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Nitrate-Rich Beetroot Juice Selectively Lowers Ambulatory Pressures and LDL Cholesterol in Uncontrolled but not Controlled Hypertension: a Pilot Study.

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Abstract:	Background Dietary nitrate has been shown to increase nitrate/nitrite levels in multiple populations, with potential blood pressure lowering effects. However, there are few reports among hypertensives. Aims We aimed to assess the effect of daily nitrate in subject with controlled hypertension vs. uncontrolled hypertension. Methods On day 0, hypertensives wore an ambulatory BP monitor (ABPM) for 24h and fasting blood was taken. Subjects then consumed concentrated beetroot juice (12.9mmol nitrate) for 14 consecutive days. On day 14 subjects consumed their last nitrate dose after fasting blood was drawn and again had an ABPM for 24h. Results According to baseline ABPM, 11 subjects had controlled BP while 8 had uncontrolled BP. There were similar, significant increases in serum nitrate/nitrite in both groups. We observed little change in BP variables among controlled hypertensives. However, there were reductions in BP variables in uncontrolled hypertensives where decreases in nighttime DBP (-6 ± 4.8mmHg), arterial stiffness (-0.08 ± 0.03 ambulatory arterial stiffness index) and LDL (-0.36 ± 0.42mmol/L) reached significance (p=003, 0.05 and 0.046 respectively). Conclusions Our results support the existing data suggesting an anti-hypertensive effect of nitrate-containing beetroot juice, but only among those with uncontrolled hypertension.

Abstract

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Aims

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According to baseline ABPM, 11 subjects had controlled BP while 8 had uncontrolled BP. There were similar, significant increases in serum nitrate/nitrite in both groups. We observed little change in BP variables among controlled hypertensives. However, there were reductions in BP variables in uncontrolled hypertensives where decreases in nighttime DBP (-6 ± 4.8mmHg), arterial stiffness (-0.08 ± 0.03 ambulatory arterial stiffness index) and LDL (-0.36 ± 0.42mmol/L) reached significance (p=003, 0.05 and 0.046 respectively).

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Title Page

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Abbreviations:

AASI = ambulatory arterial stiffness index;

ABPM = ambulatory blood pressure;

BMI = body mass index;

BP = blood pressure;

BRJ = beetroot juice;

FMD = flow mediated dilation;

HTN = hypertension;

NO = nitric oxide;

NOS = nitric oxide synthase.

Abstract

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Dietary nitrate has been shown to increase nitrate/nitrite levels in multiple populations, with potential blood pressure lowering effects. However, there are few reports among hypertensives.

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According to baseline ABPM, 11 subjects had controlled BP while 8 had uncontrolled BP. There were similar, significant increases in serum nitrate/nitrite in both groups. We observed little change in BP variables among controlled hypertensives. However, there were reductions in BP variables in uncontrolled hypertensives where decreases in nighttime DBP (-6 ± 4.8mmHg), arterial stiffness (-0.08 ± 0.03 ambulatory arterial stiffness index) and LDL (-0.36 ± 0.42mmol/L) reached significance (p=003, 0.05 and 0.046 respectively).

Conclusions

Our results support the existing data suggesting an anti-hypertensive effect of nitrate-containing beetroot juice, but only among those with uncontrolled hypertension.

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

Introduction

Nitric oxide (NO) is a pluripotent molecule with diverse systemic effects, including systemic vasodilation [1] and blood pressure (BP) regulation [2]. NO bioavailability is reflected by levels of its metabolites; nitrate and nitrite [3]. Multiple studies have shown that NO metabolites are significantly lower in hypertension (HTN) compared to matched controls [4-7]. Additionally, serum NO levels have been reported to correlate negatively with both systolic and diastolic BP [7] and depend on dietary intake in HTN and ischemic stroke [8]. Therefore, dietary interventions to increase either the bioavailability or bioactivity of NO may have clinical utility in HTN.

Until recently it was assumed that the only route for NO synthesis *in vivo* was via NO synthase (NOS) acting on its substrate, L-arginine. However, nitrite derived from dietary inorganic-nitrate has been shown to be a substrate for NOS-independent production of NO [9]. This involves both enzymatic and non-enzymatic reduction of nitrate to nitrite and to NO. Indeed, dietary nitrate has been shown to act as a precursor, in a dose-dependent manner, to nitrite and hence NO (10). Dietary nitrate has multiple cardioprotective effects as reviewed previously [11-13] and several authors have suggested that dietary nitrate is the major component responsible for the cardioprotective effect of vegetables [11, 13]. Further, a 2013 meta-analysis concluded that dietary nitrate can reduce systolic BP by 4.4mmHg ($p<0.001$) and diastolic BP by 1.1mmHg ($p=0.06$) among those without hypertension (14). There is a lack of data regarding dietary nitrate supplementation among hypertensives. However, two recent randomized, double-blind, placebo-controlled trials of nitrate supplementation among hypertensives have emerged but 24h ABPM results were conflicting [15,16].

In this proof of concept study, we wanted to assess the effect of 14d dietary nitrate on ambulatory BP, arterial stiffness, serum nitrate/nitrite, lipids as well as renal and liver indices among treated hypertensives. We further wanted to assess any differences between controlled hypertensives and uncontrolled hypertensives as well as any potential adverse outcomes.

Materials and Methods

Study design

During this uncontrolled, pilot study, subjects were tested on two separate occasions, baseline (day 1) and endpoint (day 15) (Fig. 1). On both days, in an identical manner and at the same time of day, fasting blood was drawn, the subject was fitted with an ambulatory blood pressure monitor (ABPM) for 24h and demographics, including body mass index as well as habitual dietary, exercise, smoking, alcohol and medication habits were recorded.

Study participants

We conducted an uncontrolled, pilot study of daily nitrate supplementation in clinically stable, Caucasian hypertensive outpatients, established on diverse antihypertensive regimens. We excluded subjects with kidney disease or diabetes and those on organic nitrates.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved the Human Research Ethics Committee of Connolly Hospital, Dublin. Informed consent was obtained from all individual participants included in the study.

Beetroot juice

All subjects were asked to consume 140ml beetroot juice (BRJ) daily for 14 consecutive days. We selected this dose (12.9mmol nitrate) as the nitrate content is attainable with a diet rich in vegetables [17]. During the trial all subjects were provided with written and verbal instructions not to alter dietary, tobacco, alcohol,

exercise or medication habits and not to use mouthwash, which is a known inhibitor of dietary nitrate reduction to nitrate [18]. Subjects took their 14th and final dose of BRJ after their second fasted blood sample, while wearing the ABPM for a second occasion. Compliance with the BRJ was assessed with a daily diary.

Outcome measures

Twenty four hour ambulatory blood pressure measurement

24h ABPM was performed on day 1 and day 15 using the non-invasive ABPM Spacelabs 90207 machine (Spacelabs Healthcare Ltd., Issaquah, WA, USA). For each assessment, the ABPM was fitted on the upper left arm by a researcher (CPK) in the morning after 10 minutes of quiet rest.

An initial BP reading was taken in the clinic and subjects were asked to return to the clinic wearing the ABPM 24h later. The device was programmed to record BP every 30mins between the hours of 07.00 and 23.00 and every 60mins from 23.00 to 07.00. Participants were advised that they could carry out their usual activities but to avoid strenuous exercise. For each BP measurement, volunteers were asked to hang their arm loosely down the side of their body while keeping still until the end of the measurement. If active, the volunteers were instructed to stop and remain stationary while the measurement was being recorded. Proprietary software was used to download readings and produce 24h, daytime (0700–2300 hours) and nighttime (23:00– 07:00 hours) mean BP readings (90256 ABP Report Management System; Spacelabs Healthcare). We defined uncontrolled HTN as either baseline 24h SBP >130mmHg or 24h DBP >80mmHg as recorded with APBM and controlled BP as <130/80mmHg [19]. Ambulatory arterial stiffness index (AASI) was derived from

individual 24h ABPM whereby, regression slope of diastolic BP on systolic BP was computed. AASI was defined as 1 minus the regression slope, as previously described [21].

Biochemical analysis

On day 1 and 15, two fasting, venous blood samples were drawn into serum tubes (Sarstedt Monovette Serum Z) which have a low nitrate/nitrite content before ABPM set up. One tube was analyzed locally in our clinical laboratory for routine lipid parameters (total cholesterol, LDL, HDL), liver (albumin, calcium, urea) and renal (sodium, potassium, creatinine) parameters.

The second tube was centrifuged at 4,000RPM and 4°C for 10m immediately after phlebotomy. Serum was subsequently extracted into Eppendorf tubes and frozen at −80°C and later analyzed for NO metabolites (nitrate/nitrite), which reflect NO bioavailability [3] as previously described [20].

Statistical methods

For this pilot study we did not perform a power calculation. Paired t-tests were used to compare baseline and post BRJ values for each group while unpaired t-tests were used to compare controlled and uncontrolled HTN. Results were expressed as mean ± standard deviation. All statistical tests were conducted at the two-sided 0.05 significance level using SAS (version 9.0).

Results

Study population

Of the 55 subjects we screened, twenty subjects were recruited. There was a single drop out (female, 61y, BMI=33.6kg/m^2) due to acute complication of an underlying pulmonary issue (unrelated to study). The baseline characteristics of the 19 subjects who completed this pilot study are displayed in table 1. Both groups were composed mainly of males. There was no difference in baseline BMI but the uncontrolled group were significantly younger (49 vs. 60.9y; p=0.023). There was little difference in anti-hypertensive use among the groups (1.5 agents per subject in controlled vs. 1.1 agents per subject in uncontrolled). Throughout the study, there was no reported change in prescribed medication, or habitual diet/exercise between visits.

Table 1: Baseline characteristics

	Controlled (n 11)		Uncontrolled (n 8)		P-value
	Mean	SD	Mean	SD	
Age (years)	60.9	9.1	49.3	14.5	0.023
Male gender n (%)	10 (91)		7 (88)		-
BMI (kg/m^2)	30.8	2.4	29.5	6.0	0.3
Weight category*					
Healthy range *n* (%)	0 (0)		2 (25)		-
Overweight *n* (%)	3 (27)		3 (38)		-
Obese *n* (%)	8 (73)		3 (38)		-
Smoking status (*n*)					
Current smoker	0		2 (25)		-
Ex-smoker	7 (64)		4 (50)		-
LLNS	4 (36)		2 (25)		-
Baseline systolic BP	124	14.0	136	11.0	0.035
Baseline diastolic BP	73	9.0	89	8.0	0.0004
Average No. BP meds	1.5		1.1		-
Aspirin *n*	2		4		-
Statin *n*	4		4		-
Co-morbidities					
Cerebrovascular disease *n*	4		1		-
Coronary artery disease *n*	2		2		-
Hypercholesterolaemia *n*	4		2		-

BP, blood pressure; LLNS, life-long non-smoker

Biochemistry

Baseline serum nitrate was 116% higher in the uncontrolled group compared to the controlled group but this was not significant (23.6 vs. 50.9μM; p=0.09). In contrast, baseline serum nitrite was 14% lower in the in the uncontrolled group compared to the controlled group. Again this was not significant (98.2 vs. 86.2nM; p=0.25) (table 2).

Table 2: Biochemical indices before and after nitrate supplementation (BRJ)

	Controlled				Uncontrolled				
	Pre-BRJ	Post-BRJ	Absolute change	* P-value	Pre-BRJ	Post-BRJ	Absolute change	* P-value	# P-value
Nitrate (µM)	23.6	181.6	158	0.0018	50.9	155.3	104.4	0.016	0.38
Nitrite (nM)	98.2	174.3	76.1	0.011	86.2	163.7	77.5	0.007	0.89

BRJ, beetroot juice
* p-values are derived from paired t-tests of the absolute change within each group
# p-values derived from unpaired t-tests of the absolute changes between groups.

Fasting serum nitrate increased by 770% and 310% in the controlled and uncontrolled groups respectively. Similarly, serum nitrite increased 177% and 190% in the controlled and uncontrolled groups respectively. However, these differences did not reach statistical significance (table 2). Mean LDL concentrations decreased in 7 of 8 subjects in the uncontrolled group (and this reduction was significant compared to the controlled group (3.35 ± 0.47 to 2.99 ± 0.64 vs 2.14 ± 0.67 to 2.47 ± 0.87; p=0.023). There were no significant differences in any other lipid, liver or renal parameters within or between groups (data not shown).

Ambulatory blood pressure

Average ABPM wear time was 23.3h at baseline and 22.8 at endpoint, with a mean of 34.5 successful readings taken per subject at both baseline and endpoint. Mean group values for 24h, day and night systolic and diastolic BP as well as ambulatory arterial stiffness index (AASI) are displayed in table 3. Consumption of dietary nitrate was not associated with decreased 24h, day or night BP in the controlled hypertensives. In contrast after nitrate consumption, there were reductions in night BP and arterial stiffness in the uncontrolled hypertensives (table 3).

Table 3: Ambulatory blood pressure monitor results

	Controlled				Uncontrolled				
	Pre-BRJ	Post-BRJ	Absolute change	* P-value	Pre-BRJ	Post-BRJ	Absolute change	* P-value	# P-value
24h SBP	123.7 ± 13.7	126.8 ±16.6	3.1	0.2	135.7	133.1	-2.6	0.29	0.09
24h DBP	72.3 ± 8.7	75 ± 10.7	2.7	0.078	88.8	86.1	-2.7	0.15	0.026
Day SBP	126.2 ± 13.7	127.5 ± 17.6	1.3	0.42	136.4	136.1	-0.3	0.48	0.33
Day DBP	74.5 ± 9.4	75.7 ± 10	1.2	0.39	89.8	88.9	-1	0.42	0.2
Night SBP	118.2 ± 15.4	121.1 ± 15.8	2.9	0.37	125.8	119	-6.8	0.11	0.07
Night DBP	66.5 ± 9.1	72.1 ± 12.8	5.6	0.08	80.3	74.3	-6	0.03	0.0058
Night SBP max	134.8 ± 20	139.6 ± 19	4.8	0.37	145.3 ± 9.6	135 ± 11.6	-10.3	0.1	0.064
Night DBP max	79.5 ± 20.4	85.4 ± 17	5.9	0.18	94.5 ± 6.3	88.1 ± 13.3	-6.4	0.26	0.083
AASI	0.43 ± 0.2	0.48 ± 0.18	0.05	0.13	0.42 ± 0.13	0.34 ± 0.11	-0.08	0.05	0.1
%SBP Dipping	6.2 ± 9.1	4 ± 11.1	-2.2	0.64	9.2 ± 4	13.7 ± 5.3	4.5	0.13	0.21
%DBP Dipping	11 ± 8.1	5.1 ± 12.8	-5.9	0.21	11.7 ± 6.6	17.5 ± 8	5.8	0.09	0.04

AASI, ambulatory arterial stiffness index; BRJ, beetroot juice; DBP, diastolic blood pressure; SBP, systolic blood pressure.
* p-values are derived from paired t-tests of the absolute change within each group.
# p-values derived from unpaired t-tests of the absolute changes between groups.

Adverse events

There were no reported adverse events. The BRJ was well tolerated and as reported previously [16], 8 subjects (38%) reported transient, red/pink urine (beetruria).

Discussion

In this proof of concept study, we demonstrate that daily, nitrate-rich beetroot juice for 14d led to increased NO bioavailability in both controlled and uncontrolled HTN and reductions night BP, AASI as well as LDL cholesterol in uncontrolled hypertensives only. Further, the intervention was well-tolerated, safe and did not lead to excessive BP lowering in controlled HTN.

Nitrate-rich beetroot juice was associated with significant increases in both serum nitrate and nitrite in controlled and uncontrolled hypertensives. These increases were less than observed in previous, acute studies utilizing the same dose of nitrate [16,17]. In this context it is important to note that we collected fasting blood samples and therefore the BRJ would have been consumed 12-24h prior to blood collection. Considering the peak increase in plasma nitrate and nitrite due to exogenous nitrate occurs 2-3h following ingestion [22] it is understandable that the increases in serum nitrate and nitrite we observed were blunted compared to previous studies. It is noteworthy that all subjects had fasting blood samples taken on both day 1 and 15 and were then fitted with the ABPM before consuming BRJ – therefore ABPM recordings were conducted while nitrate was bioactive.

Here, we observed decreases in BP profiles in uncontrolled hypertensives only. The effects were most apparent in nighttime DBP ($p=0.03$), DBP dipping ($p=0.09$). Although, other variables did not reach statistical significance, this proof of concept study included a small sample and was not powered to detect statistically significant results. In this context, our observations are clinically significant. Since the discovery that dietary nitrate can increase NO bioavailability, much research has focused on its

anti-hypertensive potential. A 2013 meta-analysis concluded that dietary nitrate can reduce systolic BP by 4.4mmHg ($p<0.001$) and diastolic BP by 1.1mmHg ($p=0.06$) in those without hypertension [14]. Despite much recent interest in the anti-hypertensive effect of dietary nitrate, most of the trials in this meta-analysis were of short duration (2h to 15d) and assessed young, healthy adults. There is a lack of interventional data among hypertensives. A 2013 pilot study, provided evidence that dietary nitrate was a plausible antihypertensive agent. In a small cohort of untreated, stage 1 hypertensives exogenous nitrate increased plasma nitrite 150% and this was associated with decreases in mean 24h in systolic- (-11.2mmHg; $p<0.001$) and diastolic-blood pressure (-9.6mmHg; $p<0.001$) [23]. Two recent, well-conducted trials provided conflicting evidence, one demonstrating benefit [15] and another, no effect [16]. These differences may be due to differing dosing regimens, intervention periods and patient demographics including BMI, age and medications. Our results suggest that any reduction of BP in treated hypertensives may be greatest among those with higher initial BP and after ≥7d of nitrate dosing. This observation is consistent with previous studies [15, 16, 24].

Plasma nitrite reflects flow mediated dilation (FMD) [25] and through its bioactivation to NO is recognized to be a critical pathway regulating basal vascular tone, arterial stiffness and BP [25, 26]. Therefore altering plasma nitrite, including by dietary means, has potential to affect endothelial function and arterial stiffness. In addition to BP reduction, we also observed a significant reduction in arterial stiffness ($p=0.05$). Previous randomized, controlled crossover trials have demonstrated that dietary nitrate can significantly decrease arterial stiffness and significantly improve endothelial function in healthy subjects acutely (2-6 hours) in conjunction with

significantly increased NO metabolites [22-31] These studies utilized nitrate doses varying from 1.1-22.5mmol. Further, 20 healthy overweight/ slightly obese men were randomized to a high fat meal with nitrate-rich BRJ (8.1mmol nitrate) or nitrate-depleted BRJ. Postprandial impairment in FMD was improved after the nitrate-rich BRJ compared with placebo (-0.37% vs -1.56%; p=0.03) [31]. Further, the effect appears to be maintained as evidenced by longer trials (7d) [30]. However, 7d of a high-nitrate diet (4.84mmol nitrate/day from green leafy vegetables) compared to a low-nitrate diet did not affect multiple BP variables or arterial stiffness among 38 middle aged adults with high–normal BP (SBP=120-139mmHg) [32]. Nevertheless, a double-blind, randomized, controlled trial of drug-treated (n=34) and drug-naïve (n=34) hypertensives demonstrated that 28d of 6.4mmol nitrate improved endothelial function (FMD) by ~20% (P<0.001) and reduced arterial stiffness (as assessed by pulse wave velocity) by 0.59 m/s (0.24-0.93; P<0.01) [15].

We also demonstrate for the first time that 14d dietary nitrate significantly decreased serum LDL among 8 subjects with uncontrolled HTN. According to the Third Report of The National Cholesterol Education Program [33], baseline LDL levels in the uncontrolled hypertensives were in the 'borderline high' category. After 14d dietary nitrate, LDL levels were 'near optimal/above optimal' category (p=0.046). This reduction was specific to the uncontrolled HTN group and was significant compared to controlled hypertensives (p=0.023). This observation is interesting, particularly in light of our small sample size, short intervention period and considering there was no change in diet, exercise or medications, including antihyperlipidaemic medications. We cannot however, rule out a type one error, particularly because we are not aware of any human intervention study involving provision of dietary nitrate which reported

cholesterol or its subfractions. In this context, it is interesting that decreased basal plasma nitrate/nitrite level has been reported in hypercholesterolemic subjects with suspected coronary artery disease but not in normocholesterolemic subjects [34]. Hypercholesterolemia may reduce the NO bioavailability and several explanations have been offered for this, including decreased availability of L-arginine, the substrate for NOS [35]; decreased synthesis of NO through degeneration of endothelial G-protein or G-protein-dependent pathways [36] and reduced expression of endothelial NOS [37, 38]. Further, plasma nitrate/nitrite levels have previously been reported to correlate negatively with both total cholesterol ($r = -0.40$, $p<0.01$) and LDL cholesterol levels ($r=-0.37$, $p<0.003$) [34]. Interestingly statins, widely prescribed for their cholesterol lowering properties activate endothelial NOS [39]. Further, there is evidence that 8 weeks of dietary nitrate (100 mg/L in drinking water) reduced LDL cholesterol in normal (1.12 to 0.75mmol/L; $p<0.05$) and diabetic rats (1.12 to 0.46mmol/L; $p<0.05$) compared to normal and diabetic rats without nitrate [40]. Although, our study cannot provide mechanistic insight for the LDL reductions, our data provide preliminary evidence for the first time that dietary nitrate reduces LDL levels in uncontrolled hypertensive patients. This observation is consistent with preliminary research suggesting that nitrate targets a novel pathway to enhance fat metabolism and/or energy utilization [41] and decreases lipid levels in animal models [40-42].

NO has multiple roles in cardio-metabolic regulation. Several comprehensive reviews have highlighted the diverse cardioprotective effects of dietary nitrate [11-13]. Some authors have even suggested that dietary nitrate is the major component responsible for the cardioprotective effect of vegetables [11, 12]. It has been demonstrated that

dietary nitrate acts as a precursor to NO in a dose-dependent mannerwhereby a single serving of a nitrate-rich vegetables contains more nitrate than what is formed endogenously by the all three NOS isoforms combined in 24h [9]. Dietary nitrate increases vasodilation as well as inhibiting production of mitochondrial reactive oxygen species and platelet aggregation. Despite the complex nature of NO and the multiple contributors to NO bioavailability (e.g. underlying pathology, medication use, serum lipids, tobacco exposure, exercise, alcohol intake), diet has been shown to be the major influencer of serum NO in patients with HTN and ischemic stroke [8]. In this context, our results and those of others should not be considering surprising.

This trial has several key strengths. The use of 24h ABPM provides a robust, reliable method of determining BP. We asked subjects to maintain their typical dietary, exercise, alcohol, tobacco and medication habits throughout this study. Therefore our observations closely reflect that effect of supplementary nitrate to the everyday lives of hypertensives. The increases in serum nitrate and nitrite confirmed compliance with the intervention.

This pilot study did not include a control arm or a placebo. Therefore, it is possible that any observed effect was simply regression to the mean. It is also possible that any effect observed here may be due to non-nitrate components of beetroot juice. However, emerging trials utilizing nitrate-rich beetroot juice and identical, nitrate-depleted beetroot juice have demonstrated no physiological effect of nitrate-depleted beetroot juice. Further, our results are consistent with a recent meta-analysis [14] and a double-blin, randomized, placebo-controlled trial [15]. Therefore we suggest that our observations are due to dietary nitrate. The number of participants in our study (n

= 19) may be considered small. However, many previous studies investigating chronic nitrate intake on BP had similar numbers [16, 24].

In this pilot study, we observed significant decreases in night DBP, AASI and LDL cholesterol in conjunction with increased serum NO metabolites. These effects were confined to subjects with uncontrolled BP, suggesting that the physiological effects of exogenous nitrate may be greatest in these patients. Considering the conflicting data in the area, our pilot results should be confirmed with well-designed trials, particularly regarding LDL.

Ethical approval:

"All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

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