Influence of Alkali Supplementation on Circulating microRNA Expression

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INTRODUCTION

- There is growing evidence that supplementation with an alkaline salt, potassium bicarbonate ($KHCO_3$), in an amount that neutralizes the acid load of the diet, significantly reduces urinary excretion of N-telopeptide (NTX; marker of bone resorption), calcium (Ca), and nitrogen (N; marker of muscle breakdown) implicating beneficial bone and muscle effects in older men and women.
- Preliminary studies suggest that bone and muscle physiology might be reflected in changes in circulating microRNA levels – small non-coding RNAs involved in regulation of gene expression – which can be easily measured in the serum.
- These serum microRNAs could serve as newer biomarkers of bone and muscle function providing additional information on biological pathways affected by various interventions.

OBJECTIVES

- To examine whether 3-months of oral $KHCO_3$ supplementation (81 mmol/d) vs. placebo alters serum levels of microRNAs involved in various bone and muscle functions (**Table 1**).
- To investigate associations between changes in serum microRNA levels and changes in established indices of bone and muscle metabolism by treatment group.

METHODS

Study design

- Three-month randomized, double-blind, placebo-controlled trial to determine optimal dose of $KHCO_3$ (81 vs. 120 mmol/d) vs. placebo to maximally suppress bone resorption as measured by urinary NTX .

Subject Selection

- Healthy ambulatory men and women age 60 years and older with an estimated glomerular filtration rate (GFR) of at least 50 mL·min^{-1}·1.73 m^2 (**Table 2**). Subjects encouraged to maintain diet and physical activity stable during the trial.
- The main trial enrolled 244 adults and 233 completed the 3-mo study.
- Based on the main study finding that $KHCO_3$ 81 mmol/d had more favorable effects than the 120 mmol/d dose on the main bone outcomes, we selected 24 subjects (n=12 in the $KHCO_3$ 81 mmol/d group; n=12 in the placebo group) for this secondary analysis.
- *A priori* criteria for selection were based on our main findings and included the following: a) baseline urinary net acid excretion (NAE) of 5 mmol or greater as an indicator of higher endogenous renal net acid status at baseline, b) equal numbers of men and women in the two groups.
- Protocol approved by the Tufts Medical Center-Tufts University Institutional Review Board, and written informed consent was obtained from each subject..

Study Pills

- $KHCO_3$ 81 mmol/d or matching placebo administered as 2 capsules (13.5 mmol/capsule) after each meal 3 times daily. A calcium and vitamin D supplement was supplied to all participants.
- Adherence measured by pill counts and NAE.

Anthropometric, Dietary, and Biochemical Measurements

- Baseline lean body mass measured by dual energy x-ray absorptiometry (DXA) at baseline.
- Urinary NAE, NTX, Ca, and N were measured at baseline and 3 months.
- GFR, serum P1NP (bone formation marker), and serum IGF-1 were measured at baseline and 3 months.
- Serum 25-hydroxyvitamin D level was measured at baseline.

microRNA (miR) Measurements

- RNA extracted from serum using a miRVana™ PARIS™ RNA Purification Kit (AM1556; Ambion Inc.).
- miR of interest (**Table 1**) analyzed using TaqMan® MicroRNA Assays (4427975; Applied Biosystems) following previously described multiplex reverse transcription (RT) and pre-amplification protocol.
- RT and pre-amplification conducted in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA).
- RT-qPCR amplifications conducted using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).
- All miRs normalized to U6. Fold changes calculated from baseline values using the ΔΔ cycle threshold ($\Delta\Delta C_T$) method.

RESULTS

- All characteristics (**Table 2**) and biochemical measurements (data not shown) did not differ significantly in the 2 groups at baseline.
- Three-month changes in urinary NAE differed by group as expected ($KHCO_3$ = -47±9 mmol; Placebo = -5±5 mmol; P<0.01).
- As seen in our larger cohort in the main study, $KHCO_3$ resulted in statistically significant declines in urinary NTX ($KHCO_3$ = -158±31 Nmol; Placebo = -47±28 Nmol; P<0.01), urinary Ca ($KHCO_3$ = -23±19 mmol; Placebo = 38±18 mmol; P<0.01), and serum PINP ($KHCO_3$ = -8±3 Nmol/L; Placebo = -2±3 Nmol/L; P=0.03) compared to placebo over 3 months.
- As seen in our larger cohort in the main study, there were no statistically significant differences in urinary N and serum IGF-1 level in response to $KHCO_3$ vs. placebo (data not shown).
- $KHCO_3$ supplementation resulted in statistically significant differences in the fold change in serum miR-21 and miR-133b compared to placebo (**Table 3; Fig 1**).
- There were significant inverse associations between fold changes in miR-21 and miR-133b and 3-month changes in urinary NAE, NTX, and Ca (**Fig 2**).

TABLE 1. Circulating miRs and proposed function

microRNA	Function	
miR-1	Myogenesis, IGF-1 signaling	Muscle
miR-206	Myogenesis	Muscle
miR-486	AKT signaling, Atrophy	Muscle
miR-133a	Myogenesis, IGF-1 signaling, Runx2 (osteogenesis)	Muscle
miR-133b	Myogenesis, IGF-1 signaling, osteoblast differentiation	Muscle
miR-21	Osteogenesis	Bone
miR-122	BMPR1A, bone remodeling	Bone
miR-125	Osteoblast differentiation	Bone
miR-422	Biomarker for BMD level	Bone

TABLE 2. Baseline characteristics by group (Mean ±SEM)

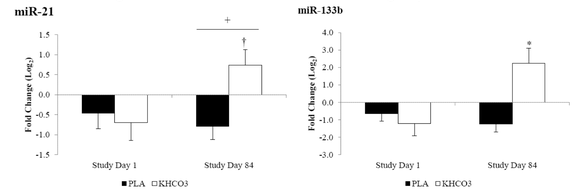
	Placebo (*n* =12)	$KHCO_3$ (*n* =12)	*P*-value
Age (yrs)	67 ± 2	67 ± 1	0.87
Height (cm)	168.7 ± 2.8	170.9 ± 4.9	0.59
Weight (kg)	69.3 ± 3.1	75.8 ± 4.9	0.28
Body Mass Index (kg·m^2)	24.3 ± 0.7	25.6 ± 1.1	0.33
Fat Mass (kg)	19.1 ± 2.1	21.9 ± 2.4	0.39
Fat-Free Mass (kg)	48.8 ± 3.1	50.2 ± 4.1	0.79
GFR (mL·min^1·1.73 m^2)	73.1 ± 2.8	71.0 ± 2.2	0.57

TABLE 3. Fold change in miRs by treatment group (Mean ±SEM)

	Time	Placebo	KHCO3	*P*- Value		
				Time	Treatment	Time-by-Treatment
miR-1	Study Day 1	-1.00 ± 0.66	-0.91 ± 0.67	0.47	0.23	0.55
	Study Day 84	-1.03 ± 0.68	0.08 ± 1.07			
miR-21	Study Day 1	-0.46 ± 0.38	-0.78 ± 0.34	0.23	**0.05**	0.07
	Study Day 84	-0.70 ± 0.45	0.74 ± 0.39			
miR-122	Study Day 1	-0.88 ± 0.61	-0.06 ± 0.62	0.44	0.43	0.75
	Study Day 84	-0.87 ± 0.54	-0.53 ± 0.46			
miR-125	Study Day 1	-0.49 ± 0.39	-0.39 ± 0.42	0.49	0.74	0.61
	Study Day 84	-0.67 ± 0.50	0.03 ± 0.56			
miR-133a	Study Day 1	-0.94 ± 0.52	-0.46 ± 0.35	0.14	0.23	0.67
	Study Day 84	-0.58 ± 0.43	0.27 ± 0.44			
miR-133b	Study Day 1	-0.65 ± 0.43	-1.23 ± 0.45	0.09	< 0.01	**0.02**
	Study Day 84	-1.23 ± 0.69	2.26 ± 0.85			
miR-206	Study Day 1	-0.71 ± 0.53	-0.28 ± 0.74	0.95	0.62	0.64
	Study Day 84	-0.57 ±0.51	-1.00 ± 0.65			
miR-422	Study Day 1	-0.63 ± 0.44	-1.52 ± 0.85	0.37	0.92	0.74
	Study Day 84	-0.91 ± 0.45	-1.33 ± 0.58			
miR-486	Study Day 1	-0.90 ± 0.51	-1.24 ± 0.34	0.56	0.19	0.25
	Study Day 84	-1.11 ± 0.49	-0.08 ± 0.55			

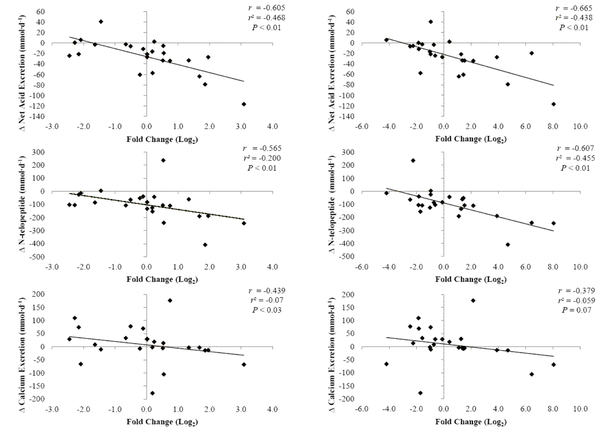
RESULTS continued

Fig 1. Fold change in miR-21 and miR-133b by treatment group



Values mean ± SEM. Data log transformed for normality
Statistical Analysis: Mixed Model Repeated Measures ANOVA with Bonferroni adjustment to determine pairwise comparisons
+Treatment Effect; $P = 0.05$
†Time-by-Treatment; $P = 0.07$
*Time-by-Treatment; $P = 0.02$

Fig 2. Associations between fold changes in miR-21 and miR-133b and 3-mo changes in UNAE, UNTX, and UCa



Values mean ± SEM. Data log transformed for normality
Statistical Analysis: Spearman rho's correlation coefficient

SUMMARY and CONCLUSIONS

- Reducing renal acid load by way of $KHCO_3$ supplementation at 81 mmol/d significantly increased expression of circulating miR-21 and miR-133b – miRs involved in osteogenesis (miR-21), osteoblast differentiation (miR-133b) and myogenesis (miR-133b).
- Furthermore, there were strong associations between increases in both miR-21 and 133b and decreases in bone resorption (NTX) and calcium excretion. Given that circulating miRs 21 and 133b have been positively associated with bone mineral density by DXA and/or inversely associated with osteoporotic fractures, the direction of change in these miRs in our study is consistent with potential beneficial effects on bone health.
- Although circulating miR-133b has a role in skeletal muscle, it was not associated with nitrogen excretion in this subgroup on self-selected diets.
- The broader significance and role of these circulating miRNAs as biomarkers of bone and muscle health are still under investigation and larger studies are needed to verify these preliminary results.