, we believe that the standardization of the experimental protocols on the quantitative analysis of NAD⁺ metabolites and precursors, developed in our laboratories for whole blood, plasma, and urine, will allow for the efficient comparison of the effect of oral supplementation with different NAD⁺ precursors on NAD⁺ metabolites levels (30). A liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of the NAD⁺ metabolome in various biological matrices will be used, with the hydrophilic interaction chromatography (HILIC) enabling the separation and detection of 18 metabolites belonging to pathways leading to NAD⁺ biosynthesis, including precursors, intermediates, and catabolites. Due to the known instability of redox cofactors, a standardized sample preparation procedure was developed to handle a variety of biological matrices. Initial human whole blood baseline levels of the highest concentration of the NAD⁺ metabolites measured via LC-MS/MS (30) are summarized in Table 2.

Table 2. Baseline concentration of selected metabolites determined in whole blood in 9 healthy donors (30).

Metabolites	NADP ⁺	NAD ⁺	NADH	NADP	NAM	NMN
Blood						
Concentration						
($\mu\text{M} \pm \text{SD}$)	13 ± 5.9	10 ± 1.0	8 ± 0.6	7 ± 1.0	5 ± 1.4	2 ± 0.2

1.3 Scientific rationale, significance, and new information to be gained

There are no prospective studies directly comparing oral supplementation of the 3 NAD⁺ precursors, NAM, NR and NMN, on their ability to increase NAD⁺ level in human blood. Moreover, while chronic oral intake of NAM has been assessed in humans, its impact on blood NAD⁺ concentration has not yet been thoroughly evaluated. Consequently, a rigorously conducted prospective study directly comparing NR, NAM, and NMN is needed to determine the optimal strategy for increasing blood NAD⁺ level through supplementation with precursors.

The primary objective of this research study is to evaluate daily oral intake for 14 days of 3 NAD⁺ precursors vitamin B3 analogues (NAM, NR, and NMN, all commercially available as dietary supplements), on whole blood NAD⁺ level in a healthy study population. The nominal dose of the 3 precursors to be tested will provide similar 'NAD⁺ equivalent'. As the molecular weight of NAM (122 g/mol) is approximately half of those for NR (255 g/mol) and NMN (334 g/mol), the daily dose used will be NAM (500 mg), NR (1000 mg) and NMN (1000 mg).

Secondary and exploratory objectives would assist in elucidating additional biological effects of NAD⁺ precursors:

- ✓ Measurement of NAD⁺ metabolites in whole blood, plasma and urine will provide a more detailed understanding on the potential differences between the 3 NAD⁺ precursors and their complex metabolism (5,8,9).
- ✓ Potential antioxidant effect of the NAD⁺ precursors will be determined by the measure of the redox ratio NAD⁺/NADH and NADP⁺/NADPH (31) and complementarily by plasma oxidative stress molecular markers (32).
- ✓ The measure of blood methyl donor and acceptor such as methionine and homocysteine could differentiate NAM versus the other precursors. NAM was shown to consume methyl to produce MeNAM in an acute dose study (17), while NR was reported to not affect homocysteine level with chronic administration (22). The effect of NMN with respect to methyl donor and acceptor capacity is not known.
- ✓ NAD⁺ serves as a pivotal regulator of gene expression involved in reactions that coordinate genomic stability, epigenetic status, and RNA processing through NAD⁺ - dependent enzymes (33). Whole blood has shown increased sensitivity to subtle and possibly systemic transcriptomic changes (34), thus we aim to use a whole blood transcriptomic approach to explore the global gene expression changes in blood before and after oral NAD⁺ precursors administration.
- ✓ NAD⁺ is involved in many key pathways related to energy metabolism interacting with various plasma metabolites. Using a non-targeted metabolomic approach, we will

determine to which extent NAD⁺ precursors can affect the plasma and urine metabolic signatures.

1.4 Safety and tolerability of oral NAD⁺ precursors

Chronic oral administration of NAD⁺ precursors has been associated with favorable safety and tolerability. NAM has been administered in long-term safely up to 3000 mg per day (14). Similarly, NR has been safely administered up to 2000 mg over 12 weeks (8, 9). Studies reported NMN supplementation as safe and well tolerated (27,28) with the highest dose tested being 1200 mg over 6 weeks (29). As such, the dosing in this study (NAM 500 mg/day, NR 1000 mg/day, NMN 1000 mg/day) for a total of 14 consecutive days is considered safe. Of note, NAM, NR and NMN have not been associated with any of the known niacin side effects such as hot flushes and tingling sensation of extremities (13). Potential side effects reported following oral administration of NAD⁺ precursors include mild headache and mild gastrointestinal symptoms, such as nausea, vomiting, and/ or diarrhea.

2. PROJECT OBJECTIVES AND DESIGN

2.1 Hypothesis and primary objectives

The main hypothesis is that daily oral intake for 14 days of a NAD⁺ precursors will lead to differential effects on the NAD⁺ metabolome measured in whole blood of healthy volunteers. The 3 precursors tested at a 'NAD⁺ equivalent dose' will be NAM (500 mg daily dose), NR (1000 mg daily dose) and NMN (1000 mg daily dose) administered once daily for 14 consecutive days.

The primary objectives are:

- ✓ To determine the extent of increase in NAD⁺ level in whole blood, for each NAD⁺ precursor compared to placebo
- ✓ To compare the extent of increase in NAD⁺ level in whole blood across the 3 NAD⁺ precursors

2.2 Primary endpoints

- ✓ Changes in whole blood NAD⁺ level at Day 14 (pre-dose) - interventions vs placebo
3 comparisons: NAM vs placebo, NR vs placebo, NMN versus placebo
- ✓ Co-primary endpoint: Changes in whole blood NAD⁺ level at Day 14 (pre-dose) across interventions
3 comparisons: NAM vs NR, NAM vs NMN, NR vs NMN

2.3 Secondary objectives

The secondary objective will be to determine to which extent the NAD⁺ precursors affect the NAD⁺ metabolome in whole blood. Evaluation of the pharmacokinetic changes will be determined by PK profiling following dosing on the first and last day of the dosing period.

2.4 Secondary endpoints

- ✓ Changes in whole blood NAD⁺ metabolites level at Day 14 (pre-dose) - interventions vs placebo, and across interventions
- ✓ Change in the NAD⁺ redox ratio (NAD⁺/NADH) and the NADP ratio (NADP⁺/NADPH) at Day 14 (pre-dose) - interventions vs placebo, and across interventions
- ✓ Change in whole blood NAD⁺ metabolites iAUC and C_{max} over 4 hours at Day 1 and at Day 14 - interventions vs placebo, and across interventions

2.5 Exploratory objectives and endpoints

To enhance the understanding of the metabolic and elimination pathways of the various NAD⁺ precursors, changes in NAD⁺ metabolome in plasma and urine will be evaluated – intervention vs placebo (and across interventions). For standardizing the dilution of urine, creatinine levels will be measured.

Changes in oxidative stress molecular markers will be determined by measuring plasma N-acetylcysteine, cysteine, and glutathione oxidized over 4 hours at Day 1 and at Day 14.

To explore how the global gene expression changes in blood before and after chronic NAD⁺ precursor intake, a transcriptomic differential analysis at baseline Day 14 versus Day 1 will be conducted. Similarly, a metabolomic comparison in plasma and urine will be performed.

Correlations across NAD⁺ metabolites levels and the other measures (baseline and intervention) will be performed to gain insight on the NAD⁺ metabolome, its impact on other metabolic pathways, and NAD⁺ precursors metabolism in human.

2.6 Project design

This is a randomized, open-label, placebo controlled, 4-arm parallel group study. It will be conducted at a single site (Clinical Innovation Lab, Nestle Research, Lausanne).

3. PROJECT POPULATION AND STUDY PROCEDURES

3.1 Project population

This project targets 68 healthy adult men and women. Each arm of 17 participants corresponding to placebo, NAM, NR and NMN groups (Figure 2) will be stratified for age (18 to 35 and 36 to 50) and sex (35).

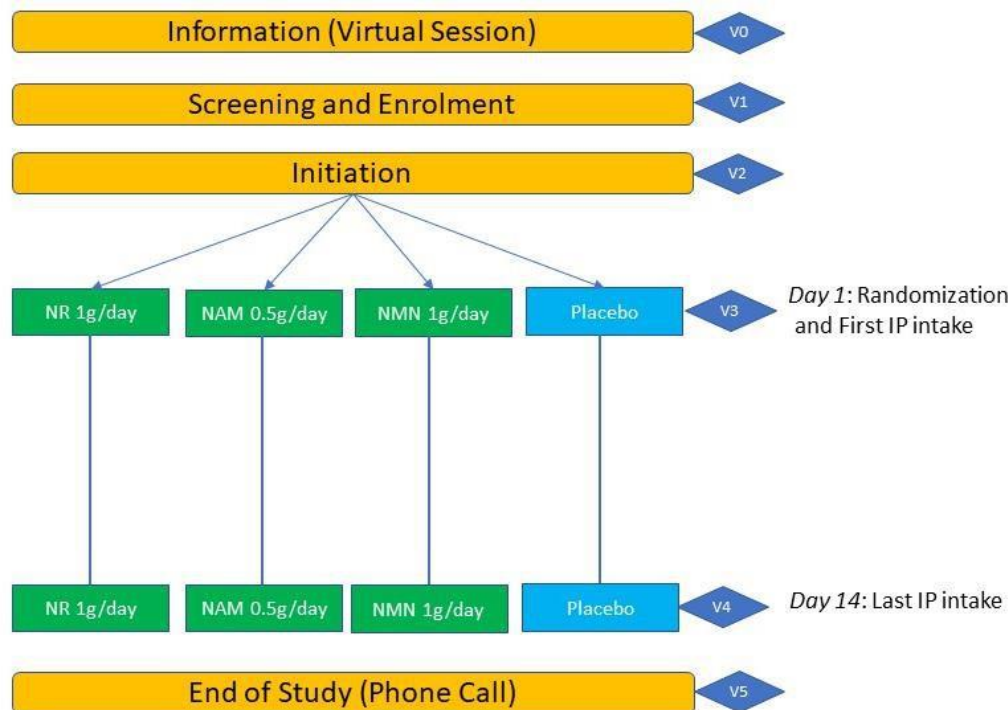


Figure 2. Study design. IP: investigation product; NAM: nicotinamide; NR: nicotinamide riboside; NMN: nicotinamide mononucleotide; g: gram; V: Visit.

3.2 Inclusion criteria

1. Healthy male and female aged 18-50 years, inclusive, at enrolment
2. Body mass index (BMI) between 18.5 to 27.0 kg/m².
3. Able to understand and to sign a written informed consent prior to study enrolment.
4. Willing and able to comply with the requirements for participation in this study.

3.3 Exclusion Criteria

1. Known history of allergy or intolerance to the investigational products.
2. Any chronic medical condition and/or history of significant medical condition (e.g. malignancy, gastrointestinal or metabolic disease, chronic inflammatory condition,

uncontrolled diabetes, cardiovascular disease) and/or psychiatric condition (e.g. depression, psychotic disorders, chronic insomnia), which in the opinion of the site physician/ investigator may risk participant wellbeing/ safety, impede participant compliance with study procedures or ability to complete the study and/ or could confound the primary objectives of the study.

3. Any acute illness or any recent medical intervention including vaccination within 14 days before the first dose of investigational product.
4. Female participants who are pregnant or intending to become pregnant, lactating and/or breastfeeding. Women of childbearing potential who are not currently using medically effective forms of contraception. Medically effective forms of contraception include hormonal methods, such as the contraceptive pill or implant (stable use for at least 3 months), an intrauterine device in use at least 30 days prior to enrolment, or barrier methods such as diaphragm or condom, in use at least 14 days prior to enrolment.
5. Use of prescription drugs known to potentially interact with NAD precursors (e.g., tetracycline antibiotics, anticoagulants, bile-acid sequestrants, such as colestipol, colesevelam, cholestyramine) within 14 days or 5 half-lives (whichever is longer) prior to the first dose of investigational product. Analgesic medicines such as paracetamol and/or non-steroidal anti-inflammatories (e.g., ibuprofen, diclofenac) may be exceptionally used as needed or for short-term treatment (≤ 3 days) up to 24 hours prior to the first dose of investigational product.
6. Use of multivitamin/ multimineral supplements, NAD⁺ precursor supplementation (e.g., niacin, nicotinic acid or niacinamide), L-tryptophan supplementation and/ or any over-the-counter (OTC) medication promoting "healthy aging" or "anti-aging" or "longevity" up to 30 days before first dose of investigational product.
7. On a self-restricted diet, controlled diet or special therapeutic diet (e.g., low carbohydrate diet, ketogenic diet or intermittent fasting) up to 30 days before first dose of investigational product.
8. Average alcohol consumption greater than 2 standard drinks per day over a week for males, and greater than 1 standard drink per day over a week for females. One standard drink contains 10-12 g of ethanol. Examples of standard drinks are one beer can (300 ml), one glass of wine (100 ml) or one glass of schnaps (30 ml).
9. Current smoker (e.g., cigarette, tobacco, cannabis) who exceeds 5 cigarettes per week.
10. Performing shift work or trans-meridian travel greater than two time zones within 14 days prior to the first dose of investigational product.

11. Currently participating in another research study.

12. Family or hierarchical relationships with the Clinical Innovation Lab (CIL) team.

3.4 Recruitment, informed consent form and screening

Recruitment within Nestlé entities

An advert of the study will be sent out by email within Nestlé entities as well as being available via an internal online CIL portal hosted on the Nestlé Intranet. The advertisement describes the study and contains links to informed consent form, information letter, study flyer and to the recording of the information session (Visit 0). The candidates will be free to contact the study coordinator or designee at CIL for any further information, and those who are willing to participate can book an appointment for a screening visit using an internal booking system (only accessible by Nestlé employees).

Recruitment through a research recruitment agency

The potential external candidates may also be contacted through a recruitment agency, for example MIS Trend (Lausanne). A document will be provided by the research team to the agency. This document is a summary of the research project protocol, includes a pre-selection questionnaire, as well as an oral consent form (see "*document de pré-selection agence de recrutement*"). A member of the recruiting agency will use this document during a phone call with potential candidates, identified from an existing listing managed by the agency, to explain the study outline and complete a pre-selection questionnaire.

Potential candidates will receive a copy of the pre-selection questionnaire signed by both parties. The agency will then give the details of the study coordinator or designee (phone number, email) to selected candidates willing to participate in the study. The selected candidates will then be free to contact the study coordinator or designee, who will give them an appointment for the information session or provide them IRB-approved study information documents (information session slides or recording).

Screening visit and informed consent procedure (Visit 1) – 30 min, on site, CIL

The medical doctor or his/ her designee will remind the participant of the study procedures. During this visit, the participant will sign 2 original informed consent forms that will be countersigned by the medical doctor or his/ her designee. The participant will then get one original signed informed consent, while the second original copy will be kept at the site. Female participants will undergo a dipstick pregnancy test to exclude pregnancy. Demographic data (year of birth, sex, height, weight) and medical history (general health status, current medications if any, past medical and surgical history, any allergies/intolerances) will be recorded.

3.5 Study procedures

The description of the study assessments and procedures planned at each visit and during the intervention period is summarized in the Appendix B. The timelines of the study are presented considering an investigational product administration period of 14 days. Figure 3 depicts the activities at each visit.

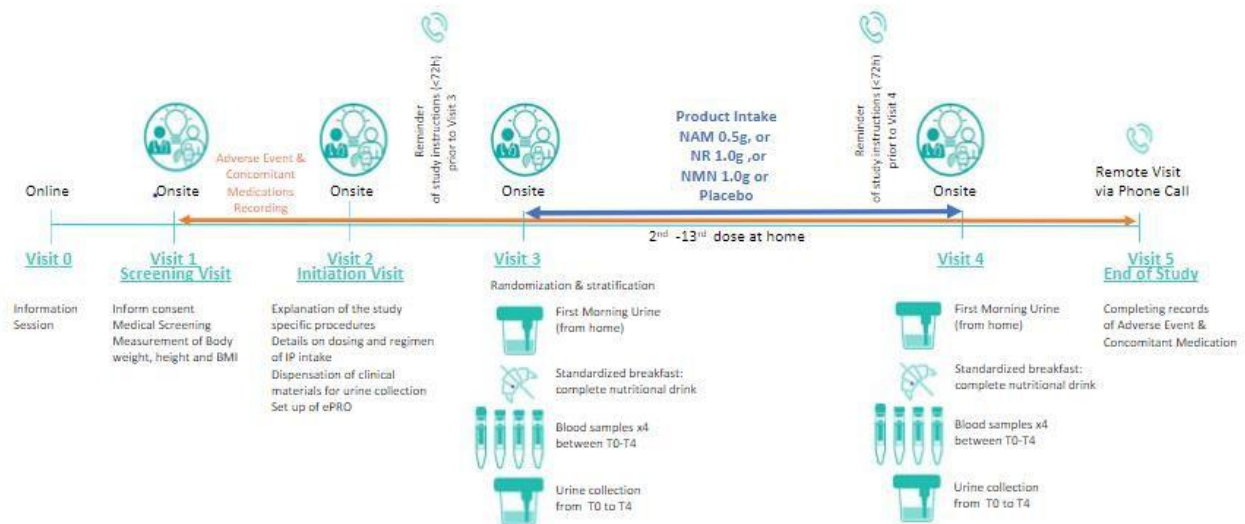


Figure 3. Description of activities at each visit.

3.6 Research project visits and assessments

- ✓ **Visit 0 (V0) - Information Session (approximately 30 minutes, remotely, online videoconference):** See section 3.4 “Recruitment, informed consent form and screening” above.
- ✓ **Visit 1 (V1) - Informed Consent Form signature and screening (approximately 30 minutes, on site, CIL):** See section 3.4 “Recruitment, informed consent form and screening” above.
- ✓ **Visit 2 (V2) - Initiation visit (approximately 30 minutes, on site, CIL)**

During the initiation visit, the participants will be given guidance on:

- Study specific requirements for dietary and activity restrictions
- Details on investigational product intake i.e., dosing and regimen
- Explanation including a leaflet on how to collect a first morning urine sample prior to Visit 3 (V3) and Visit 4 (V4); in addition, the required investigational materials will be dispensed.

After the above explanations, the participants will create their personal electronic Patient Reported Outcome (ePRO) account in the Patient Cloud Application on their smartphone, and will be given instructions to use the Application to answer the following questionnaires:

- Dietary and Activity diary
- Investigational Product Intake diary
- Adverse Events and Concomitant Medication diary
- ✓ **Phone calls 1 (between Day -3 and Day -1) and 2 (between Days 11 and Day 13) (approximately 5 minutes)**

These phone calls up to 72 hours before V3 and V4 serve as reminders to confirm attendance and ensure study instructions are followed by the participants, as below:

- Consumption of their regular diet within 24 hours before planned investigational product intake and fasting for at least 8 hours before planned IP administration at V3 and V4
- No consumption of caffeine containing products (e.g., coffee, tea, energy drinks, dark chocolate) within 24 hours before planned investigational product intake at V3 and V4
- No moderate-intensity* or vigorous-intensity** physical activity within 24 hours before planned investigational product intake at V3 and V4

**Activities such as brisk walking, dancing, or raking the yard, slow swimming, that will raise heart rate and breathing rate. **Activities such as jogging, running, carrying heavy objects or other loads upstairs, shovelling snow, participating in a fitness class, and fast swimming, that will lead to hard and fast breathing (37,38).*

- ✓ **Visit 3 (V3) – First investigational product (IP) intake (Day 1, approximately 5 hours, on site, CIL)**
1. At home, the participant will collect the first morning urine sample as per study instructions and bring it to the investigational site.
 2. Upon arrival at the investigational site, the urine sample brought by the participant will be directly frozen.
 3. Next, the study staff will confirm the prerequisites (see above Phone calls 1 and 2).
 4. The participant will then consume a standardized breakfast consisting of a complete nutrition drink (Boost, 360 Calories: Appendix A). Of note, this can be taken with water ad-libitum. The standardized breakfast must be taken at least 2 hours prior to first blood sampling at baseline time (T baseline). During the study day, drinking water ad-libitum is allowed but no other liquid nor food should be consumed.
 5. Next, stratification and randomization will take place.

6. A venous catheter will be inserted in the forearm and a pre-dose blood sample will be withdrawn at T baseline. Immediately after blood sampling, the investigational product will be orally consumed with 200 ml of water (T0h).
7. Blood samples post-IP intake will subsequently be withdrawn at 1 hour (h) (T1h), 2 h (T2h) and 4 h (T4h) (Figure 4).
8. Between T0h - T4h, all urine post IP intake will be collected for metabolic analysis
9. After the last blood sampling at T4h, the venous catheter will be carefully removed, and the participants will be offered a snack.

	6-8 am	Baseline	T0	T 1h	T 2h	T 4h
Whole blood		X		X	X	X
Plasma		X		X	X	X
Urine	X			X		
Breakfast		X				
NAD precursor			X			

Figure 4. Description of procedures at Visit 3 and Visit 4.

- ✓ **Intervention period (self-administration of IP on Day 2 to Day 13)**
 - At home, from Day 2 to Day 13, the participant should self-administer the IP at the end or within 30 min after their usual breakfast every 24 hours. If they do not consume breakfast, IP should still be administered in the morning at a specific recurrent time every day. In case they forget, they should still take the IP during the day until 8 pm. No overnight fasting is required before self-administered IP supplementation.
 - Participants should follow their regular diet and physical activity throughout the intervention period.

✓ **Visit 4 (V4) - Last IP intake (Day 14, approximately 5 hours, on site, CIL)**

V4 will follow the same procedures as depicted above in V3 and Figure 4; except for step 5, stratification and randomization, that has already taken place at V3 and will not occur again at V4.

✓ **Visit 5 (V5) – End of Study (Day 16, Phone call, around 10 minutes)**

End of Study Visit will involve a phone call to verify the reporting of, if any, adverse events (AEs) and concomitant medications during the study period (reported in ePRO by the participant).

3.7 Withdrawal and discontinuation

Participants' involvement in this research project is voluntary. They can end their participation at any time. They do not have to justify the withdrawal of their consent or any termination of their participation. De-identified, coded data and samples may be used for future research. Each participant will be informed about this possibility in a separate information letter. Participants' involvement in this further use is also voluntary, independent from the study, and can end at any time.

If the participants disagree to consent for re-use (i.e., coded data or samples), they can still participate to the study and their data will be used in the frame of this research project only. In case of study withdrawal, data and samples collected up to that point will be used and analyzed in a coded way so that identity is not displayed (link between code and identity kept confidential at site).

If the participants agree and consent to re-use data or samples, any request for further use will be considered as a new research project and will be submitted to the Ethics Committee for review and approval. In case of study withdrawal, data and samples collected up to that point will be used and analyzed as de-identified, coded data only (link between code and identity kept confidential at site).

In case of further use withdrawal, after the end of the study (last participant last visit), the data will be inactivated immediately, and the samples will be destroyed or anonymized. It is impossible to make data fully anonymous (i.e., no link between identity and assigned code) and so the data will stay coded in Nestlé system but will not be used furthermore.

3.8 Screen failures

Screen failures are defined as participants who consent to participate in the study but are not subsequently enrolled i.e., do not meet the enrollment criteria. Screen failure participants will be coded under CHE001-SCR-XXXX format into the eCRF. No other data than informed consent date and inclusion/exclusion criteria will be collected for these participants.

3.9 Participant's discontinuation criteria

3.9.1 Participant's discontinuation from trial

A participant may be discontinued from the trial for the following reasons:

1. A participant can withdraw consent to participate in the trial at any time without reprisal (See Declaration of Helsinki ref. 3).
2. Investigator's decision to discontinue a participant from the trial if, in the opinion of the Investigator, continuation in the trial would be detrimental to the participant's wellbeing. Should

the Investigator's decision be based on an AE or a serious adverse event (SE), this event must be reported in the appropriate manner.

If the participant withdraws consent from the study, no further evaluations should be performed, and no additional data should be collected. The sponsor may retain and continue to use any data collected before such withdrawal of consent unless the participant specifically withdraws his/her consent to the treatment of personal data.

The reason for premature discontinuation should always be documented. The investigator should request the participant to return all unused study products and follow-up with the participant regarding any unresolved adverse events.

3.9.2 Participant's discontinuation from trial product

A participant may be definitively discontinued from the trial product for the following reasons:

1. Participant's decision to withdraw from the trial product.
2. Investigator's decision to discontinue participant from trial product if, to the opinion of the Investigator, continuation of exposure to trial product would be detrimental to the participant's wellbeing. Should the Investigator's decision be based on an AE or a SE, this event must be reported in the appropriate manner.

Any participant discontinued from trial product remains in the trial. The participant is not exposed anymore to the trial product but should continue to attend all trial procedures as per protocol.

3.10 Participant's replacement

In case of larger non-inclusion rate than planned (10%), recruitment will be adjusted accordingly to ensure inclusion of 60 subjects into the Full Analysis Set (FAS). Recruitment will be capped to 88 subjects which corresponds to a non-inclusion rate of approximately 30%.

3.11 Duties of research participants

For the duration of the study participants will be asked:

- To comply with the study procedures, especially investigational product intake, completion of questionnaires and to follow the dietary and activity restrictions,
- To inform the research team of any medication or supplementation taken,
- To attend the study visits either on site or remotely,
- To give the research team accurate information about any undesirable side-effects they may experience.

3.12 Premature exclusion from the study

The medical responsible of the study or his/her designee may decide to exclude participants from the study during any time point, including before the end of the study, in the sole interest of their health as well as of the health of other participants and study team (e.g., in case of suspicion of confirmed or potential infection by COVID-19 virus). If the decision to prematurely exclude is based on an AE or a SE, this event must be reported to (details in section 6.2 and 6.3).

3.13 Dosing regimen, route of administration, compliance

The duration and timing of intake for all investigational products and placebo is for 14 consecutive days, orally once daily, at a specific recurrent time every day, recommendable in the morning (regardless of breakfast consumption).

The 3 investigational products are:

- Nicotinamide (NAM) from Pure Encapsulations, LLC (1 capsule of 500 mg a day)
- Nicotinamide Riboside (NR) from Tru Niagen Pro (2 capsules of 500 mg a day)
- Nicotinamide Mono Nucleotide (NMN) from UltraHealth (2 capsules of 500 mg a day)

The placebo is:

- Microcrystalline cellulose from Pure Encapsulations, LLC (1 capsule of 500 mg a day)

The investigational and placebo products are already commercially available and have been validated as safe and tolerable for human oral administration. Refer to Appendix D: DESCRIPTION OF THE NAD⁺ PRECURSORS AND PLACEBO CAPSULES (ALL CAPSULES ARE SIZE (00))

Investigational product and placebo intake will be overseen by study site staff on the first day (Day 1, V3) and last day of dosing (Day 14, V4), which will take place at the CIL. Following intake on these days, study site staff will record compliance with investigational product in ePRO.

Participants will self-administer investigational product at home from Day 2 to Day 13. During this period, they will record their daily compliance in ePRO.

Participants will be asked to return any remaining investigational product for safe disposal when they return for dosing at the site on the last day of dosing (Day 14, V4).

Participants will be considered compliant if they have been compliant for ≥ 12 days and with confirmed intake on Day 1 and Day 14.

4. DATA MANAGEMENT

4.1 Data Capture

Data will be entered from the source document into an electronic Case Report Forms (eCRF - web database) entitled RAVE, solution of iMedidata. Data should be entered in eCRF within 7 days after the subject's visit. At the end of the study, the Investigator must sign each eCRF electronically.

4.2 Access rights

Designated site personnel will be provided with a personal username and password to access the eCRF database. This username/ password pair should be used by a single individual only; passwords must not be shared with any other person.

4.3 Audit trail

The clinical data management systems developed for this study comply with the Good Clinical Practices (GCP) predicate rule requirements, laws, and regulations (Personal data protection) and allow an audit of actions performed by users. Thus, all data modifications will be documented in an audit trail file. After the last verification, the relevant data will be locked into the database. The system is also compliant with the e-signature laws (particularly the Title 21 CFR Part 11 of the Code of Federal Regulations); in that saving the data is equivalent to a handwritten signature.

During the study, the participants will capture the responses into online questionnaires within 'Electronic Patient reported Outcome' (ePRO) solution, developed by iMedidata.

iMedidata Patient Cloud is a secure, password-protected application, allowing participants to complete questionnaires via a tablet or smartphone. The coded data from these questionnaires are transmitted directly to RAVE via iMedidata Cloud. At the end of the study, the coded data will be electronically transferred into the Nestlé secure data repository. The system used is iMedidata and is compliant with Swiss regulation. Medidata's privacy notice and terms of use can be found in Annex I and II.

4.4 Data sources

For this study, the following data sources are identified:

- Investigator's site data

Site personnel will directly enter these data into the eCRF via manual data entry. The exhaustive list of clinical data collected at each visit will be described in the Data Listing document.

The Data Listing document is written by the Clinical Data Manager and approved by the Clinical Team.

· Participant's reported outcome

During the study duration, the participants will capture the responses into online ePRO questionnaires on product compliance, adverse events, concomitant medications, diet and physical activity 24h prior to V3 and V4.

The «Adverse Events and Concomitant Medications Diary» ePRO notebook will be considered as a source document and entered by the participant. Records will be verified in the eCRF by the study site staff.

The «Investigational Product Intake» diary will be considered as a source document and entered by the participant. The date and time of the product intake will be collected.

The «Dietary and physical activity» questionnaire will be considered as a source document and entered by the participant overseen by the study site staff on the day of visits in the ePRO.

· External data

In addition to the data recorded in the eCRF, external data such as the clinical laboratory results might be electronically collected. Prior to this, the laboratory results will be entered into Labkey database to be transferred into Life Science Analytics Framework (LSAF) for analysis and storage.

4.5 Data validation

The data manager will validate all data entered into the eCRF. All computerized edit checks programmed to validate the data, at least on primary outcome data are described in the Data Validation Plan document, written by the Clinical Data Manager.

Data discrepancies will trigger automatic queries, directly displayed on the screen when data entered are saved. If necessary, manual queries will be sent to the Investigator for clarification. All these discrepancies / queries have to be answered or confirmed by the site.

4.6 Coding

Type of data	Dataset	Dictionary
Adverse Events	AE	MedDRA version 24.1 or later
Serious Adverse Events	SE	MedDRA version 24.1 or later

4.7 Database Lock

The database will be locked after review, query resolution, signatures of the eCRF and determination that clinical database is ready for analysis.

Once the study is finished and database is locked, the data manager should follow the database unlock/ relock process to document any further change.

5. STATISTICS AND METHODOLOGY

5.1 Background

The sample size of this trial is based on 3 published studies (Table 3) on the effect of chronic dosing of NR on blood NAD⁺ level. The estimates of the most conservative scenario (21) have been retained for the calculations.

Table 3. Effect of chronic NR intervention on blood NAD⁺ level.

	NAD ⁺ level baseline (µM)	+/- SD	NAD ⁺ level (µM) after 1000 mg NR intervention (duration)	+/- SD	Percent change
Ref 21	27	5	50 (9 days)	20	+ 85%
Ref 22	31	8	73 (2 w)	34	+ 133%
Ref 23	20	5	54 (3w)	18	+ 170%

5.2 Effects to be estimated

The trial primary outcome is whole blood NAD⁺ level in µM at Day 14 (pre-dose). The units under investigation will be individual participants.

Six tests of hypotheses will be conducted:

1. Superiority of NAM, NR, NMN versus Placebo measured as a greater whole blood NAD⁺ level at Day 14 (pre-dose) corrected for baseline (Day 1 pre-dose), sex and age (Full analysis set).
2. Superiority of NAM vs NR vs NMN measured as a greater whole blood NAD⁺ level at Day 14 (pre-dose) corrected for baseline (Day 1 pre-dose), sex and age (Full analysis set).

Superiority of NAM, NR and/or NMN vs Placebo will be declared if the lower bound of the corresponding confidence interval of the whole blood NAD⁺ level difference at Day 14 (pre-dose) is greater than 0.

For the IPs comparison, superiority of IP#1 over IP#2 will be declared if the corresponding confidence interval of the whole blood NAD⁺ difference at Day 14 (pre-dose) excludes the value zero and the point estimate goes in the direction of a greater level in IP#1 group than IP#2 group.

Confidence intervals will be computed at an alpha level equal to $1-(0.05/3)$.

Estimations calculated on the full analysis set should remain consistent with the estimations made on the per protocol set for robust conclusions.

5.3 Sample size calculation

In order to control for the family wise error rate at 5% maximum, the primary and co-primary endpoints will be evaluated in a hierarchical order. The next hypothesis test will be conducted if and only if the preceding one led to rejection of the null hypothesis when evaluated on the Full Analysis Set (FAS):

1. Superiority of whole blood NAD⁺ level at Day 14 (pre-dose) in NAM vs Placebo or NR vs Placebo or NMN vs Placebo.
2. Superiority of whole blood NAD⁺ level at Day 14 (pre-dose) in NAM vs NR or NAM vs NMN or NR vs NMN.

The first test is a combination of three hypotheses. In order to control for the inflation of the family wise error rate at this level, a Bonferroni correction will be applied. Assuming a standard deviation of NAD⁺ at Day 1 (pre-dose) of 5 µM and at Day 14 (pre-dose) of 20 µM (based on the variability observed in reference 21; Table 3), a difference of +23 µM of NAD⁺ level in whole blood between active arms and placebo, 80% power and $5/3=1.66\%$ alpha one would need to measure the NAD⁺ level at Day 14 (pre-dose) of 12 subjects in each arms (NAM, NR, NMN and Placebo).

The second test is a combination of three hypotheses. In order to control for the inflation of the family wise error rate at this level, a Bonferroni correction will be applied. Assuming a standard deviation of NAD⁺ at Day 1 (pre-dose) of 5 µM and at Day 14 (pre-dose) of 20 µM (based on the variability observed in reference 17; Table 3), a minimum difference of +19 µM of NAD⁺ level at Day 14 (pre-dose) in whole blood, one would need to measure the NAD⁺ level at Day 14 (pre-dose) of 15 subjects in each active arms (NAM, NR, NMN).

As a summary, one would need to target 15 subjects in each active arms and 12 subjects in the placebo arm within the full-analysis set. In order to maintain an equal allocation ratio for the randomization, the target is set to 15 subjects per arms.

Assuming a drop-out rate of 10% in each arm, a total of 68 (17 per arms) subjects will need to be recruited to ensure that at least 60 complete the trial and are included into the full analysis set.

In case of larger non-inclusion rate than planned (10%), recruitment will be adjusted accordingly to ensure inclusion of 60 subjects into the Full Analysis Set (FAS). Recruitment will be capped to 88 subjects which corresponds to a non-inclusion rate of approximately 30%.

Calculations were performed using SAS 9.4 software, using the PROC POWER function.

5.4 Randomization

Subjects will be randomized before the first intake in a 1:1:1:1 ratio to one of the four arms (NAM, NR, NMN and Placebo). The randomization will be stratified based on sex (Female/Male) and Age (18 to 35 and 36 to 50).

The randomization will be performed using Medidata Randomization and Trial Supply Management (RTSM) tool, using the dynamic allocation method and setting the second best probability to 15%.

5.5 Datasets to be analysed

Intention-to-treat (ITT) analysis population

All randomized subjects will be included in the intention to treat analysis population. ITT driven analyses will be conducted using the assigned intervention.

Safety analysis set (SAF) analysis population

Safety data (e.g., adverse events) will be analyzed in the SAF which consists of all subjects in the ITT population with documentation of at least one intake of one investigational product (including placebo). SAF driven analyses will be conducted according to the product consumed irrespective of the randomization assignment.

Full analysis set (FAS) analysis population

Efficacy data will be analyzed in the FAS dataset which ideally represents the analysis population that is as close as possible to the ITT principle. FAS analysis population consists of all subjects in the SAF population with available primary endpoint baseline (Day 1 pre-dose whole blood NAD⁺ level), sex, age, and post baseline primary endpoint (Day 14 pre-dose whole blood NAD⁺ level). FAS driven analyses will be conducted using the assigned intervention.

Per Protocol (PP) analysis population

The Per Protocol (PP) dataset consists of subjects from the FAS without non-adherence to the assigned intervention which are believed to impact the primary analyses such as product intake non-compliance and unauthorized concomitant medication intake. The list of subjects excluded from the PP will be documented prior to trial analysis. PP driven analyses will be conducted using the assigned intervention.

5.6 Statistical analyses

Primary analyses

The trial primary outcomes are differences in whole blood NAD⁺ level at day 14 (pre-dose) between interventions and placebo, as well as across interventions.

The differences NAM-Placebo, NR-Placebo, NMN-Placebo, NAM-NR, NAM-NMN and NR-NMN will be estimated using one single ANCOVA linear model with baseline whole blood NAD⁺ level (Day 1 pre-dose), sex, age and assigned intervention as covariates. Primary analyses will be conducted on the Full Analysis Set (FAS).

The 6 tests of null-hypotheses (no difference between the tested elements) will be conducted using the two-sided 98,33% (i.e.100-5/3) confidence intervals of the mean estimated differences.

In order to control for the false positive inflation coming from multiple testing, the comparisons between interventions (NAM-NR, NAM-NMN and NR-NMN) will be interpreted if and only if at least one of the comparisons between interventions and placebo are statistically significant.

A sensitivity analysis of the primary endpoint will be conducted repeating the main analysis on the Per Protocol population.

Secondary analyses

Secondary analyses will be conducted on the ITT analysis set.

Changes in whole blood NAD⁺ metabolites level [NAD⁺, NAD⁺H, NAM, NR, NMN, NAD⁺P, NAD⁺PH, MeNaM and MeXPY (combination of Me2PY and Me4PY)] at day 14 (pre-dose) - interventions vs placebo, and across interventions will be evaluated using one single ANCOVA model per metabolite with the corresponding metabolite baseline whole blood level (Day 1 pre-dose), sex, age, and assigned intervention as covariates.

The same method will be used in order to compare NAD redox ratio (NAD⁺/NADH) and the NADP ratio (NADP⁺/NADPH) at Day 14 (pre-dose) - interventions vs placebo, and across interventions.

iAUC and C_{max} of each NAD⁺ metabolites will be evaluated at Day 1 and Day 14 for each subject. Both parameters will be analyzed using a separated mixed model for repeated measurement (MMRM) including the visit (Day 1, Day 14), the baseline whole blood level of the metabolite (Day 1 pre-dose), sex, age, and assigned intervention as fixed effects as well as visits as random effect in order to model the correlation between repeated measures within subjects.

An unstructured correlation matrix will be used, and the degree of freedom will be estimated using the method of Kenward- Roger.

Acute effect of IPs will be estimated from these models comparing the iAUC and C_{max} at Day 1. Chronic effect of IPs will be estimated from these models comparing the iAUC and C_{max} at Day 14.

Exploratory analyses

The secondary statistical analyses will be repeated on plasma NAD⁺ metabolites and on oxidative stress molecular markers (for example plasma N-acetylcysteine, cysteine, and glutathione oxidized) including the comparison of Day 14 pre-dose level, the comparison of ratios as well as the chronic and acute effect measured on iAUC and C_{max}. Two urine samples will be collected: i) a first morning urine sample (pre-dose) and ii) a second urine sample between T1 h to T4 h post IP intake at Visit 3 (Day 1) and Visit 4 (Day 14). The change in standardized urine NAD⁺ metabolites over creatinine level (ratio) after chronic treatment will be evaluated with the same model as the first secondary endpoint.

Transcriptomics and a more global untargeted metabolomic analysis of whole blood and plasma will be performed, respectively, if the primary analysis shows that whole blood NAD⁺ level is impacted by the NAD⁺ precursor supplementation. The change of transcripts and metabolites from pre-dose at both Day 1 and Day 14 will then be explored, accordingly.

Association between:

- NAD⁺ metabolites levels in whole blood, plasma, and urine (pre-dose) measured at Day 1 and Day 14
- iAUC and C_{max} of NAD⁺ metabolites over 4 hours of kinetic in whole blood and plasma measured at Day 1 and Day 14
- oxidative stress molecular markers measured at Day 1 and Day 14
- NAD⁺ metabolome, transcriptomic and metabolomic data

will be evaluated using Partial Pearson coefficient adjusted for sex and age (regardless to group assignment). Due to the high number of statistical tests conducted (p value of the test of correlation coefficient against the value 0), a Benjamini Hochberg false discovery rate correction (at level 5%) will be applied on the set of p values generated and presented in the correlation matrix.

Missing values and outliers

Due to the limited sample size per arms and the absence of intermediate measurements of the primary outcome, there are no credible missing data imputation methods to apply in case of large extent of missing information.

Indeed, the use of a mixed model for longitudinal data is only possible when repeated measurements are conducted. Multiple imputation methods would require a large number of measured values in order to proceed to credible imputation on the missing ones.

If outliers are identified on data pertaining to the primary endpoints, the planned ANCOVA will be replaced by its robust version using Sandwich estimator of the variance.

Every decision on missing data and outliers handling will be documented in the data review meeting minutes and finalized along with the trial statistical analysis plan.

6. REGULATORY ASPECTS AND SAFETY

6.1 Local regulations / Declaration of Helsinki / Ethical considerations

This research project will be conducted in accordance with the protocol, the Declaration of Helsinki (3), the principles of Good Clinical Practice, the Human Research Act (HRA) and the Human Research Ordinance (HRO) (1) as well as other locally relevant regulations. The Project Leader acknowledges his/her responsibilities as both the Project Leader and the Sponsor.

6.2 Notification of safety and protective measures (HRO Art. 20)

The project leader is promptly notified (within 24 hours) if immediate safety and protective measures must be taken during the conduct of the research project. The Ethics Committee will be notified via BASEC of these measures and of the circumstances within 7 days (day1 starts when project leader/ sponsor is made aware of the safety concern).

6.3 Serious events (HRO Art. 21)

A serious event cannot be excluded from being attributable to the research project (investigational product intake, collection of biological material or collection of personal health data). In case of a serious event related to study product or study procedures, the research project should be interrupted, and the Ethics Committee should be notified on the circumstances via BASEC within 7 days according to HRO Art. 21.

6.4 Definition and assessment of Serious Events (SEs) (HRO Art. 21)

A SE is defined as any adverse event where it cannot be excluded that the event is attributable to the investigational product, sampling of biological material or the collection of personal health data, and which:

- a. requires inpatient treatment not envisaged in the protocol or extends a current hospital stay;
- b. results in permanent or significant incapacity or disability; or
- c. is life-threatening or results in death.

6.5 Reporting of SEs

Upon awareness of an SE, the site staff must complete the SE form in the e-CRF within 24 hours. Notification does not depend on whether there is a causal relationship with the study product or not, all SEs must be entered within 24 hours after awareness.

Upon data entry, a first email will be automatically sent to the Clinical Safety Manager at the following email address (sae.declaration@rdls.nestle.com). Following emails will be automatically sent when changes or additional information are entered in the e-CRF.

In case the e-CRF is not functional, the Clinical Safety Manager must be notified of any SE within 24 hours after awareness by the site per fax +4121 785 9491 or per email sae.declaration@rdls.nestle.com, by using the paper form. All SEs occurring until the last trial visit will be similarly reported.

The project leader shall notify the Ethics Committee of a serious event within 7 days. In addition, the project leader shall report to the committee on the connection between the event and the investigational product intake, collection of health-related personal data or the sampling of biological material. At the same time, he or she shall submit proposals concerning the next steps to be taken.

The Ethics Committee shall reach a decision on the continuation of the research project within 30 days after receipt of the report.

All SEs must be followed up until the outcome is resolved or stable. In case of SE(s) persisting beyond study termination, a follow-up visit may be required. If further analyses are required for the evaluation of a potential cause-effect relationship between the study procedure(s) and the SE, all examinations and laboratory analyses will be documented in the source document of the Investigator file at the research site.

Serious events will be monitored and kept in the Investigator file as source documents, in conformity with the HRO regulation, art. 21.

6.6 Amendments

Substantial changes to the project set-up, the protocol and relevant project documents will be submitted to the Ethics Committee for approval according to HRO Art. 18 before implementation. Exceptions are measures that have to be taken immediately in order to protect the participants. Substantial amendments are changes that affect the safety, health, rights and obligations of project participants, changes in the protocol that affect project objective(s) or central research topic (category B only), changes of project site(s) or of project leader and sponsor. Note: List of substantial changes is available on www.swissethics.ch.

6.7 End of project

Upon project completion or discontinuation, the Ethics Committee is notified within 90 days.

The test products will be destroyed after the last subject last visit in agreement with the project manager. Certificates of destruction will be issued and filed in the trial master file (TMF).

6.8 Financial compensation

A financial compensation of 940 CHF will be given to participants. In case of withdrawal from the research project, the compensation will be calculated on a *pro-rata* basis.

Transportation fees will be refunded 0.70 CHF per km from home or work office to investigational site for non-Nestlé employees and Nestlé employees not working at Vers-Chez-Les Blanc. The refunded maximal distance will be 100 km outward and return per visit. If study participants travel by public transport, they will be reimbursed the price of their travel ticket.

The compensation and transportation fees will be transferred on the banking account of the participant.

6.9 Funding

The research project will be funded by Société des Produits Nestlé SA.

6.10 Insurance

Société des Produits Nestlé SA has taken out an insurance with Zürich Compagnie d'Assurance SA that will cover any damage that may occur during the research project.

7. FURTHER ASPECTS

7.1 Risk-Benefit Assessment

No direct benefit to health is expected from taking part in this study. This research protocol is of minimal risk. The tested products have been administered previously in humans at higher or equal doses evaluated in the present study (NAM 500 mg/day, NR 1000 mg/day, NMN 1000 mg/day) and proven to be safe and tolerated for human administration (see section 1.4). The blood samples will be taken by well-trained nurses. Venipuncture can cause minimal pain and rarely a local infection, but this risk is minimized by the careful disinfection of the skin and the use of sterile, disposable equipment. Participants may experience temporary bruising around the puncture site that should disappear within a few days. The daily amount of withdrawn blood is no more than 40 mL in total and other biological samplings i.e., urine collection is non-invasive; neither will impact participants' health.

8. QUALITY CONTROL AND DATA PROTECTION

8.1 Quality measures

For quality assurance, the Ethics Committee may visit the research sites. Direct access to the source data and all project related files and documents must be granted on such occasions.

An internal quality control of the essential research project documents submitted to the Ethics Committee is ensured.

8.2 Data recording and source data

The data recording and source data flow is presented in Figure 5.

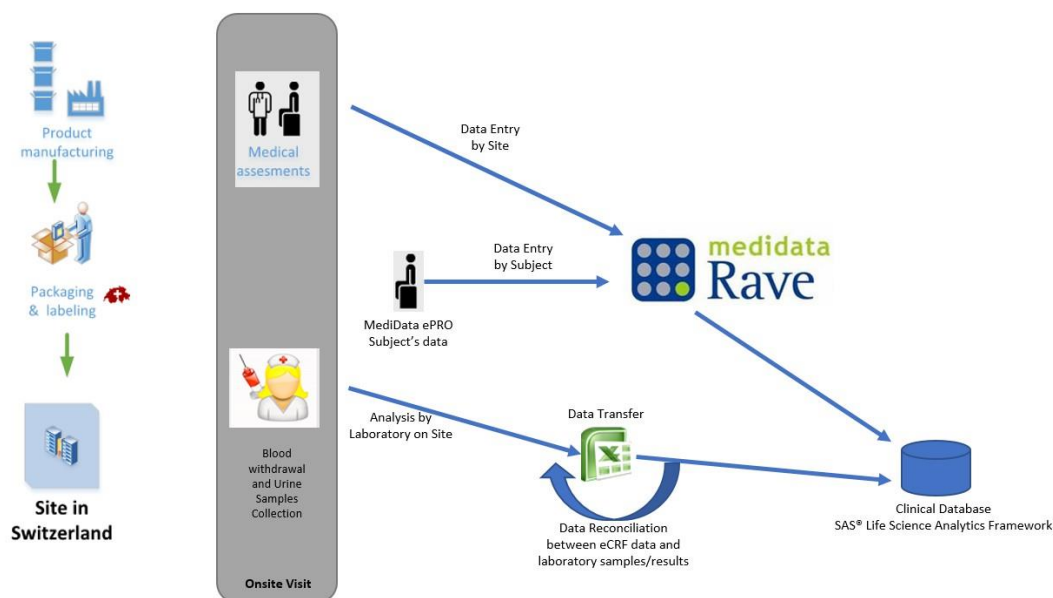


Figure 5. Study Data Flow.

8.3 Participant Data Rights

The study team will implement and maintain appropriate technical and organizational measures to ensure accessibility to participants of their personal data collected during the research project. Upon request, the study team will ensure to provide extracts of personal data to participants.

8.4 Participant Confidentiality

The processing of personal and medical data will follow the Swiss regulation in matter of data privacy and in conformity with the ORH (Human Research Ordinance) regulation, Art. 5. The study team will ensure protection of Participant's personal data and that all reports, publications, and any other disclosures, except where required by laws.

Those data will be coded to ensure their confidentiality: Participants are identified only by the investigational site identification number (CHE001 for the Clinical Innovation Lab) and a participant identification number (00XX, the XXth participant in the study). All participant records will be kept safely in an access-controlled area. Identification code lists linking participant names to participant identification numbers should preferably be stored separate from participant records. Health related data will not be released without the written permission of the participant, except for experts representing Nestlé in charge of inspecting in the frame of a so-called monitoring or audit and for external inspectors in case of potential inspection. The scientists exploiting research project results will only have access to coded data.

In case a participant is not included in the project, his/her preselection data will be destroyed at the end of the study.

In case of revocation of the consent before the study is completed, it is impossible to anonymize the data collected up to that point. Collected data will be coded and kept in our system.

8.5 Participant data' handling

8.5.1 Data Reporting and Case Report Forms (CRFs)

The study team will accurately, completely and in a timely manner record collected data from source documents into a secured database, e-CRF (iMedidata Rave™). The clinical data management system complies with the Good Clinical Practices (GCP) predicate rule requirements, laws, and regulations (personal data protection) and allows an audit of actions performed by users. Thus, all data modifications will be documented in an audit trail file. After the last verification, the relevant data will be locked into the database. The system is also compliant with the e-signature laws (particularly the Title 21 CFR Part 11 of the Code of Federal Regulations); in that saving the data is equivalent to a handwritten signature. Designated site personnel will be provided with a personal username and password to access the eCRF. Usernames and passwords must not be shared with any other person.

8.5.2 Electronic Patient Reported Outcome

Electronic Patient Reported Outcome (ePRO) data are reported by the participant via iMedidata Patient Cloud. These data include information from the following: investigational product intake diary, adverse events and concomitant medications diary reported by the participant throughout the study, as well as dietary and physical activity questionnaires filled at visit days by the participants overseen by the study site staff.

iMedidata Patient Cloud is a secure, password-protected application, allowing participants to complete questionnaires via a tablet or smartphone. The coded data from these questionnaires are transmitted directly to the iMedidata (see data flow in section 8.2). At the end of the study, the

coded data will be electronically transferred into the Nestlé secure data repository. The system used is iMedidata and is compliant with Swiss regulation. Medidata's privacy notice and terms of use are available.

8.6 Confidentiality and coding

Project data will be handled with uttermost discretion and is only accessible to authorized personnel who require the data to fulfil their duties within the scope of the research project. On the CRFs and other project specific documents, participants are only identified by a unique participant number.

Biological material (blood and urine) in this project is not identified by participant name but by a unique participant number. Biological material is appropriately stored in a restricted area only accessible to authorized personnel.

The samples may be transferred abroad and analyzed by Nestlé or its collaborators in the scope of the study. The storage and analysis of samples will be detailed in the study laboratory manual. If the subjects' consent to participate in the Nestlé Research Biobank (formally called the Nestlé Center for Biological Resources, NCBR), the samples or aliquots will be stored at Nestlé Research in Switzerland and used for other scientific research not defined at the time of sample collection. If the subjects do not consent for biobanking, the samples will be used only for the study and destroyed within maximum 10 years after the Last subject Last visit.

8.6.1 Sample biobanking and study data re-use

The sample will be bio-banked upon further use consent from the subjects in order to conduct scientific research and will be declared to the relevant legal and regulatory bodies in accordance with applicable legislation.

Any new analysis of samples and study data not determined at the time of sample collection will be approved by the relevant ethical committee(s). The samples of biobank will be stored at the Nestlé Research Center. The biobank may be moved to abroad or Switzerland if required.

8.7 Data Protection requirements and Data Breaches reporting

The study team will (a) comply with all applicable data protection laws and recommendations (36) and (b) implement and maintain appropriate technical and organizational measures against the unauthorized or unlawful acquisition, access to and/or processing of personal data and against the accidental loss, disclosure, or destruction of, or damage to personal data.

The study team involved in the processing of personal data have undergone adequate training in the care, protection and handling of such information and have committed themselves to confidentiality or are under an appropriate statutory obligation of confidentiality.

Société des Produits Nestlé SA is entitled to inspect or appoint representatives to inspect all facilities, equipment, documents and electronic data related to the processing of personal data by the study team upon giving at least seven (7) days' notice.

The study team shall promptly notify the Nestlé department of Cyber security of any act or omission or other matter of becoming aware that is likely to have an adverse effect on the security of the information technology systems.

Where the study team receives any request, complaint, notice or communication which relates directly or indirectly to the processing of the personal data, or to either party's compliance, it shall promptly notify Nestlé department of Cyber Security.

The study team will, promptly upon request, provide Nestlé department of Cyber Security with all information and reasonable assistance necessary to enable Société des Produits Nestlé SA to comply with, and demonstrate compliance with, applicable data protection laws, in particular with respect to (a) giving effect to the rights of data subjects, (b) conducting data protection impact assessments; (c) obtaining any necessary authorizations from any relevant governmental authorities; and (d) notifying governmental authorities and/or data subjects of any data breaches affecting Personal Data processed during the study.

The study team shall not appoint any third party, except the Authorized Subcontractors, to process personal data on its behalf without Société des Produits Nestlé SA prior, express written authorization, such authorization not to be unreasonably withheld.

The study team will not transfer any personal data to any recipient located outside of Switzerland without the prior written consent authorization of Société des Produits Nestlé SA.

8.8 Retention and destruction of study data and biological material

8.8.1 Data Reuse

In addition to the current study, the participants will be proposed to consider providing consent for "further re-use" of their biological samples and data in the broader scope of Nestlé Center of Biological Resources (NCBR); if they consent to this, the samples and health related data will be handled as described in the biobank regulations (CERVD opinion - Req. 2020-00307).

If they do not consent, the data will be used only in the frame of this research. No data will be further used without the Ethics Committee approval and the participants' consent.

The dissent, the consent as well as potential NCBR withdrawal of each participant will be tracked via the Electronic Data Capture (EDC) Medidata RAVE.

8.8.2 Archiving and Destruction of study data and biological material

The investigational site files, including the list of codes, will be kept, and archived separately in a secure repository compliant to Nestlé policies with respect to data privacy by the research team for 25 years, even if the candidate is not included in the research project. The Trial Master file will be kept separately for 25 years as per Nestlé policy.

Data and/or samples will be destroyed upon request of the participant, unless it compromised the conduction of the study.

Data collected before the withdrawal will be used for analyses. Biological material might be destroyed, and personal data might be inactivated from the database field if requested by the participant.

In case of revocation of the consent before the study is completed, it remains impossible to fully delete the data collected up to that point. Collected data will be coded, inactivated, and kept in our system audit trail.

8.9 Research Project completion

According to HRO regulation Art. 22, after research project completion or termination, the medical expert will inform, within 90 days, the Ethics Committee of the end of the study, using an end of research report.

9. PUBLICATION / DECLARATION OF INTEREST

This trial is registered in www.clinicaltrial.gov.

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3. Declaration of Helsinki (<https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects>)
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APPENDIX A: COMPOSITION OF THE STANDARDIZED COMPLETE BREAKFAST NUTRITION DRINK

Nutrition product: Boost Plus (237 mL per bottle) – 360 Calories

Nutrition Information		8 fl oz (1 Carton)	%DV*
Calories	kcal	360	**
Calories from Fat	kcal	130	**
Total Fat	g	14	22
Saturated Fat	g	2	10
Trans Fat	g	0	**
Cholesterol	mg	10	3
Sodium	mg (mEq)	200 (8.7)	8
Potassium	mg (mEq)	360 (9.2)	10
Total Carbohydrate	g	45	15
Dietary Fiber	g	3	12
Sugars	g	22	**
Protein	g	14	28
Vitamin A***	IU	1250	25
Vitamin C†	mg	60	100
Calcium†	mg	350	35
Iron	mg	4.5	25
Vitamin D†	IU	240	60
Vitamin E	IU	30	100
Vitamin K†	mcg	32	40
Thiamin	mg	0.38	25
Riboflavin	mg	0.43	25
Niacin	mg	4	20
Vitamin B ₆	mg	0.7	35
Folic Acid	mcg	100	25
Vitamin B ₁₂	mcg	2.1	35
Biotin	mcg	75	25
Pantothenic Acid	mg	2.5	25
Phosphorus†	mg	300	30
Iodine	mcg	38	25
Magnesium†	mg	100	25
Zinc†	mg	4.5	30
Selenium	mcg	18	25
Copper	mg	0.5	25
Manganese	mg	0.7	35
Chromium	mcg	30	25
Molybdenum	mcg	18.75	25
Chloride	mg (mEq)	272 (7.7)	8
Choline	mg	55	10
Water	ml	185	

*Daily Value
 **DV not established
 ***Includes 50% of vitamin A activity from beta carotene
 †CalcLock™ blend

APPENDIX B: SCHEDULE OF ASSESSMENTS

Study procedures / assessments	V0	V1	V2		V3	Intervention period										V4				V5
Info session (virtual)		Screening & Enrolment	Initiation visit	Phone call 1 between Day -3 and -1	Start of IP intake											Phone call 2 between Day 11 and 13			Last IP intake	Study end (phone)
Visit windows (Days)	D -20 to -10		D - 15 to -5	D1		D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D16	
Information session	X																			
Informed consent	X																			
Inclusion / exclusion criteria	X																			
Demographics / Medical history	X																			
Urine dipstick pregnancy test	X																			
ePRO account setup		X																		
Stratification/Randomization				X																
Breakfast intake on-site				X													X			
IP and placebo intake and compliance (ePRO) on-site for PK analysis at T0 h				X													X			
IP and placebo dispensation				X																
Self-administration of IP or placebo					X		X	X	X	X	X	X	X	X	X	X	X			
Compliance of self-administered IP and placebo intake by participant (ePRO)					X		X	X	X	X	X	X	X	X	X	X	X			
Laboratory assessments				X																
i) Pre-dose PK blood sampling at T baseline																				
ii) Post IP intake (T0h) PK blood sampling at T1h, T2h, T4h																				
iii) First morning urine sample (at home)																				
iv) Collection of urine post IP intake between T0h - T4h																				
Check Dietary compliance (ePRO)				X													X			
Check Physical activity compliance (ePRO)				X													X			
AE/SE/Concomitant medication (ePRO)	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Abbreviations: bHCG, beta Human Chorionic Gonadotropin; ePRO, electronic Patient Reported Outcomes; IP, investigational product; eCRF, electronic Case Report Form; PK, pharmacokinetics; AE, adverse event; SE, serious event.																				

APPENDIX C: REVIEW OF THE HUMAN PHARMACOKINETIC DATA ON THE EFFECT OF NAM, NR AND NMN

1. NAM human pharmacokinetics summary

Following oral capsule administration, NAM is absorbed throughout the gastrointestinal tract, reaching peak blood concentration within 1 hour. NAM concentration decreases rapidly in blood and is distributed to the periphery with a high hepatic extraction ratio (14).

Hepatic methylation using methionine as a methyl donor is important in the detoxification of NAM. The resulting product, namely N-methyl-NAM (MeNAM) is excreted in urine, whereas NAM is reabsorbed by the renal tubules. For this reason, only small amount of non-methylated NAM appears in urine. MeNAM is further oxidized in the liver and the end products are N-methyl-2-pyridone-5-carboxamide (Me2py) and N-methyl-4-pyridone-5-carboxamide (Me4py).

Despite numerous studies, there is limited data on the potential of NAM supplementation to increase NAD⁺ concentrations in human blood or other organs. Chronic oral daily doses up to 3000 milligrams (mg) per day are reported to be safe and tolerated (14).

- ✓ In a pilot study, 27 adults (age 52 to 72 years) undergoing cardiac surgery were administered NAM orally (either 1000 mg or 3000 mg/day over 3 consecutive days). Findings indicated an increase in the serum concentration of NAM (~20 µM), NMN (~1 µM) and MeNAM (~3 µM) within the first day of administration (15).
- ✓ In 6 healthy participants (age 26 to 35 years), oral intake of a natural extract containing 200 mg NAM, increased whole blood NAM, with an observed maximum concentration (C_{max}) of ~26 µM and corresponding time to maximum concentration (T_{max}) of ~30 min. An acute increase in blood NAD⁺ or NMN levels could not be detected over 4 hours. Baseline whole blood level of NAM, NMN, and NAD⁺ prior to supplementation were 0.84 µM, 2.3 µM, and 26.1 µM, respectively (16).
- ✓ A single oral dose of NAM (300 mg) administered to 10 healthy young male participants (age 20 to 23 years) resulted in an increase in plasma NAM (T_{max} ~1.5 h, C_{max} ~30 µM) and MeNAM (T_{max} ~1.5 h, C_{max} ~ 4 µM) (17).
- ✓ In a randomized, double-blind, controlled study conducted in 14 elderly adults (age 72.9 ± 4.0 years) (mean ± standard deviation (SD)) with impaired physical mobility, oral daily supplementation of NAM at a dose of 200 mg for 32 days did not lead to any change in muscle NAD⁺ metabolites content (NAM, NMN, NR, NAD⁺, NADH) aside a 2.5-fold increase in MeNAM level. The level of blood NAD⁺ metabolites were not measured (18).

Based on the above studies, it is anticipated that an acute oral dose of NAM (500 mg) will rapidly increase whole blood levels of NAM, MeNAM and NMN within 4 hours. The anticipated effect on other NAD⁺ metabolites such as NAD⁺, NADH or NR remains unclear.

2. NR human pharmacokinetics summary

Studies reporting on the pharmacokinetics (PK) of NR are summarized below. These studies support the chronic daily dosing of NR in humans up to 1000 mg per day.

- ✓ In the first human PK analysis of three different single oral doses of NR (100, 300 and 1000 mg) (19), peripheral blood monocyte (PBMC) NAD⁺ metabolites from 12 healthy subjects were quantified. Within the first 4 hours, a statistically significant increase in MeNAM and Me2Py was already detected at 1 hour post administration of a 1000 mg oral dose. Of note, no increase was detected for NAD⁺, NMN, NAM, or nicotinic acid adenine dinucleotide (NAAD) at this dose. At the lower doses, no statistically significant change in NAD⁺ metabolite levels were detected. PBMC baseline levels reported for NAM, NAD⁺, NMN and MeNAM, were approximately 22, 12, 1.5 and 1 µM, respectively.
- ✓ Twelve young adult males (22.9 ± 1.0 years) and twelve elderly adult males (mean age 71.5 ± 1.0 years) received NR (500 mg) or placebo in a single-dose, double-blind cross-over study (20). NADH and NADPH were measured in blood erythrocytes before and 2 h after oral administration of NR or placebo. NR supplementation increased NADH (51% young; 59% elderly) and NADPH (32% young; 38% elderly) levels compared to placebo. No other NAD⁺ metabolites were measured.
- ✓ In a non-randomized, open-label PK study of 8 healthy volunteers (33 ± 8 years), 250 mg NR was orally administered once daily on Days 1 and 2, then up-titrated to a dose of 1000 mg twice daily on Days 7 and 8 (21). On the morning of Day 9, PK sampling commenced at defined time points pre-dose and post oral dose of 1000 mg NR. Whole-blood levels of NR and NAD⁺ were analyzed. A peak increase in NR over baseline was detected on Day 9 post-dose (C_{max} 0.029 vs C_{baseline} 0.023 µM, T_{max} 3 h). A significant baseline increase was observed for NAD⁺ (baseline on Day 9: 50 µM versus on Day 0: 27 µM). However, on Day 9, 1000 mg NR intake did not produce a peak in whole blood NAD⁺ concentration. No significant change in baseline NMN and NR concentration between Day 9 and Day 0 was detected. No other NAD⁺ metabolites were measured.
- ✓ To evaluate the PK and dose-dependency of NR oral availability in overweight, but otherwise healthy men and women (age 40 to 60 years), a 8-week randomized, double-blind, placebo-controlled study was conducted (22). Consumption of 100, 300 and 1000 mg NR (35 subjects per group) significantly increased whole blood NAD⁺ levels in a dose-

dependent manner (from 31 to ~74 μM at 1000 mg) within 2 weeks. The increases were maintained throughout the remainder of the study. In plasma, an increase in NAM from 0.18 to 0.36 μM , and in MeNAM from 0.019 to 0.169 μM was observed at week 56.

- ✓ Twelve healthy elderly males (age 72 to 78 years) were orally supplemented with 1000 mg NR once daily for 21 days in a placebo-controlled, randomized, double-blind, crossover study, with a washout period of 21 days between phases (23). Whole blood analysis pre-dose and 14 hours after the last dose, indicated statistically significant increases in NAD⁺, Me2Py, NMN, Me4py, MeNAM and NAAD (Table A). No increase was observed for NAM, NADP or NR. In muscle, no increase in NAD⁺ was detected. In urine, the main metabolites detected were Me2py, MeNAM and Me4py.

Table A. Change in whole blood NAD⁺ and metabolites after 21 days of 1000 mg NR daily intake (mean +/- SD) (19).

	Baseline concentration (μM)	Post supplementation concentration (μM)
NAD⁺	20.0 +/- 5.2	54.0 +/- 18.3
Me2py	1.38 +/- 0.48	7.61 +/- 1.54
NMN	1.14 +/- 0.23	1.67 +/- 0.31
Me4py	0.56 +/- 0.18	3.85 +/- 1.02
MeNAM	0.10 +/- 0.05	0.79 +/- 0.36
NAAD	0.04 +/- 0.02	0.27 +/- 0.22
NAM	10.24 +/- 1.59	10.45 +/- 1.45
NADP	6.77 +/- 1.12	7.28 +/- 1.13
NR	0.19 +/- 0.07	0.17 +/- 0.04

- ✓ In a randomized, double-blind, placebo-controlled, cross-over study, 30 healthy middle-aged and older adults (age 55 to 79 years) received 500 mg twice daily oral supplementation of NR and placebo for 6 weeks. Increases in NAD⁺ and NAAD level detected in PBMC were observed (24). No change was recorded for NADP, NAM, NR and NMN.
- ✓ In a 7-day double-blinded, randomized, counter-balanced, crossover design study, eight male participants (23 ± 4 years) received 1000 mg of NR or cellulose placebo supplementation. NR supplementation was safe and did not alter whole-body metabolism or substrate utilization at rest or during exercise. NR augmented the skeletal muscle NAD⁺

metabolome, increasing the concentration of NAR and NAMN as well as the methylated products Me2PY and Me4PY, although skeletal muscle NAD⁺ concentration remained unchanged (25).

3. NMN human pharmacokinetics summary

The recent commercial availability of NMN supplements has been associated with a series of published human PK evaluations. These studies support chronic daily dosing up to 1200 mg per day.

- ✓ A single-arm non-randomized study was conducted in 10 healthy participants (47.9 ± 6.0 years). Single oral ascending doses of NMN (100, 250, and 500 mg) were administered with a one-week washout between each ascending dose increment (26). A dose-dependent increase of Me2py and Me4py was detected in plasma with a steady increase over 5 h. NAM also increased but in a non-dose-dependent manner. The analytical method did not allow for the detection of plasma NMN levels.
- ✓ In a 10-week, randomized, placebo-controlled, double-blinded study, 25 postmenopausal women (61 ± 5 years) with prediabetes, and overweight or obese, were supplemented with 250 mg once daily oral NMN or placebo (27). An increase in NAD⁺ was detected in PBMC, while Me2py and Me4py were increased in plasma. At week 10, acute intake of NMN did not result in a peak increase of NAD⁺. In muscle, MeNAM, Me2py and Me4py increased significantly, while the levels of NAD⁺ and NAM did not change.
- ✓ Ten elderly males (71.1 ± 3.9) were administered 250 mg NMN daily for 12 weeks in a placebo-controlled, randomized, double blind, parallel-group study (28). Whole blood analysis revealed a significant increase in NAD⁺, NMN, NR, NAMN, and nicotinic acid riboside (NAR). No change was detected for NA and NAM.
- ✓ In a six-week randomized, double-blind, placebo-controlled, four-arm study, 48 young and middle-aged (35.6 ± 6.1) recreationally trained runners were randomized into four groups: 300, 600, 1200 mg/day NMN and placebo (29). The intervention was safe at all doses and increased the aerobic capacity during exercise. Blood NAD⁺ levels were not determined.

**APPENDIX D: DESCRIPTION OF THE NAD+ PRECURSORS AND PLACEBO CAPSULES
(ALL CAPSULES ARE SIZE (00))**

Commercial Product name	Ingredient	Amount per capsule	Manufacturer	Quality of product	Link to product	Commercial Product
Niacinamide	Niacinamide (NAM)	500 mg	Pure Encapsulation (a Nestlé Health Science company)	GMO free, Vegan, Vegetarian Certified by independent laboratories	Niacinamide Supplements - Pure	
True Niagen Pro	Nicotinamide Riboside (NR)	500 mg	ChromaDex Inc.	NSF Certified Sport NSF International Certified for Sport	Tru-Niagen-PRO-500mg	
PUR NMN	Nicotinamide Mononucleotide (NMN)	500 mg	UltraHeath LLC	Pharmaceutical grade supplement	PUR NMN UltraHeath	
PLACEBO	Microcrystalline cellulose	500 mg	Pure Encapsulation (a Nestlé Health Science company)	GMO free, Vegan, Vegetarian Certified by independent laboratories		NA

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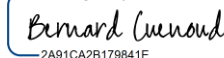
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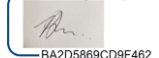
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Statistical Analysis Plan

Trial N°	2201NR
Project I2L Code N°	DUND-103047

Effect of Oral NAD⁺ Precursors Administration on Blood NAD⁺ Concentration in Healthy Adults

By: Clinical Research Unit / Biostatistics
Date: Dec 15th, 2022
Version: Final
N° 1.0
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Template version written by A. Tavenard on the 12th of April 2013

1 INTRODUCTION

1.1 Background

Nicotinamide adenine dinucleotide (NAD) is a coenzyme playing a central role in human metabolic pathways. Found in all living cells, NAD is involved in redox reactions, carrying electrons from one reaction to another through its two forms: an oxidized (NAD⁺) and reduced (NADH) form. NAD⁺ is also used in other cellular processes, most notably as a substrate of enzymes in post-translational proteins modifications. Given the pivotal role of NAD⁺ in human physiology, studying its metabolism has become essential to monitor genetic and/ or drug-induced perturbations related to metabolic status and diseases such as ageing, cancer, obesity or cardiometabolic diseases (1,2).

NAD metabolism, transport, and function are complex and dynamic. NAD can be metabolized into several molecules, including nicotinamide adenine dinucleotide phosphate (NADP) and nicotinic acid adenine dinucleotide phosphate (NAADP). The metabolites play key roles in energy transduction and cell signaling. Products of NAD liver metabolism, such as nicotinamide (NAM) and n-methyl-nicotinamide (MeNAM), have also emerged as key regulators of energy metabolism, epigenetics, and disease pathophysiology, highlighting the involvement of NAD metabolites in several metabolic pathways apart from the traditionally described oxidation-reduction reactions (3).

Cellular levels of NAD metabolites and the NAD redox ratio (NAD⁺/NADH), appear to decrease during chronological aging (6), and in several other pathological conditions such as adult-onset mitochondrial myopathy (9), schizophrenia (7), and bipolar disorder (8).

Moreover, dysregulation in NAD metabolism has emerged as a contributing factor in the pathogenesis of several disease states therefore therapeutic strategies to increase NAD⁺ levels have been proposed to address NAD related dysregulation (4).

A recognized approach to increase NAD⁺ level is through oral supplementation of its precursors promoting NAD⁺ synthesis *in vivo*. NAD⁺ precursors exist in multiple forms, including nicotinic acid (NA), nicotinamide (NAM), nicotinamide riboside (NR), and nicotinamide mono nucleotide (NMN). However, it is unclear how the various precursors compare in their ability to increase NAD⁺ levels. Moreover, a subset of recent studies indicates that the effects of NAD⁺ supplementation in humans might be less pronounced than what was observed in preclinical studies (1,4,5).

1.2 Objectives

1.2.1 Primary objective

The main hypothesis is that daily oral intake for 14 days of a NAD⁺ precursors will lead to differential effects on the NAD⁺ metabolome measured in whole blood of healthy volunteers. The 3 precursors tested at a 'NAD⁺ equivalent dose' will be NAM (500 mg daily dose), NR (1000 mg daily dose) and NMN (1000 mg daily dose) administered once daily for 14 consecutive days.

The primary objectives are:

- To determine the extent of increase in NAD⁺ level in whole blood, for each NAD⁺ precursor compared to placebo
- To compare the extent of increase in NAD⁺ level in whole blood across the 3 NAD⁺ precursors

1.2.2 Secondary objectives

The secondary objective will be to determine to which extent the NAD⁺ precursors affect the NAD⁺ metabolome in whole blood. Evaluation of the pharmacokinetic changes will be determined by PK profiling following dosing on the first and last day of the dosing period.

1.2.3 Exploratory objectives

The metabolic and elimination pathways of the various NAD⁺ precursors and global gene expression changes in blood before and after chronic NAD⁺ precursor intake will be investigated.

1.3 Endpoints

1.3.1 Primary endpoints

- Changes in whole blood NAD⁺ level at Day 14 (pre-dose) - interventions vs placebo
3 comparisons: NAM vs placebo, NR vs placebo, NMN versus placebo
- Co-primary endpoint: Changes in whole blood NAD⁺ level at Day 14 (pre-dose) across interventions
3 comparisons: NAM vs NR, NAM vs NMN, NR vs NMN

1.3.2 Secondary endpoints

- Changes in whole blood NAD⁺ metabolites level at Day 14 (pre-dose) - interventions vs placebo, and across interventions
- Change in the NAD⁺ redox ratio (NAD⁺/NADH) and the NADP ratio (NADP⁺/NADPH) at Day 14 (pre-dose) - interventions vs placebo, and across interventions
- Change in whole blood NAD⁺ metabolites iAUC and C_{max} over 4 hours at Day 1 and at Day 14 - interventions vs placebo, and across interventions

1.3.3 Exploratory endpoints

Changes in NAD⁺ metabolome in plasma and urine will be evaluated – intervention vs placebo (and across interventions). For standardizing the dilution of urine, creatinine levels will be measured.

Changes in oxidative stress molecular markers will be determined by measuring plasma N-acetylcysteine, cysteine, and glutathione oxidized over 4 hours at Day 1 and at Day 14.

Transcriptomic differential at baseline Day 14 versus Day 1 will be conducted. Similarly, a metabolomic comparison in plasma and urine will be performed.

Correlations across NAD⁺ metabolites levels and the other measures (baseline and intervention) will be performed.

2 BLIND DATA REVIEW

The blind data review will focus on feasibility of the planned analyses and verification of the protocol assumptions. The trial results (group difference estimation) will not be discussed during the blind data review process.

2.1 Disposition of subjects

Frequency table showing disposition of study subjects will present counts and percentages of the following groups of subjects:

- • Screened
- • Screening failures
- • Enrolled
- • Completed the study

Percentages will be calculated out of enrolled subjects. For Screened subjects and Screening failures, only counts will be presented.

Reasons for discontinuation will be presented in frequency table where percentages will be calculated out of all discontinued subjects. Screening failures will not be counted as discontinued subject.

Discontinued study subjects will be listed. The listing will include subject ID, group where the patient belongs, age in days when subject discontinued the study, last visit performed and reasons for discontinuation.

2.2 Protocol deviations

Subject's protocol deviations as automatically recorded in eCRF and manually added will be summarized in a frequency table, on ITT population.

Deviations having a major impact on primary endpoints will be flagged and will lead to PP population exclusion.

The amount of minor, major and leading to PP exclusion deviations will be discussed during the blind data review. Unbalance between randomized groups will be investigated.

2.3 Unresolved data queries

Unresolved data queries (unlikely or impossible data) will be identified and listed as part of the blind data review meeting minutes. Their impact on statistical analysis will be evaluated with a sensitivity analysis, either by precluding those observations, or using more appropriate robust estimators. The difference of estimation between the planned and the sensitivity analysis will be discussed in the final statistical report.

2.4 Blinded feasibility check

All inferential models specified in this document have underlying structural hypotheses (data distribution, proportionality of hazards, etc). Those hypotheses will be tested as part of the blind data review process. In case of hypothesis rejection, the SAP will be updated with a more appropriate method.

Moreover, at the time of writing SAP, urine, plasma, metabolomics, and transcriptomics data weren't available. Thus, after receiving this data if the written analysis in this document isn't feasible, a second version of SAP with a revised analysis will be released. The between group estimations will not be used as a driver for method modification. The reason for method modification will be highlighted in the BDRM minutes.

3 ANALYSIS POPULATIONS

3.1 Intention-to-treat (ITT) analysis population

All randomized subjects will be included in the intention to treat analysis population. ITT driven analyses will be conducted using the assigned intervention.

3.2 Safety analysis set (SAF) analysis population

Safety data (e.g., adverse events) will be analyzed in the SAF which consists of all subjects in the ITT population with documentation of at least one intake of one investigational product (including placebo). SAF driven analyses will be conducted according to the product consumed irrespective of the randomization assignment.

3.3 Full analysis set (FAS) analysis population

Efficacy data will be analyzed in the FAS dataset which ideally represents the analysis population that is as close as possible to the ITT principle. FAS analysis population consists of all subjects in the ITT population with available primary endpoint baseline (Day 1 pre-dose whole

blood NAD+ level), sex, age, and post baseline primary endpoint (Day 14 pre-dose whole blood NAD+ level). FAS driven analyses will be conducted using the assigned intervention.

3.4 Per Protocol (PP) analysis population

The Per Protocol (PP) dataset consists of subjects from the FAS with no protocol deviation impacting the primary analyses such as product intake non-compliance and unauthorized concomitant medication intake. Participants will be considered compliant if they have been compliant for ≥ 12 days and with confirmed intake on Day 1 and Day 14 and two missed days must not be consecutive during days D11-D13.

The list of subjects excluded from the PP will be documented prior to trial analysis. PP driven analyses will be conducted using the assigned intervention.

4 STATISTICAL ANALYSIS PLAN

4.1 Disposition and Analysis Populations

The disposition table will present:

- Number of subjects
 - Screened
 - Randomized
 - Who completed/did not complete

The analysis populations will present:

- Number of subjects in each statistical population (as defined in section **Error! Reference source not found.**) (ITT, SAF, FAS, PP).

4.2 Screening, Baseline, and demographical information

Demographical information collected are the following:

- Gender
- Age
- Weight, height, and BMI

4.3 Medical History

Medical history is collected at screening visit. Number of subjects by exact reported terms will be provided on the ITT population.

4.4 Concomitant medications

Concomitant medications of all enrolled subjects will be listed (condition or event, date of start and end, ongoing). Whether the medication led to an exclusion from PP set will be clearly indicated with a dedicated flag (Y/N variable).

The concomitant medications will not be tabulated, due to the absence of WHO-Drug dictionary coding (one item might be reported with different reported terms, thus summary statistics by reported term makes no sense).

4.5 Protocol deviations

Protocol deviations are collected over the trial and will be summarized on the ITT:

- Number of Major Protocol deviations
 - Breakdown by reported deviation term.
- Number of Minor Protocol deviations
 - Breakdown by reported deviation term.
- Number of protocol deviations impacting the primary endpoint (as identified to derive the per protocol population).
 - Breakdown by reported deviation term.

4.6 Primary endpoint(s)

The trial primary outcome is whole blood NAD⁺ level in μM at Day 14 (pre-dose). The units under investigation will be individual participants.

Six tests of hypotheses will be conducted:

1. Superiority of NAM, NR, NMN versus Placebo measured as a greater whole blood NAD⁺ level at Day 14 (pre-dose) corrected for baseline (Day 1 pre-dose), sex and age (Full analysis set).
2. Superiority of NAM vs NR vs NMN measured as a greater whole blood NAD⁺ level at Day 14 (pre-dose) corrected for baseline (Day 1 pre-dose), sex and age (Full analysis set).

Superiority of NAM, NR and/or NMN vs Placebo will be declared if the lower bound of the corresponding confidence interval of the whole blood NAD⁺ level difference at Day 14 (pre-dose) is greater than 0.

For the IPs comparison, superiority of IP#1 over IP#2 will be declared if the corresponding confidence interval of the whole blood NAD⁺ difference at Day 14 (pre-dose) excludes the value zero and the point estimate goes in the direction of a greater level in IP#1 group than IP#2 group.

Confidence intervals will be computed at an alpha level equal to $1-(0.05/3)$.

In order to control for the family wise error rate at 5% maximum, the primary and co-primary endpoints will be evaluated in a hierarchical order. The next hypothesis test will be conducted if and only if the preceding one led to rejection of the null hypothesis when evaluated on the Full Analysis Set (FAS).

The first test is a combination of three hypotheses. In order to control for the inflation of the family wise error rate at this level, a Bonferroni correction will be applied.

4.6.1 Main analysis of primary endpoint(s)

The trial primary outcomes are differences of change in whole blood NAD⁺ level at day 14 (pre-dose) from day 1 (pre-dose) between interventions and placebo, as well as across interventions.

The differences NAM-Placebo, NR-Placebo, NMN-Placebo, NAM-NR, NAM-NMN and NR-NMN will be estimated using one single ANCOVA linear model with baseline whole blood NAD⁺ level (Day 1 pre-dose), sex, age and assigned intervention as covariates. Primary analyses will be conducted on the Full Analysis Set (FAS).

The 6 tests of null-hypotheses (no difference between the tested elements) will be conducted using the two-sided 98,33% (i.e., $100 - 5/3$) confidence intervals of the mean estimated differences.

In order to control for the false positive inflation coming from multiple testing, the comparisons between interventions (NAM-NR, NAM-NMN and NR-NMN) will be interpreted if and only if at least one of the comparisons between interventions and placebo are statistically significant.

In dataset, NAD⁺ can be recognized by LBTEST = NICOTINAMIDE ADENINE DINUCLEOTIDE and Unit = UM.

4.6.2 Supportive analysis of primary endpoint(s)

A sensitivity analysis of the primary endpoint will be conducted repeating the main analysis on the Per Protocol population. Estimations calculated on the full analysis set should remain consistent with the estimations made on the per protocol set for robust conclusions.

4.7 Secondary endpoint(s)

All secondary endpoints' analyses will be performed using the ITT set.

4.7.1 Main analysis of secondary endpoint(s)

Secondary analyses will be conducted on the ITT analysis set.

Changes in whole blood NAD⁺ metabolites level (NAD⁺, NAD⁺H, NAM, NR, NMN, NAD⁺P, NAD⁺PH, MeNaM and MeXPY (combination of Me2PY and Me4PY)) at day 14 (pre-dose) from day 1 (pre-dose) interventions vs placebo, and across interventions will be evaluated using one single ANCOVA model per metabolite with the corresponding metabolite baseline whole blood level (Day 1 pre-dose), sex, age, and assigned intervention as covariates.

The same method will be used in order to compare NAD redox ratio (NAD⁺/NADH) and the NADP ratio (NADP⁺/NADPH) at Day 14 (pre-dose) - interventions vs placebo, and across interventions. Since the distributions of ratios are frequently skewed, natural logs will be used. Then, the results will be converted back to the original scales to facilitate interpretation; thus, the CIs will be not necessarily symmetrically distributed around the overall summary estimate.

iAUC and C_{max} of each NAD⁺ metabolites will be evaluated at Day 1 and Day 14 for each subject. Both parameters will be analysed using a separated mixed model for repeated measurement (MMRM) including the visit (Day 1, Day 14), the baseline whole blood level of the metabolite (Day 1 pre-dose), sex, age, and assigned intervention as fixed effects as well as

subject as random effect in order to model the correlation between repeated measures within subjects.

An unstructured correlation matrix will be used, and the degree of freedom will be estimated using the method of Kenward- Roger. Acute effect of IPs will be estimated from these models comparing the iAUC and Cmax at Day 1. Chronic effect of IPs will be estimated from these models comparing the iAUC and Cmax at Day 14.

The name of each test in dataset and corresponding abbreviation is listed below:

METHYL NICOTINAMIDE (MeNAM)
NICOTINAMIDE (NAM)
NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD)
NICOTINAMIDE ADENINE DINUCLEOTIDE PH RED (NADPH)
NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPH (NADP)
NICOTINAMIDE ADENINE DINUCLEOTIDE REDUCE (NADH)
NICOTINAMIDE MONONUCLEOTIDE (NMN)
NICOTINAMIDE RIBOSIDE (NR)
SUM OF ME2PY AND ME4PY (MeXPY)

4.7.2 Supportive analysis of secondary endpoint(s)

In the presence of outliers, robust ANCOVA with the same covariates will be applied to the secondary endpoints as a sensitivity analysis.

4.8 Exploratory analyses

The secondary statistical analyses will be repeated on plasma NAD⁺ metabolites and on oxidative stress molecular markers (for example plasma N-acetylcysteine, cysteine, and glutathione oxidized) including the comparison of Day 14 pre-dose level, the comparison of ratios as well as the chronic and acute effect measured on iAUC and Cmax. Two urine samples : i) a first morning urine sample (pre-dose) and ii) a second urine sample between T1 h to T4 h post IP intake at Visit 3 (Day 1) and Visit 4 (Day 14), will also be analyzed. The change in standardized urine NAD⁺ metabolites over creatinine level (ratio) after chronic treatment will be evaluated with the same model as the first secondary endpoint.

Transcriptomics and a more global untargeted metabolomic analysis of whole blood and plasma will be performed, respectively, if the primary analysis shows that whole blood NAD⁺ level is impacted by the NAD⁺ precursor supplementation. The change of transcripts and metabolites from pre-dose at both Day 1 and Day 14 will then be explored, accordingly.

Association between:

- NAD⁺ metabolites levels in whole blood, plasma, and urine (pre-dose) measured at Day 1 and Day 14

- iAUC and Cmax of NAD+ metabolites over 4 hours of kinetic in whole blood and plasma measured at Day 1 and Day 14
- oxidative stress molecular markers measured at Day 1 and Day 14
- NAD+ metabolome, transcriptomic and metabolomic data

will be evaluated using Partial Pearson coefficient adjusted for sex and age (regardless to group assignment). Due to the high number of statistical tests conducted (p value of the test of correlation coefficient against the value 0), a Benjamini Hochberg false discovery rate correction (at level 5%) will be applied on the set of p values generated and presented in the correlation matrix.

Moreover, the differences of total active NAD between interventions and placebo, as well as across interventions will be estimated using ANCOVA linear model with baseline whole blood total active NAD level (Day 1 pre-dose), sex, age and assigned intervention as covariates. This endpoint wasn't mentioned in the protocol but as we provide NAD precursors, it might be that the overall active NAD is elevated, not just NAD+ and NADH.

4.9 Safety analysis

All safety analysis will be conducted by default on the SAF analysis set.

4.9.1 Exposure to trial treatment

The duration and timing of intake for all investigational products and placebo is for 14 consecutive days, orally once daily, at a specific recurrent time every day, recommendable in the morning (regardless of breakfast consumption).

Compliance will be summarized by arms and is defined in section Per Protocol (PP) analysis population^{3.4}

4.9.2 Adverse events

The occurrence of adverse events (AEs) will be summarized in tables presenting frequency of individual AEs (count and percentage) and frequency of subjects with at least one AE (count and percentage), separately for each feeding regimen and overall.

A first summary table will display these frequencies for any AEs, and by characteristics of AEs:

Seriousness (No / Yes)

Relationship to the product (Unrelated / Unlikely / Probable / Related)

Severity (Mild / Moderate / Severe)

Caused study discontinuation (No / Yes)

A second table will display these frequencies of AEs (irrespective of seriousness) by MedDRA System Organ Class (SOC) and Preferred Term (PT).

4.9.3 Deaths and Serious adverse events

The number of subjects with serious adverse events and with at least one serious adverse event leading to death will be tabulated.

4.10 Other methodological aspects

4.10.1 Covariates and subgroups

The covariates included in the planned models are defined as:

- Gender (Male / Female) (ref: Female)
- Age (years)(continuous)
- Group (NAM / NMN / NR / Placebo) (ref: Placebo)

4.10.2 Handling of missing data

Due to the limited sample size per arms and the absence of intermediate measurements of the primary outcome, there are no credible missing data imputation methods to apply in case of large extent of missing information.

Indeed, the use of a mixed model for longitudinal data is only possible when repeated measurements are conducted. Multiple imputation methods would require a large number of measured values in order to proceed to credible imputation on the missing ones.

If outliers are identified on data pertaining to the primary endpoints, the planned ANCOVA will be replaced by its robust version using Sandwich estimator of the variance.

4.10.3 Unresolved queries

All queries were resolved.

4.10.4 Handling of outliers and unresolved queries

The metabolites level with the values < LLOQ will be impute by the LLOQ/2.

4.10.5 Pooling of centers in a multi-centric trial

Not applicable to this monocentric trial.

4.10.6 Multiplicity adjustments

The 6 tests of null-hypotheses (no difference between the tested elements) will be conducted using the two-sided 98,33% (i.e., $100-5/3$) confidence intervals of the mean estimated differences.

In order to control for the false positive inflation coming from multiple testing, the comparisons between interventions (NAM-NR, NAM-NMN and NR-NMN) will be interpreted if and only if at least one of the comparisons between interventions and placebo are statistically significant.

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