Title Page

Title: Dietary nitrate lowers ambulatory blood pressure in treated, uncontrolled hypertension: a 7d, double-blind, randomized, placebo-controlled, crossover trial.

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Summary conflict of interest statements: NONE.

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Running head: Dietary nitrate lowers blood pressure.

Keywords: dietary nitrate, nitrite, nitric oxide, hypertension, blood pressure.

Clinical trials: This study was registered at clinicaltrials.gov as NCT02597010.

Abbreviations list: ABPM, ambulatory blood pressure; BMI, body mass index; BP, blood pressure; BRJ, beetroot juice; CVD = cardiovascular disease; FMD, flow mediated dilation; HTN, hypertension; NO, nitric oxide; NOS, nitric oxide synthase.

Abstract

Dietary nitrate has been shown to increase nitrate/nitrite levels and decrease blood pressure (BP) in multiple populations. There are few reports among hypertensives and these reports have provided conflicting evidence. We aimed to assess the effect of daily nitrate compared to placebo in subjects with uncontrolled hypertension.

On day 0, hypertensives wore an ambulatory BP monitor (ABPM) for 24h and blood was taken. Subjects were then randomized to 7d nitrate-rich beetroot juice (12.9mmol nitrate) followed by 7d nitrate-depleted beetroot juice (0.5mmol nitrate) or vice versa. ABPM and blood was assessed before and after both conditions.

20 subjects with treated yet uncontrolled hypertension entered and completed the trial (mean age = 62.5y, mean BMI = 30.7kg/m^2). Baseline BP was 137/80 ± 7/7mmHg. Dietary nitrate was well tolerated and resulted in significantly increased plasma nitrite ($p=0.0004$) and decreased 24h SBP and DBP compared to placebo (-8mmHg; $p=0.012$ and -4mmHg; $p=0.018$ respectively).

Our results support the existing data suggesting an anti-hypertensive effect of dietary nitrate in treated yet uncontrolled hypertensives. Targeted dietary strategies appear promising contributors to BP control.

Hypertension (HTN) affects >one billion people. Despite advances, blood pressure (BP) remains uncontrolled in ~50% of individuals treated with medication[1,2]. There is a need for novel, effective therapies for primary and secondary prevention of HTN.

Nitric oxide (NO) is a major vasodilating molecule and plays a critical role in vascular homeostasis and BP regulation[3]. Reduced NO bioavailability, either through decreased production or increased consumption, has been associated with endothelial dysfunction and implicated in the development of prehypertension[4] and HTN[5]. Multiple studies have demonstrated that plasma and/or urinary NO metabolites are significantly lower in hypertensives compared to matched controls[6-11]. Additionally, NO levels correlate positively with vascular function[11] and peak brachial artery dilation[12], but negatively with BP[7,9]. Thus, the restoration of NO signalling provides an attractive mechanism to control HTN.

Dietary nitrate has been suggested to be an important contributor to NO signalling in humans, with the first human report of an anti-hypertensive effect in 2006. A 3d double-blind, crossover study of 0.1mmol/kg per day sodium nitrate resulted in a significant 3.7mmHg reduction in diastolic BP in young, healthy volunteers[13]. A 2008 study demonstrated marked hypotensive effect of nitrate-rich beetroot juice, (-10.4/8mmHg) which correlated with increased plasma nitrite[14].

Since then, research regarding dietary nitrate and CVD has increased. Indeed, several reviews have detailed the existing pre-clinical/clinical evidence as well as possible mechanisms for dietary nitrate and its potential role in BP regulation[15-19]. A 2013 systematic review and meta-analysis concluded that dietary nitrate can reduce systolic

BP by 4.4mmHg ($p < 0.001$) and diastolic BP by 1.1mmHg ($p = 0.06$)[16]. This and another review[17] noted that inverse associations between nitrate dose of SBP reduction ($r^2=0.45$; $p=0.033$). However, it is important to note that these reviews focused mostly on normotensive, healthy, normal-weight males in acute interventions and there is a lack of data regarding hypertensives.

Nonetheless, high habitual intake of dietary nitrate has been inversely associated with HTN risk[20,21]. Further, a proof of principal study in 15 untreated, grade 1 hypertensives demonstrated that a single nitrate dose (3.5mmol as beetroot juice), increased plasma nitrite by 150% and decreased 24h BP (-11.2/-9.6mmHg; $p<0.001$) as well as arterial stiffness compared to control ($p<0.05$)[22]. In a subsequent unblinded and uncontrolled, pilot study, we demonstrated that 14d dietary nitrate could increase serum NO in controlled and uncontrolled hypertensives but selectively lowered BP and arterial stiffness in uncontrolled hypertensives only[22]. However, recent RCTs of dietary nitrate in hypertensives provided inconsistent results. One of these RCTs demonstrated that 4w of 6.45mmol dietary nitrate significantly reduced BP[23] However, a subsequent crossover RCT reported no effect of one week of the same nitrate dose[24].

Considering these inconsistencies, we wanted to follow on from our pilot study and assess, in a more rigorous manner, the effect of 7d daily dietary nitrate on ambulatory BP, serum nitrate/nitrite, lipids and renal indices among treated but uncontrolled hypertensives.

Methods

Trial design

This was a 7d, double-blind, randomized, placebo-controlled, crossover trial to assess the effect of dietary nitrate. Subjects were tested on three separate occasions, baseline (day 1), midpoint, (day 8) and endpoint (day 15) – before and after each intervention period (Figure 1).

Study Participants

Subjects with known or suspected uncontrolled hypertension, established on diverse antihypertensive regimens, were recruited from specialist clinics and invited to wear an ABPM for 24h. We excluded subjects with controlled hypertension (<130/80mmHg) as well as those with kidney disease, diabetes, cognitive impairment, or sleep apnoea and those taking organic nitrates. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Research Ethics Committee of Connolly Hospital, Dublin, Ireland.

Intervention

This trial utilized nitrate-rich beetroot juice (NO_3-) and nitrate-depleted beetroot juice (placebo; PL) as previously described[26]. Independent testing and analysis of the NO_3- and PL were conducted previously[26] and the specific compositions are displayed in table 1.

A physician not involved in data gathering (LC) generated the allocation sequence, while a nutrition researcher enrolled subjects (CPK). After baseline assessments, subjects were randomized to consume 140ml NO_3 (12.9mmol nitrate) at ~9am each

154 morning for 7d followed by PL (0.5mmol nitrate) for 7d or the crossover condition. We
155 selected this dose as the nitrate content is attainable with a diet rich in vegetables[27].
156
157 During the trial, all subjects were provided with written and verbal instructions not to
158 alter behaviours which are known to influence NO kinetics, including dietary,
159 tobacco, alcohol, exercise or medication habits and not to use mouthwash or
160 antibiotics, which are known inhibitors of dietary nitrate reduction to nitrite.

161 Subjects took their 7th and final dose of each beverage 2-3h before their midpoint and
162 endpoint assessments. In this manner, the juice was most active during waking hours
163 and testing coincided with peak physiological and biochemical effect[23,28]. Compliance
164 with the juice was assessed with a daily diary.
165
166 **Outcome measures**
167 On all 3 testing days (days 1, 8 and 15) in an identical manner and at the same time of
168 day, non-fasting blood was drawn and subjects were fitted with an ambulatory BP
169 monitor for 24h as previously described[22].
170
171 **Lifestyle assessment**
172 Weekly dietary nitrate intake was estimated at each time-point using the 'Nitrate Veg
173 Table'[29]. This table asks subjects to report how many times in the previous week they
174 consumed foods rich in nitrate and provides a composite score for weekly nitrate intake.
175 At each time-point, we also asked detailed questions regarding the subjects exercise,
176 alcohol, medication, tobacco and alcohol habits.
177
178 **Biochemical analysis**

On testing days, venous blood samples were drawn into serum and plasma tubes which have a low nitrate/nitrite content before ABPM set up. Serum was analysed locally for routine lipid (total cholesterol, LDL, HDL) and renal (sodium, potassium, creatinine) parameters.

The half-life of NO is <1 second[30]. Therefore, direct determination of NO *in vivo* is difficult. However, measurement of NO metabolites (nitrate/nitrite) in biological fluids reflects NO bioavailability[31]. Therefore, the plasma tube was centrifuged at 4,000RPM and 4°C for 10m immediately after phlebotomy. Plasma was subsequently immediately extracted into Eppendorf cry vials, frozen at −80°C and later analysed for nitrate/nitrite with the current gold standard, ozone-based chemiluminescence analysis (NO analyser, NOA280i, Sievers) which is the most accurate and sensitive NO metabolite detection method as previously described[32].

Statistical methods

The primary outcome was 24h SBP. Our sample size was based on the primary outcome of mean 24h ambulatory BP. At $\alpha = 0.05$, we estimated that 19 participants would provide 80% power, assuming an SD of 3.8mmHg, to detect a 2.6mmHg difference in the mean 24h ambulatory BP based on our pilot study[23].

The difference between baseline and post-interventions was calculated for all variables, denoted as ΔNO_3- and ΔPL. Ambulatory blood pressures were analyzed by using mixed models in SAS 9.0 software by using the PROC MIXED command (SAS Institute Inc.). The subject was included as a random factor in each model. Fixed effects included the treatment (active or placebo) and the order of treatments to

account for a possible "carry over" effectThe statistical difference between ΔNO_3- and ΔPL for other outcome variables was calculated using paired t-tests. Results were expressed as mean ± standard deviation. All statistical tests were conducted at the two-sided 0.05 significance level.

Results

Study population

Of 93 subjects screened, 20 were recruited. There were no dropouts. Baseline characteristics are displayed in table 2. The cohort were mainly male (65%), Caucasian (90%), obese (mean BMI = 30kg/m^2) and most had history of CVD. All subjects were prescribed ≥1 antihypertensive medication. There were no reported adverse events. The juices were well tolerated and as reported previously[14,23,28], 14 subjects (70%) reported transient, red/pink urine (beetruria).

Lifestyle results

Throughout the trial, there was no reported change in prescribed medication, or habitual tobacco/alcohol/exercise habits between visits. Dietary nitrate intakes were constant on both a group and individual level at all 3 time-points (12.2 nitrate 'units' weekly.

Biochemical results

Plasma nitrite levels increased significantly after NO_3- (p=0.001) but not after PL (p=0.3) (table 3). There was no difference at any stage between lipid, or renal parameters (data not shown).

Ambulatory blood pressure results

Average ABPM wear time was similar at all 3 time-points with an average of >30 successful readings taken. Mean group values for 24h, day and night systolic and diastolic BP decreased after NO_3- (table 3). There was no observed treatment effect regarding either SBP or DBP (24h, day or night).

Discussion

Daily dietary nitrate for 7d was well tolerated and led to increased NO metabolites and reduced 24h and day BP. Our trial extends the growing literature regarding the hypotensive effect of dietary nitrate since we recruited highly selected individuals with uncontrolled hypertension but already established on anti-hypertensive medication. Further, we controlled for factors influencing NO, including diet, alcohol, smoking, exercise and medication.

We observed significant increases in plasma nitrite, which were greater here (126 to 732μM) than in our pilot study (100 to 175μM)[23]. This can be explained by the timing of phlebotomy. Here, non-fasting blood samples were taken 2-3h after subjects consumed juice, coinciding with peak NO metabolite bioavailability [14, 23,28].

We observed significant decreases in 24h (-8/-4mmHg) and day BP (-9/-4mmHg) profiles but no significant effect at night. Considering that all subjects consumed juice 2-3h before their morning clinic visit and the peak biochemical and physiological response to exogenous nitrate is reported at 2-3h[14, 23,28], the lack of effect at night is not surprising.

It is noteworthy that BP values decreased after PL as well as after NO_3-. It is possible that other bioactive components may affect BP. For example, antioxidants, flavonoids, anthyocianates, polyphenols, and betaine may upregulate NOS expression, decrease oxidative stress, and increase NO metabolite bioavailability.[33] These components and nitrate may have individual and synergistic additive effects, even at low doses.[33] Interestingly and importantly, if BP values hadn't decreased after PL, the statistical significance of the antihypertensive effect of NO_3- would have been even greater.

Despite the growing interest in dietary nitrate, there remains a lack of data regarding hypertensives. An acute unblinded, uncontrolled pilot study demonstrated that a single dose of dietary nitrate (3.5mmol) could increase plasma nitrite by 150% and decrease 24h BP (-11.2/-9.6mmHg; $p<0.001$)[22]. In a subsequent unblinded and uncontrolled, pilot study we demonstrated that 14d dietary nitrate increased serum NO metabolites in controlled and uncontrolled hypertensives but selectively lowered BP and arterial stiffness in uncontrolled hypertensives only[23]. However, two recent, well-conducted trials provided conflicting evidence. A double-blind, randomized, controlled trial of drug-treated (n=34) and drug-naïve (n=34) hypertensives demonstrated that 28d of 6.45mmol dietary nitrate significantly reduced clinic BP, home BP and 24h ABPM[24]. A subsequent randomized, placebo-controlled, double-blind crossover trial assessed 27 individuals with treated HTN after 7d of high-nitrate beetroot juice (6.45mmol) and 7d of nitrate-depleted beetroot juice (0.5mmol). Despite significant increases in nitrate/nitrite, there was no difference in home BP or 24h ABPM[25].

It is noteworthy that we utilized double the dose of nitrate in our trial compared to these trials (12.9 vs. 6.45mmol)[24,25]. We selected this dose as the nitrate content is attainable with a diet rich in vegetables[26]. For example the dietary approaches to stop hypertension (DASH), traditional Japanese, Mediterranean and vegetarian diets are all high in nitrate (~10-20mmol)[27,34,35]. Further, the nitrate content of these diets has been suggested to mediate the major cardio-protective effects[27,34,35]. National dietary data surveys show average daily nitrate intakes in the USA and Europe to be 0.5 – 3·0mmol/d[36,37], reflecting a processed diet in low in vegetables.

The inconsistencies may be due to differing dosing regimens, intervention periods and patient demographics. The authors suggest that use of anti-hypertensives may diminish the effect of exogenous nitrate. However, half of the subjects in the Kapil et al trial[23] were on anti-hypertensives as were our subjects here and in our pilot study[22]. A plausible explanation, which the authors mention[24] is that the baseline BP was quite well controlled and therefore an additional decrease is unlikely. Indeed, we observed as a similar effect in our pilot study where values in controlled hypertensives did not decrease[22]. According to a recent review, dietary nitrate appears to be most hypotensive in those with higher baseline BP[38]. We speculate that this may be due to crosstalk between endothelial NO synthase and the nitrate–nitrite–NO pathways, whereby beneficial effects of nitrate are likely to be more pronounced when NO synthase is compromised (e.g. HTN). Additionally, it has been demonstrated that the abundance and activity of a nitrite reductase enzyme is higher in those with higher BP[22]. Other factors likely to influence the effect of dietary nitrate include habitual dietary intake, baseline NO bioavailability as well as response to exogenous nitrate. Response to exogenous nitrate is complex and determined by oral microbiota, gastric pH, and oxygen tension among others[39].

Trial strengths

This was a double-blind, randomized, placebo-controlled crossover trial. The use of a placebo juice means that we could fully blind and randomize this trial. The use of ABPM provides a robust measure of BP. There were no dropouts and there was no change in multiple measured factors known to influence NO kinetics throughout the trial and no participant used antibiotics or mouthwash (both known inhibitors of nitrate reduction to nitrite) during the trial. Compliance with the BRJ was 100%.

Trial limitations

We included a small sample. However, considering the crossover design of our trial and the samples studied in previous nitrate studies combined with our sample size calculation, we feel that 20 well-characterized subjects can provide useful information in this context. This trial was of short duration (7d), designed to assess the efficacy and tolerability of daily dietary nitrate in uncontrolled hypertensives. We did not include a washout period as the pharmacokinetics of dietary nitrate reveal that biochemical and physiological effects of nitrate ingestion are absent after ~24h. Therefore, subjects completed the first arm of the study on day 8 and commenced the second arm of the study on day 9.

Conclusion

Our results suggest that dietary nitrate has anti-hypertensive effects among uncontrolled hypertensives in conjunction with increased NO metabolites. This effect is consistent with the majority of animal model, pre-clinical and clinical data. Future studies are required to ascertain the long-term effect of dietary nitrate on BP in humans, particularly in light of evidence that dietary nitrate appears more effective in those with higher baseline BP and/or those who are treatment naïve. This intriguing concept has potential implications for multiple vulnerable populations, for example those with resistant HTN and hypertensives who refuse/can't tolerate medication.

Financial support: This work was supported by the Irish Heart Foundation. The Irish Heart Foundation had no role in the design, analysis or writing of this article.

Conflict of interest: None

Authorship:

- CK made substantial contributions to study conception and design, data acquisition analysis and interpretation of data; has drafted the submitted article; has provided final approval of the version to be published; and has agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
- ED made substantial contributions to study conception and design and analysis/interpretation of data; has revised the submitted article critically for important intellectual content; has provided final approval of the version to be published.
- PJ made substantial contributions to biochemical analysis and analysis/interpretation of data; has revised the submitted article critically for important intellectual content; has provided final approval of the version to be published.
- LC made substantial contributions to study conception and design and analysis/interpretation of data; has revised the submitted article critically for important intellectual content; has provided final approval of the version to be published; and has agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Table 1. Composition of nitrate-depleted and nitrate-rich beetroot juice

	Nitrate-depleted beetroot juice	Nitrate-rich beetroot juice

Abbreviation	PL	NO_3-
Dose	140ml	140ml
Energy (kcal)	112.42	152.46
Carbohydrate (g)	24.64	32.186
Sugars (g)	24.32	30.646
Protein (g)	2.849	5.39
Fat (g)	0.308	0.308
SFA (g)	0.0077	0.0077
MUFA (g)	0.0077	0.00924
PUFA (g)	0.1386	0.1232
Sodium (mg)	139.986	177.1
Potassium (mg)	1041.04	1424.5
Calcium (mg)	13.244	12.705
Nitrate, mmol (mg)	0.19 (11.5)	12.9 (800)

Abbreviations: MUFA = monunsaturated fatty acids; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acids.

Table 2. Baseline characteristics

N=	20
Age (y)	62.5 ± 13.1

Male gender n (%)	13 (65)
BMI, kg/m^2 (range)	30.7 ± 5.8
Race n (%)	
Irish	18 (90)
Asian	1 (5)
African-American	1 (5)
Smoking status (*n*)	
Current smoker	0
Ex-smoker	8
LLNS	12
Years with HTN	7.5 ± 6.5
Baseline SBP	137 ± 7
Baseline DBP	80 ± 7
Pharmacology	
Average No. BP meds	2 ± 1 (1 to 4)
Aspirin *n*	6
Statin *n*	8
Baseline nitrate intake (units/week)	12.2 ± 7
Co-morbidities	
Cerebrovascular disease *n*	8
CAD *n*	3

Abbreviations: BMI = body mass index; CAD = coronary artery disease; HTN = hypertension; LLNS = lifelong non-smoker.

Table 3: Ambulatory blood pressure results

	Baseline	After PL	ΔPL	After NO_3-	ΔNO_3-	Differences in treatment groups (mmHg, 95% CI)	*P value
24h SBP	137 ± 7	133 ± 9	-4	129 ± 9	-8	-4.4 (-8.7 to 0.1)	0.044
24h DBP	80 ± 7	79 ± 8	-1	76 ± 8	-4	-3.2 (-6.1 to -0.3)	0.032
Day SBP	141 ± 8	138 ± 10	-3	132 ± 9	-9	-5.8 (-10.4 to -1.2)	0.016
Day DBP	83 ± 6	83 ± 9	0	79 ± 8	-4	-4.0 (-7.3 to -0.6)	0.021
Night SBP	130 ± 9	125 ± 12	-5	123 ± 11	-7	-2.1 (-8.2 to 3.9)	0.473
Night DBP	74 ± 8	71 ± 11	-3	70 ± 11	-4	-1.9 (-5.6 to 1.7)	0.284

All BP values are as mean (mmHg) ± SD
ΔPL = change in ABPM following PL compared to baseline
ΔNO_3- = change in ABPM following NO_3-compared to baseline
*P-values derived from mixed model analysis as described.
Abbreviations: ABP = ambulatory blood pressure; DBP = diastolic blood pressure; PL = placebo; SBP = systolic blood pressure.

Table 4: Biochemical results

	Baseline	After PL	ΔPL	After NO_3-	ΔNO_3-	*P-value
Nitrite (μM)	126 ± 115	131 ± 134	5	732 ± 653	581	0.0004
CRP	9.31	10.35	0.14	14		0.37
Bilirubin	13.8	14.8	-1	15.9	0.13	
ALT	38.5	32.3	-6.2	35.4	-3.1	0.1
ALP	86.6	82.5	-4.1	78.2	-8.4	0.24
GGT	55.9	55.2	-0.7	56.3	1.4	0.45
Total cholesterol	4.6	4.67	0.7	4.57	-0.03	0.25
LDL-C	2.5	2.54	0.4	2.34	-0.16	0.1
HDL-C	1.4	1.47	0.7	1.51	0.11	0.37
TAG	1.49	1.42	-0.07	1.42	-0.07	0.33
Na^+	140	140.3	0.3	140.2	0.2	0.19
K^+	4.7	4.9	0.2	5.1	0.4	0.24
Creatinine	76.9	78.9	2	77.1	0.2	0.13
Urate	331.4	323.8	-7.6	331.3	-0.1	0.26

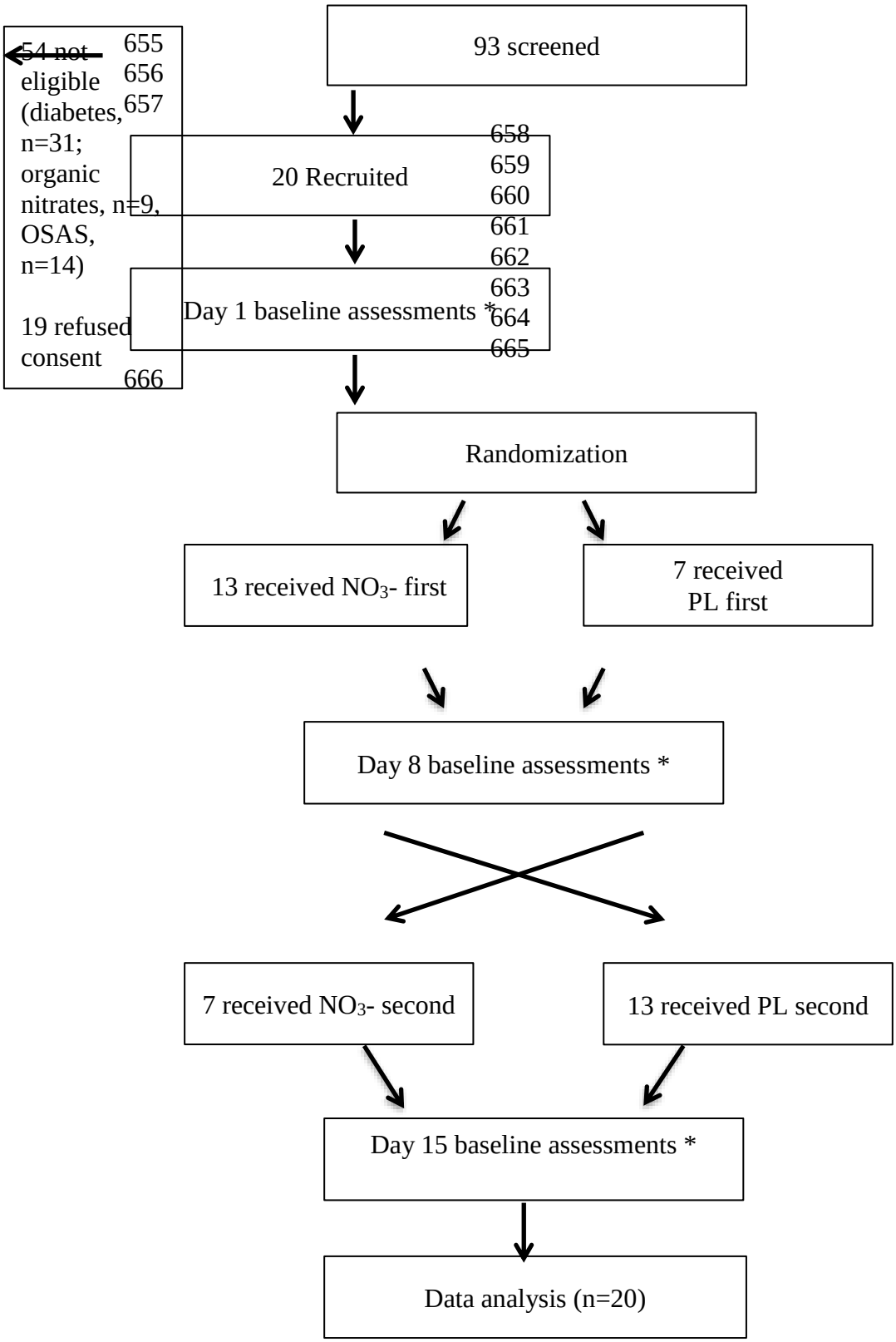
ΔPL = change following PL compared to baseline

ΔNO_3- = change following NO_3-compared to baseline;

*P-values derived from paired t-tests of ΔNO_3- vs. ΔPL

Abbreviations: ALP = Alkaline phosphatase**;** ALT = Alanine Aminotransferase**;** CRP = C reactive protein**;** GGT = Gamma-Glutamyl Transferase**;** HDL-C = high density lipoprotein**;** K^+= potassium**;** LDL-C = low density lipoprotein**;** Na^+ = sodium**;** NO_3- = nitrate rich beetroot juice; PL = placebo; TAG = Triglycerides

Figure 1: Trial design



*Assessments included 24h ABPM, blood draw and assessments of dietary nitrate intake.