Doku-Abschnitt 3

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BRIEF REPORT

Levodopa-Entacapone-Carbidopa Intestinal Gel in Parkinson's Disease: A Randomized Crossover Study

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Abstract

Background: The addition of oral entacapone to levodopa-carbidopa intestinal gel treatment leads to less conversion of levodopa to 3-O-methyldopa, thereby increasing levodopa plasma concentration. The objective of this study was to compare systemic levodopa exposure of the newly developed levodopa-entacapone-carbidopa intestinal gel after a 20% dose reduction with levodopa exposure after the usual levodopa-carbidopa intestinal gel dose in a randomized crossover trial in advanced Parkinson's disease patients.

Methods: In this 48-hour study, 11 patients treated with levodopa-carbidopa intestinal gel were randomized to a treatment sequence. Blood samples were drawn at prespecified times, and patient motor function was assessed according to the treatment response scale.

Results: Systemic exposure of levodopa did not differ significantly between treatments (ratio, 1.10 [95% confidence interval, 0.951-1.17]). Treatment response scale scores did not significantly differ between treatments ($P = 0.84$).

Conclusions: Levodopa-entacapone-carbidopa intestinal gel allowed a lower amount of levodopa administration and was well tolerated. Long-term studies are needed to confirm the results. © 2016 International Parkinson and Movement Disorder Society.

Key Words: Parkinson's disease; clinical trials; randomized; levodopa infusion; pharmacotherapy

Levodopa-carbidopa intestinal gel (LCIG; Duodopa/Duopa; AbbVie, Chicago, IL) is an effective treatment developed for patients with advanced Parkinson's disease (PD).[1] It provides a stable levodopa plasma concentration compared with oral administration because of continuous infusion into the duodenum/jejunum by a portable pump.

Addition of the cathecol-O-methyltransferase (COMT) inhibitor entacapone, blocking levodopa's second-largest pathway, leads to less levodopa conversion to 3-O-methyldopa (3-OMD), thereby increasing the levodopa plasma concentration.[2,3] Currently, only orally administered entacapone is available.[4]

Levodopa-entacapone-carbidopa intestinal gel (LECIG; LECIGon; LobSor Pharmaceuticals AB, Knivsta, Sweden) is a newly developed formulation for intestinal infusion.

The primary objective of this trial was to compare the systemic levodopa exposure between hours 0-14 ($AUC_{0\text{-}14}$) after continuous infusion of LECIG and conventional LCIG in a crossover study in advanced PD patients.

Methods

Inclusion Criteria

All patients with idiopathic PD currently on a stable LCIG treatment (<125 mL per day) for a minimum of 30 days who were aged 30 years or older with a body mass index (BMI) between 17 and 31 and had not been exposed to entacapone within 3 months of screening were eligible for inclusion. The exclusion criterion was increased fluctuation of PD symptoms within 7 days prior to screening. For additional information, see NCT02448914.

Medication

The study product, LECIG (levodopa [20 mg/mL], entacapone [20 mg/mL], and carbidopa monohydrate [5 mg/mL]; LECIGon; LobSor Pharmaceutical AB, Knivsta, Sweden), was administered via the same gastrojejunostomy tube as used for LCIG infusion.[5,6] LECIG is contained in 50-mL syringes attached to an infusion pump (Cane, Italy), together measuring

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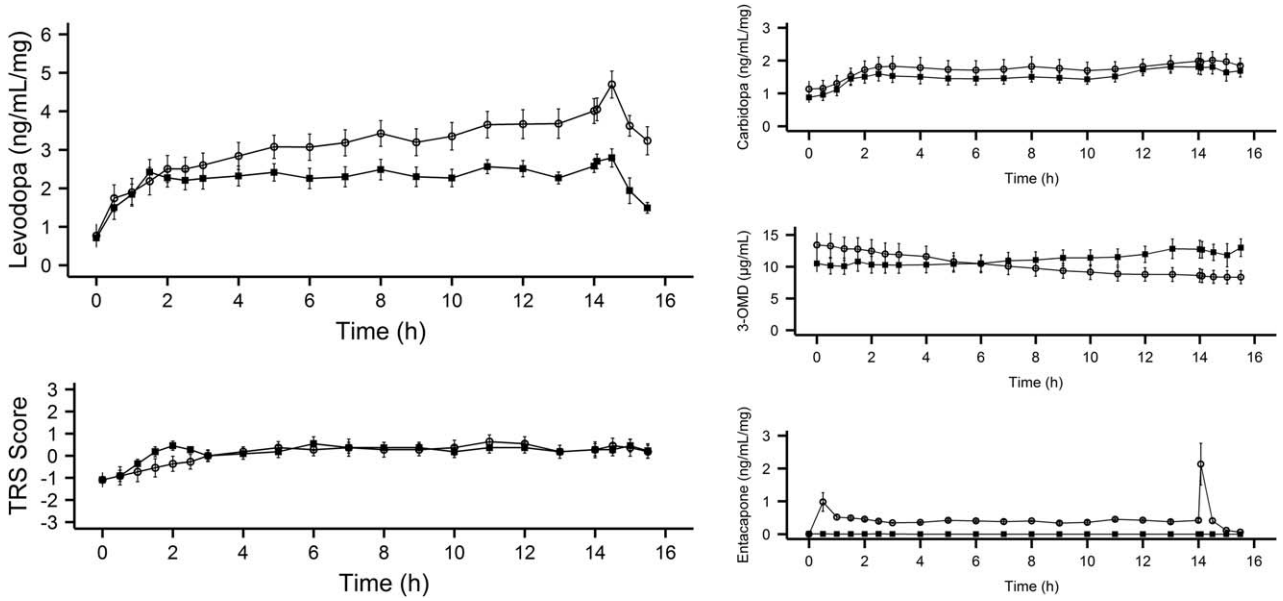

FIG. 1. Pharmacokinetic mean (±SE) dose-adjusted plasma concentrations (0-15.5 h) of levodopa (n = 11), carbidopa (n = 11), 3-O-methyldopa (n = 11) and entacapone (n = 6), and mean TRS score. Filled squares = levodopa/carbidopa infusion (LCIG), open circles = levodopa/entacapone/carbidopa infusion (LECIG); TRS: treatment response scale.

55 × 150 mm (Fig. 1). The weight of the LECIG pump is 139 g, and a full syringe weighs 88 g, resulting in a total weight of 227 g.

The reference product was LCIG (levodopa [20 mg/mL] and carbidopa monohydrate [5 mg/mL]; Duodopa; AbbVie Ltd, Chicago, IL). The LCIG pump and cassette (100 mL) measured 100 × 197 mm, and the weight of the LCIG pump system with a full cassette is approximately 550 g.

Study Design and Intervention

This randomized, open-label, 2-day crossover clinical trial was conducted at the Clinical Trial Consultants AB (CTC) center at Uppsala University Hospital between May and July 2015. The local Uppsala Ethical Review Board in Sweden approved the study, and all patients provided written informed consent.

Prior to the study start, a set of closed randomization envelopes were sent to CTC. The allocation ratio was 1:1.

Patients were randomized to receive 1 of 2 treatment sequences, LECIG/LCIG or LCIG/LECIG, over 2 consecutive days. Patients received LECIG morning doses corresponding to 80% (n = 5) or 90% (n = 6) of their individual morning dose of LCIG, 80% of the LCIG maintenance dose, and 80% of extra doses. The LECIG dose reduction was based on a previous study.[3] The duration of the infusions was 14 hours, and after the infusion stopped, the tube was flushed with water, as is needed with both treatments. Study nurses changed the syringe/cassette during the study and weighed them before and after the infusion. Oral levodopa-carbidopa immediate-release tablets were allowed after the infusion stopped and until 3 hours before the infusion started. Standardized low-protein meals were served at predefined times.

Pharmacokinetic Sampling and Motor Function Assessment

Blood samples on days 1 and 2 were drawn immediately prior to dosing, half-hourly between 0 and 3 hours, and hourly between 3 and 14 hours. A blood sample was collected within 5 minutes after flushing and then half-hourly between 14.5 and 17 hours.

During the interim analysis it was found that the entacapone was degraded by the stabilizer (sodium metabisulfite) used in the blood collection tubes; subsequently the blood samples were collected in 2 separate tubes, one with and one without stabilizer.

Trained study nurses assessed patient motor function according to the treatment response scale (TRS) at the same times as the pharmacokinetic sampling. The TRS is a 7-point scale ranging from -3 (severe parkinsonism) to 0 ("on" state without dyskinesia) to + 3 ("on" state with severe choreatic dyskinesia).[7]

Outcomes

The primary outcome was to compare the systemic exposure ($AUC_{0\text{-}14\,h}$) of levodopa after continuous infusion of LECIG and LCIG. Additional outcomes included TRS scores and safety and pharmacokinetics of levodopa, carbidopa, 3-OMD, and entacapone.

Safety Assessment

The patients were monitored for adverse events throughout the study.

TABLE 1. Pharmacokinetic parameters of LCIG and LECIG during 0 to 14 hours; mean (SD) values (n = 11)

	Treatment			
	LCIG	LECIG	*P*	Ratio LECIG/LCIG (95% CI)
Levodopa				
$AUC_{0\text{-}14}$[a] (ng·h/mL)	35,479.1 (14,693.0)	39,016.1 (17,327.6)	0.27	1.10 (0.95-1.17)
$AUC_{0\text{-}14/dose}$ (ng·h/mL)/mg	31.9 (9.4)	42.7 (14.1)	0.00013	1.34 (1.19-1.45)
C_{max}[a] (ng/mL)	3269.0 (1140.4)	3668.0 (1481.1)	0.089	1.12 (0.98-1.19)
Carbidopa				
$AUC_{0\text{-}14}$[a] (ng·h/mL)	5950.1 (3236.3)	5582.4 (3605.3)	0.03	0. 938 (0.815-0.990)
$AUC_{0\text{-}14/dose}$ (ng·h/mL)/mg	20.9 (7.7)	24.1 (11.3)	0.03	1.15 (1.02-1.22)
C_{max}[a] (ng/mL)	559.3 (292.4)	498.1 (297.8)	0.02	0.89 (0.79-0.98)
3-OMD				
$AUC_{0\text{-}14}$[a] (ng·h/mL)	154,714.1(56,931.0)	145,745.7 (61182.8)	0.21	0.94 (0.79-1.01)
$AUC_{0\text{-}14/dose}$ (ng·h/mL)/mg	—	—		
C_{max}[a] (ng/mL)	13,281.8 (4861.0)	13,518.2 (6116.2)	0.74	1.02 (0.82-1.15)
Entacapone				
$AUC_{0\text{-}14}$[a] (ng·h/mL)	—	5205.9 (1073.7)		
$AUC_{0\text{-}14/dose}$ (ng·h/mL)/mg	—	5.6 (1.1)		
C_{max}[a] (ng/mL)	0.03[b]	935.3 (550.9)		

[a]Results are presented as mean values (SD) for C_{max}, $AUC_{0\text{-}14}$, and CV.
[b]Geometric mean,

Statistical Analysis

A sample size of 15 patients was initially calculated for the study. A blinded interim analysis for sample size recalculation was done by estimating the coefficient of variation for $AUC_{0\text{-}14/dose}$ on the paired patient data. Based on this, a sample size of 11 patients was calculated.

All pharmacokinetic and statistical analyses were performed in R 3.2.2.[8] The following pharmacokinetic parameters for the analytes were estimated with the ncappc-package[9]: maximum concentration in plasma (C_{max}) and area under the plasma concentration-time curve between the hours 0 and 14 ($AUC_{0\text{-}14}$), using the trapezoid rule. For levodopa, carbidopa, and entacapone, the dose-adjusted $AUC_{0\text{-}14}$ ($AUC_{0\text{-}14/dose}$) was calculated by dividing $AUC_{0\text{-}14}$ with the total administered dose between the hours 0 and 14. Statistical comparison of $AUC_{0\text{-}14}$ and C_{max} for levodopa, carbidopa, and 3-OMD and $AUC_{0\text{-}14/dose}$ for levodopa and carbidopa was conducted with the paired, 2-tailed Student *t* test on the logarithmic values, with back-transformation to nominal values of point estimates and the 95% confidence interval (CI). The statistical comparison of the ordinal mean TRS scores was done with Wilcoxon's signed rank test.

Bioanalytical Assay

The blood sample analyses were conducted by OnTarget Chemistry, Uppsala, Sweden (at SVA laboratories), using the ultra-performance liquid chromatography/mass spectrometry method, validated in accordance with the Guideline on Bioanalytical Method Validation.[10]

Results

Patient Characteristics

Enrollment was conducted by the investigator. Of 12 screened patients 1 did not meet the inclusion criteria because of a too-high BMI. The 11 patients included had a mean age ± SD of 71.2 ± 4.1 years with 14 ± 5.1 years since diagnosis and a BMI of 23.8 ± 1.9 (Supplemental Table e-1). All 11 patients completed the study.

Pharmacokinetics and Motor Function

Systemic exposure ($AUC_{0\text{-}14}$) for levodopa did not significantly differ between treatments, but the dose-adjusted levodopa exposure ($AUC_{0\text{-}14/dose}$) was found to be significantly higher during LECIG administration compared with LCIG (Fig. 1, Table 1). Six patients had a 40% or higher increase in levodopa systemic exposure, whereas 3 patients had the expected 20% increase, and 2 patients did not reach the target systemic exposure. An incline in the levodopa LECIG plasma concentration profile during the day was observed. When increasing the morning dose of LECIG from 80% (n = 5) to 90% (n = 6), the initial levodopa plasma concentration-time profile mimicked the LCIG profile more closely (Supplemental e-Figure).

As expected, the mean $AUC_{0\text{-}14}$ for carbidopa was significantly lower with LECIG; however, $AUC_{0\text{-}14/dose}$ for carbidopa was found to be significantly higher. The 3-OMD concentration decreased during LECIG

administration. Three of 4 patients randomized to receive LCIG on day 2 had low but detectible plasma concentrations of entacapone left during the first hours of LCIG administration; maximum measured concentration was 0.03 μg/mL.

Mean TRS scores did not differ significantly between treatments ($P = 0.84$).

Adverse Events/Safety and Tolerability

Six adverse events (AEs) were reported by 2 patients (18%) after LCIG administration, and 10 adverse events were reported by 6 patients (55%) after LECIG administration. Headache was reported by 1 patient after administration of LCIG and 3 patients after LECIG administration. Five unique adverse events in 3 patients were assessed as related to study drug: nausea (1 event after each treatment), diarrhea (after LCIG administration), and dizziness and headache (both occurring after LECIG administration). All AEs were mild.

No serious or severe AEs were reported, and no AEs led to discontinuation or change in therapy. No clinically significant changes in vital signs, electrocardiograms, or physical examinations occurred.

Discussion

This is the first clinical trial performed with the levodopa-entacapone-carbidopa intestinal gel (LECIG). The results suggest that the required levodopa dose can be successfully reduced with LECIG without lowering levodopa exposure. Possible differences in COMT activity between individuals, seen as a higher-than-expected increase in $AUC_{0\text{-}14/dose}$, may have contributed to the higher variability in levodopa exposure after LECIG treatment.[11] The undesired levodopa plasma concentration increase during the day suggests that the dose for some patients could be decreased more than 20%, for example, by decreasing the flow rate of the maintenance dose in the afternoon. Mean TRS scores propose that LECIG can provide therapeutically effective plasma concentrations despite the dose reduction. The peak concentration observed after flushing the tube may be a challenge for patients who are susceptible to developing dyskinesia with small changes in dose.[12] It was observed to be most pronounced for entacapone because of its small central volume of distribution (0.08 ± 0.03 L/kg).[13]

Carbidopa has previously been reported to be a COMT substrate in vitro,[14] which may partly explain the observed higher dose-adjusted carbidopa AUC.

Entacapone displayed its coveted effect while decreasing 3-OMD plasma concentration during the LECIG treatment. The formation of 3-OMD requires methyl groups, which is hypothesized to be a part of a cascade of events that may lead to the development of neuropathy.[15] Speculatively, COMT inhibition may thus reduce the risk of this side effect.[16,17]

The reported AEs in this trial are common with both LCIG and oral administration of levodopa products and entacapone; however, this trial was short and with a small number of patients, which limited the possibility to detect rare and long-term side effects. However, entacapone is a well-established drug with known side effects and has been on the market for more than a decade, ensuring a thorough safety profile.

The main positive outcome from this treatment is the reduction in levodopa dose and 3-OMD concentration without the addition of oral entacapone several times daily. The patient population with the most to gain from this treatment is patients who have previously obtained good treatment effect and tolerability from oral entacapone.

A possibly similar effect may be achieved with opicapone, a newly developed COMT inhibitor with the advantage of once-daily administration, which has been found to be noninferior to entacapone.[18] The combination LCIG and opicapone could be a possible alternative to LECIG, but this needs to be investigated further.

Because of the short treatment time, conclusions have to be drawn with caution, and long-term comparative efficacy studies are needed to confirm the results and investigate the possible long-term side effects with the addition of entacapone. However, the present clinical trial indicates that LECIG may offer adequate therapeutic levodopa exposure at a lower dose, using a smaller pump, compared with LCIG. ■

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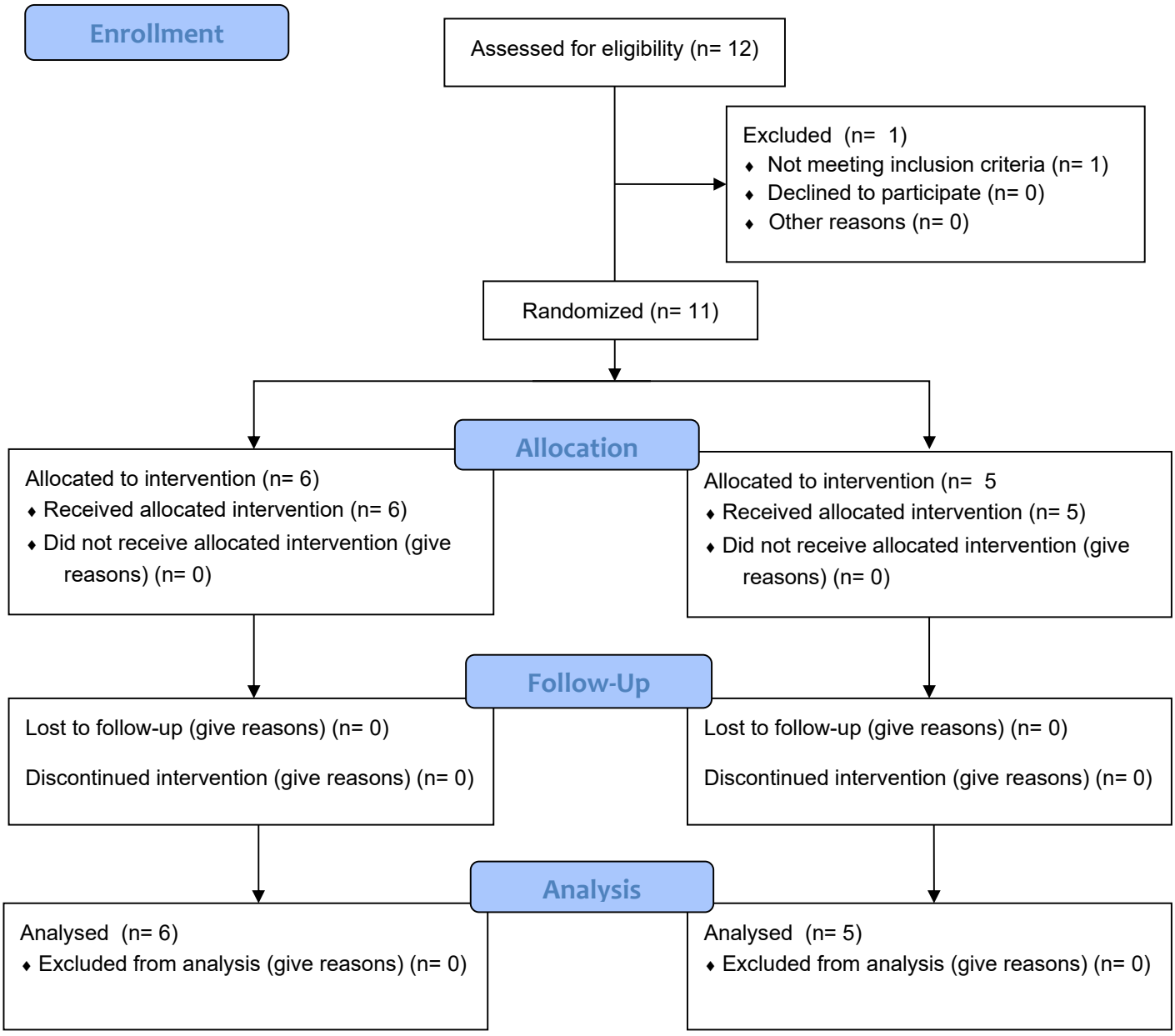
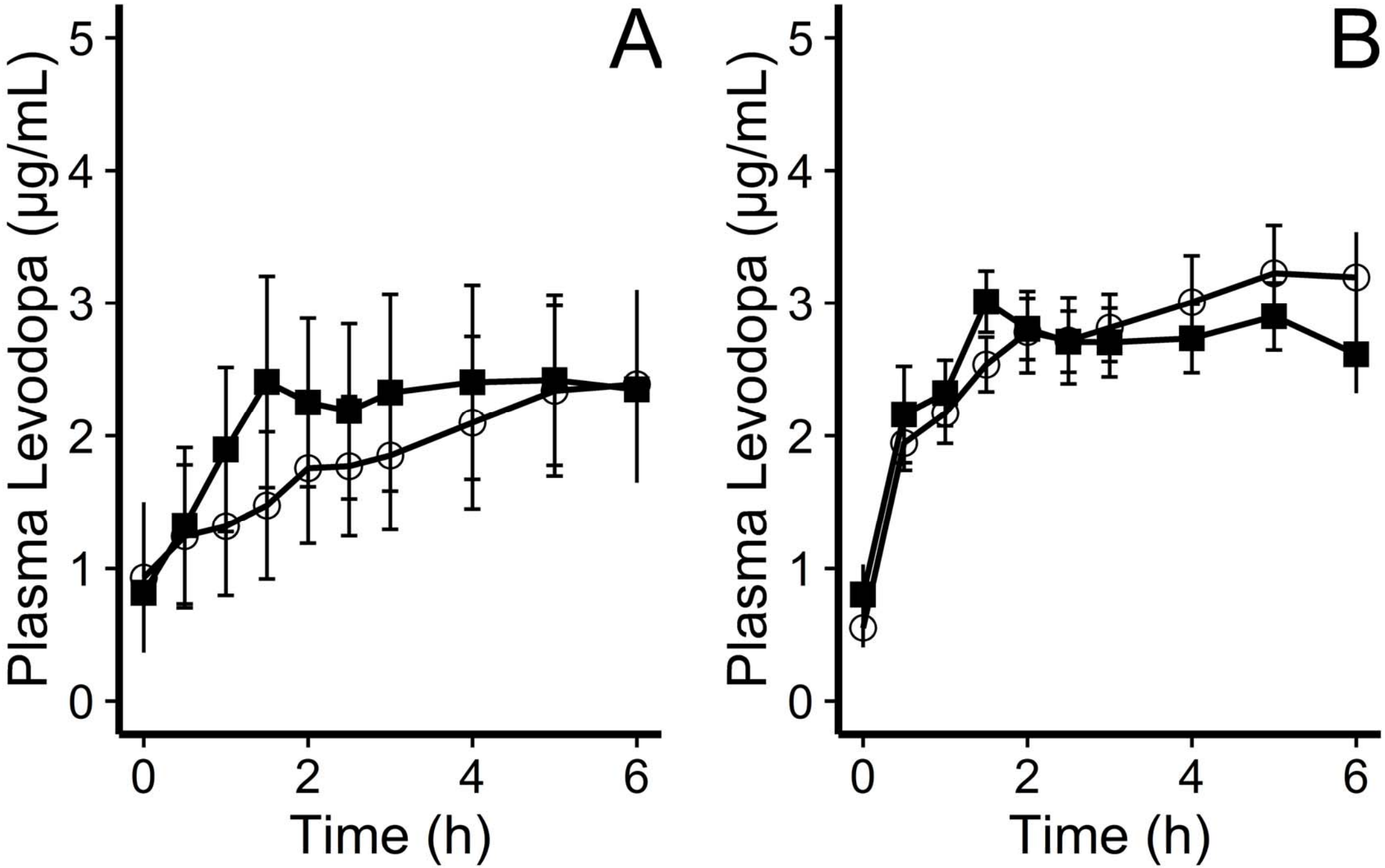
Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

CONSORT
TRANSPARENT REPORTING of TRIALS

CONSORT 2010 Flow Diagram




A
B
Plasma Levodopa (µg/mL)
Time (h)
0
1
2
3
4
5
0
2
4
6

Anhang

Studie NCT02448914: Ein- und Ausschlusskriterien

Fundstelle: https://clinicaltrials.gov/ct2/show/study/NCT02448914

Für die Studie in Frage kommende Altersgruppen: 30 Jahre und älter (Erwachsene, Ältere Erwachsene)
Geschlechter, die für die Studie in Frage kommen: Alle
Nimmt gesunde Freiwillige an: Nein

Einschlusskriterien:

- Bereit und in der Lage, eine informierte Zustimmung zu geben und vom Prüfarzt als entscheidungsfähig beurteilt
- Fortgeschrittene, auf Levodopa ansprechende idiopathische Parkinson-Krankheit, die seit mindestens 30 Tagen mit einer Duodopa-Infusion behandelt wird
- 30 Jahre alt oder älter
- BMI zwischen 17,0 und 31,0 kg/m2, jeweils einschließlich
- Einverständnis zur Anwendung adäquater kontrazeptiver Maßnahmen:

- Weibliche Patienten, die seit mehr als einem Jahr postmenopausal sind oder weibliche Patienten im gebärfähigen Alter, die während der Studie eine hocheffiziente Verhütungsmethode anwenden (d. h. eine Methode mit einer Versagensrate von weniger als 1 % [z. B. Sterilisation, Hormonimplantate, Hormonspritzen, einige Intrauterinpessare oder vasektomierter Partner]). Orale Kontrazeptiva in Kombination mit anderen Verhütungsmitteln werden akzeptiert.

- Männliche Patienten, die vasektomiert sind oder der Verwendung von Kondomen während der Studie zustimmen und eine Partnerin haben, die eine hocheffiziente Verhütungsmethode wie oben beschrieben anwendet.

Ausschlusskriterien:

- Überempfindlichkeit oder Allergie gegen das Prüfpräparat (IMP) oder gegen chemisch verwandte Produkte
- Kontraindikationen für die Anwendung von Levodopa oder Carbidopa oder Entacapon
- Bedarf an einer täglichen Gesamtdosis von Duodopa während der Studienteilnahme von mehr als 125 mL
- Erhöhte Fluktuation der klinischen Parkinson-Symptome innerhalb von 7 Tagen vor dem Screening
- Verabreichung eines Prüfpräparats innerhalb von 3 Monaten vor der Screening-Untersuchung und/oder aktuelle Teilnahme an einer anderen klinischen Studie mit einem Arzneimittel oder einem Medizinprodukt der Klasse III
- Verwendung einer verbotenen Medikation gemäß Abschnitt 9.6 des Prüfplans
- Bekannte Hepatitis B, Hepatitis C oder HIV-Infektion
- Blut- oder Plasmaspende oder größerer Blutverlust (≥500 mL) innerhalb von 3 Monaten vor dem Screening
- Positiver Urindrogentest (Amphetamin, Benzodiazepine, Tetrahydrocannabinol, Kokain oder Opiate) beim Screening
- Bekannter Alkoholmissbrauch
- Unwilligkeit, die Anforderungen des Protokolls zu erfüllen
- Andere medizinische oder soziale Gründe für den Ausschluss nach Ermessen des Prüfarztes

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Population pharmacokinetics of levodopa gel infusion in Parkinson's disease: effects of entacapone infusion and genetic polymorphism

M. Senek[1,2], D. Nyholm[1]✉ & E. I. Nielsen[2]

Levodopa-entacapone-carbidopa intestinal gel (LECIG) provides continuous drug delivery through intrajejunal infusion. The aim of this study was to characterize the population pharmacokinetics of levodopa following LECIG and levodopa-carbidopa intestinal gel (LCIG) infusion to investigate suitable translation of dose from LCIG to LECIG treatment, and the impact of common variations in the dopa-decarboxylase (DDC) and catechol-O-methyltransferase (COMT) genes on levodopa pharmacokinetics. A non-linear mixed-effects model of levodopa pharmacokinetics was developed using plasma concentration data from a double-blind, cross-over study of LCIG compared with LECIG in patients with advanced Parkinson's disease (n = 11). All patients were genotyped for rs4680 (polymorphism of the COMT gene), rs921451 and rs3837091 (polymorphisms of the DDC gene). The final model was a one compartment model with a high fixed absorption rate constant, and a first order elimination, with estimated apparent clearances (CL/F), of 27.9 L/h/70 kg for LCIG versus 17.5 L/h/70 kg for LECIG, and apparent volume of distribution of 74.4 L/70 kg. Our results thus suggest that the continuous maintenance dose of LECIG, on a population level, should be decreased by approximately 35%, to achieve similar drug exposure as with LCIG. An effect from entacapone was identified on all individuals, regardless of COMT rs4680 genotype. The individuals with higher DDC and COMT enzyme activity showed tendencies towards higher levodopa CL/F. The simultaneous administration of entacapone to LCIG administration results in a 36.5% lower apparent levodopa clearance, and there is a need for lower continuous maintenance doses, regardless of patients' COMT genotype.

Levodopa/carbidopa intestinal gel (LCIG) is a treatment developed for patients with advanced Parkinson's disease (PD) when oral treatment fails to provide sufficient stability in symptom relief[1]. Continuous infusion of drug, resulting in a more stable plasma concentration, stabilizes the symptom fluctuations (on–off phenomenon) as well as decreases the time with dyskinesia (levodopa-related involuntary movements)[2]. The levodopa/entacapone/carbidopa intestinal gel (LECIG) is a gel with the addition of entacapone[3]. Entacapone is a reversible inhibitor of catechol-O-methyltransferase (COMT), the enzyme responsible for the second major metabolic pathway of levodopa. The addition of carbidopa causes inhibition of dopa decarboxylase (DDC), which is the enzyme responsible for the largest part of levodopa's metabolism. The addition of entacapone has shown to allow lower levodopa dose administration through the inhibition of COMT, thus increasing levodopa plasma concentrations[3].

The drug-containing gel is infused directly into the small intestine, via a gastrojejunostomy tube, bypassing the stomach, and is thereby not affected by gastric emptying, which usually has a negative and erratic impact on levodopa absorption. The infusion treatment, most commonly administered only during day-time, consists of a morning bolus dose and a continuous maintenance infusion. The morning bolus dose is administered at the highest pump rate (40 mL/h) to allow levodopa to rapidly reach therapeutic plasma concentrations. When initializing LCIG treatment, the doses are based on the patients' previous oral levodopa morning dose, and total daily dose. Patients can also administer small bolus doses (extra doses) during the day, if needed.

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	Age (years)	Duration PD (years)	Duration LCIG (years)	Body weight (kg)	LCIG formulation		LECIG formulation	
					Morning dose (mg) n = 10[a]	Maintenance dose (mg)	Morning dose (mg) n = 10[a]	Maintenance dose (mg)
Mean (SD)	70 (4)	16 (4.8)	2.7 (2.7)	74 (15)	131 (56)	969 (277)	120 (49)	772 (226)
Median	70	14	1.3	73	130	1048	122	824
Min, max	63, 76	8, 23	0.2, 7.6	51, 99	41, 217	363, 1367	41, 198	279, 1107

Table 1. Patient characteristics, n = 11 (male n = 7, female n = 4). [a]One patient did not have a morning dose prescribed. SD, standard deviation; PD, Parkinson's disease; LCIG, levodopa-carbidopa intestinal gel; LECIG, levodopa-entacapone-carbidopa intestinal gel.

A previous pilot study was conducted where oral entacapone (200 mg, every 5 h) was added to LCIG treatment. With a 20% decrease in LCIG dose with the COMT inhibitor, the plasma concentrations at steady state (0.5–8 h) did not differ compared to LCIG administered alone without dose adjustment[4]. In a clinical trial investigating the infusion of LECIG using a 20% reduction of morning and maintenance infusion doses, the morning levodopa plasma concentrations were found to be lower than following infusion of LCIG, and there was a trend towards an accumulation in levodopa concentrations throughout the day[3]. This may result in insufficient symptom relief in the morning and an increased risk of dyskinesia in the latter part of the day. The LCIG treatment was highly individualized, with morning and continuous maintenance doses to meet individual patient needs. The patients were also allowed to administer extra doses if needed, which complicates the data analysis when using conventional area-based methods.

It was also observed that not all patients had the same increase in levodopa plasma concentration with the new treatment[3], and it was hypothesized that a reason for this could be differences in enzyme activity. Genetic variations in the gene encoding for the enzyme COMT (rs4680), and in the DDC promoter gene (rs921451 and rs3837091) have been suggested to affect the natural activity and/or expression of the respective enzymes, which in turn may affect the pharmacokinetics of levodopa[5,6]. Hypothetically, an individual with e.g. high COMT activity may benefit the more from the addition of entacapone, and polymorphism related to DDC might be correlated to the effect of carbidopa on levodopa pharmacokinetics.

The aim of this analysis was to investigate the impact of simultaneous entacapone infusion on levodopa pharmacokinetics using a model-based approach to provide a translation of dose from LCIG to LECIG treatment, based on previously published data[3], and to investigate the effect on levodopa pharmacokinetics by genotypes of the DDC and COMT genes.

Methods

Study population. Eleven patients were included in a randomized, open-label, 2-day crossover clinical trial (Table 1)[3]. The local Ethical Review Board in Uppsala, Sweden and the Swedish Medical Products Agency approved the study, and all patients provided written informed consent. All research was performed in accordance with relevant guidelines/regulations. For two consecutive days, patients were randomized to receive one of two treatment sequences, LECIG/LCIG or LCIG/LECIG. LECIG morning doses corresponded to 80% (n = 5) or 90% (n = 6) of their LCIG morning dose, 80% of the LCIG continuous maintenance dose, and 80% of the dose for extra bolus administrations. The treatment duration was 14 h, at which point the tube was immediately flushed with water, as is required with both treatments. When flushed, the gel left in the tube, approximately 3 mL, is infused. This volume corresponds to 60/15 mg of levodopa/carbidopa (LCIG) and 60/60/15 mg of levodopa/entacapone/carbidopa (LECIG). Oral levodopa-carbidopa immediate-release tablets were allowed as night-time medication after infusion stop and until 3 h before the infusion start. During the study, low-protein meals were served at hour 1, 4, 7, 10 and 13 after infusion start. The mean (min, max) of protein in grams at each time point was 8.8 (5.7,12), 10.8 (9.3,12), 2.1 (2.0,2.3), 10.3 (8.9,11), 5.4 (3.0, 6.3) day 1 and 8.8 (5.7,12), 10.8 (9.3,12), 2.1 (2.0,2.3), 9.9 (5.8,11), 5.3 (3.0, 6.0) day 2.

Blood samples were drawn immediately prior to dosing, half-hourly between 0 and 3 h, and hourly between 3 and 14 h. A blood sample was collected within 5 min after flushing and then half-hourly between 14.5 and 17 h.

Sequence variations of DDC and COMT genes. All patients that were included in the study submitted a blood sample for genotyping of DDC and COMT polymorphism, after providing written informed consent. Genomic DNA was extracted from the blood samples and the single nucleotide polymorphisms (SNP) rs4680 ($COMT_{SNP}$) and rs921451 (DDC_{SNP}) were analyzed by allelic discrimination TaqMan assay. The SNP in the COMT gene (rs4680)[5,7] results in the substitution of A > G, which causes the conversion of the enzyme valine (158Val, higher activity) to methionine (158Met, lower activity). The 158Val allele is associated with a higher enzymatic activity of COMT. The SNP in the DDC gene (rs921451)[6,8] results in a nucleotide substitution of T > C, which is associated with lower expression and/or activity. For identification of the DDC gene (rs3837091) polymorphism (DDC_{INSDEL}), the Sanger sequencing method was used, and the amplicons were compared to a GenBank-reference sequence. The polymorphism (rs3837091)[7] is characterized by a 4-base pair deletion (AGAG), which may cause lower expression and/or activity of DDC. For each patient, one control for each genotype was analyzed. Any difference in CL/F for the DDC_{SNP}, DDC_{INSDEL} and $COMT_{SNP}$ were graphically explored, based on empirical Bayes estimates.

Model development. *Base model.* Initially, a population pharmacokinetic model was developed with shared parameters for both treatments. Thereafter, differences in parameter estimates were successively investigated, to evaluate the impact of simultaneous entacapone infusion. One and two compartment disposition models with first order absorption were evaluated, parameterized in terms of absorption rate (ka), relative bioavailability (Frel), apparent volume of central (V_C/F) and peripheral (V_P/F) compartment, apparent clearance (CL/F) and inter-compartmental clearance (Q/F). Inter-individual variability was included assuming a log-normal distribution of structural model parameters. Bodyweight was included as a primary covariate on all disposition parameters according to the allometric power model, with allometric power exponents or 0.75 for CL/F and 1 for V/F[9]. Oral levodopa-carbidopa tablets were allowed as night time medication during the study, but only until 3 h before morning dose. Eight individuals took night-time medication 01:10–05:50 h after stop of LECIG administration and 01:43–04:28 h after stop of LCIG, and very few blood samples were collected in relation to the oral treatment. Thus, the information available was too sparse to allow for estimation of the absorption related parameters for the oral levodopa treatment. Therefore, based on a previously published levodopa pharmacokinetic model where oral levodopa-carbidopa tablet administration was compared to LCIG[10], the absorption model for oral treatment was described with a single transfer rate constant fixed to 2.4 h^{-1} with one transit compartment between the depot and central compartment, and a relative difference in Frel of 1.03. Since number of levodopa measurements below the limit of quantification was low (1.9%) these samples were handled using the M6 method[11], where LOQ/2 is assigned to the first value and subsequent samples below LOQ were ignored. The difference in levodopa parameters for LECIG were investigated as a relative difference in the estimate of CL/F, ka and F_{rel} compared to LCIG. The effect of food intake was explored both as a binary variable (yes/no), and as a continuous variable reflecting the amount of protein intake, that was assumed to decrease the drug absorption during an estimated period following food intake. For investigation of dosing regimens, a simulation dataset was created with the same number of individuals and the same demographic characteristics as the individuals included in the model development dataset. The model was used to simulate 1000 datasets, where individuals were dosed with either the same or altered dose regimens.

Data analysis and model evaluation. The population pharmacokinetic model was developed using the non-linear mixed effects modelling software NONMEM[12] (version 7.3; Icon Development Solutions, Ellicott City, MD, USA, 2009) with the first order conditional estimation method with INTERACTION (FOCEI) and a user-defined model (ADVAN13 NONMEM Subroutine). PsN[13] (version 4.7.0; Department of Pharmaceutical Biosciences, Uppsala University) was used for running models.

Parameter precision, scientific plausibility, goodness-of fit plots, prediction corrected visual predictive checks (pcVPCs)[14], and the objective function value (OFV) were used for model evaluation during the model development process. The OFV (approximates − 2 log(likelihood) of the data given the model) was utilized in likelihood ratio testing (LRT) to compare nested models (a ΔOFV of 3.84 for 1 degree of freedom, corresponding to a significance level of 0.05 was used). R[15] (version 3.4.2; R Foundation for Statistical Computing) was used for data management and Xpose[13] (version 4.6.0; Department of Pharmaceutical Biosciences, Uppsala University) was used for graphical evaluation. Parameter uncertainty of model parameters was assessed with the Sampling Importance Resampling (SIR) procedure[16]. The adequacy of the final model was evaluated using pcVPCs with 1000 replicates of the observed data.

Ethics approval. The local Ethics Review Board in Uppsala, Sweden and the Swedish Medical Products Agency approved the clinical trial. The genotyping part of the study was separately approved by the Ethics Review Board. All patients provided written informed consent.

Results

The final population pharmacokinetic model was a one-compartment model parameterized in terms of ka, Frel, Vc/F and CL/F. The estimated absorption rate constant (ka) for both treatments was very high, and was therefore fixed to 50 h^{-1}, corresponding to the lowest value which did not give a significant increase in OFV. Estimation of a two compartment model resulted in an OFV drop of 23, however the distribution phase was estimated to be very fast and the model became unstable with high uncertainty on the estimated parameters. Inter-individual variability was explored on all parameters, and found to be significant on CL/F and Vc/F. Addition of an inter-individual variability on relative bioavailability resulted an OFV drop of 5.85, but was associated with a high relative standard error (190%) and model instability, and was therefore not retained in the model. The model improved, with a difference in OFV of − 436, when the effect of entacapone was estimated as a shift in the typical value of levodopa CL/F, including an inter-individual variability in the shift parameter. The population parameter for CL/F was estimated to 27.9 L/h/70 kg for LCIG and to be 36.5% lower for LECIG, with associated inter-individual variabilities of 28% and 11%, respectively. All final model parameter estimates are given in Table 2.

The pcVPC, showing the observed and model predicted levodopa plasma concentration normalized for the variability in the independent variables, stratified on treatment is shown in Fig. 1. The observed plasma concentrations are in general well predicted by the model for both treatments.

The developed population levodopa pharmacokinetic model, which describes the time course of drug exposure in patients, was used to simulate alternative dose regimens for LECIG. In the scenarios, both morning bolus dose and continuous maintenance dose were altered. The infusion period simulated was 14 h. The scenarios included no dose adjustment (i.e. 0% lower morning dose and maintenance dose); 20% lower morning and maintenance dose and; 0% lower morning dose with a 35% lower continuous maintenance dose, compared to LCIG. Figure 2 shows a comparison of the levodopa plasma concentration of the three LECIG scenarios compared to LCIG administration. The levodopa plasma concentration is displayed as the median and the 10th and

Parameter	Point estimates (%RSE)[b] [% Shrinkage]	SIR (%RSE)[b] [95% CI]
CL/F_{LCIG} (L/h/70 kg)	27.9 (7.31)	28.1 (5.82) [25.1; 31.5]
$CL/F_{LECIG,Shift}$[a]	− 0.365 (5.24)	− 0.364 (4.48) [-0.391; − 0.328]
V_C/F (L/70 kg)	74.5 (7.60)	75.0 (8.60) [63.3; 87.8]
ka (h^{-1})	50 FIX	–
ktr_{oral} (h^{-1})	2.4 FIX	–
$F_{rel,LCIG/LECIG}$	1 FIX	–
$F_{rel,oral}$	1.03 FIX	–
$IIV_{CL/F,LCIG}$	27.9 (19.8) [1E−10]	28.6 (14.8) [21.2; 36.2]
$IIV_{CL/F,LECIG,Shift}$[a]	11.4 (23.5) [22.6]	12.0 (30.1) [4.49; 17.9]
IIV_{VC}	34.4 (17.0) [0.264]	35.6 (17.2) [24.2; 45.7]
Proportional error (%)	11.0 (27.4)	11.1 (8.96) [3.24; 13.1]
Addititive error (µg/mL)	0.316 (10.2)	0.316 (6.14) [0.278; 0.354]

Table 2. Parameter estimates for the final population pharmacokinetic model of LCIG and LECIG, and results from the SIR evaluation. [a]Shift in CL/F for LECIG, $CL/F_i = TVCL/F_{LCIG} \times e^{CL,LCIG} \times \left(\frac{Weight}{70}\right)^{0.75} \times \left(1 + TVCL_{LECIG,Shift} \times e^{CL,LECIG,Shift}\right)$. [b]NONMEM point estimate and the associated % relative standard error (% RSE, reported on the approximate standard deviation scale (SE/variance estimate)/2). CI, confidence interval; IIV, inter-individual variability (CV%). SIR, sampling importance resampling.

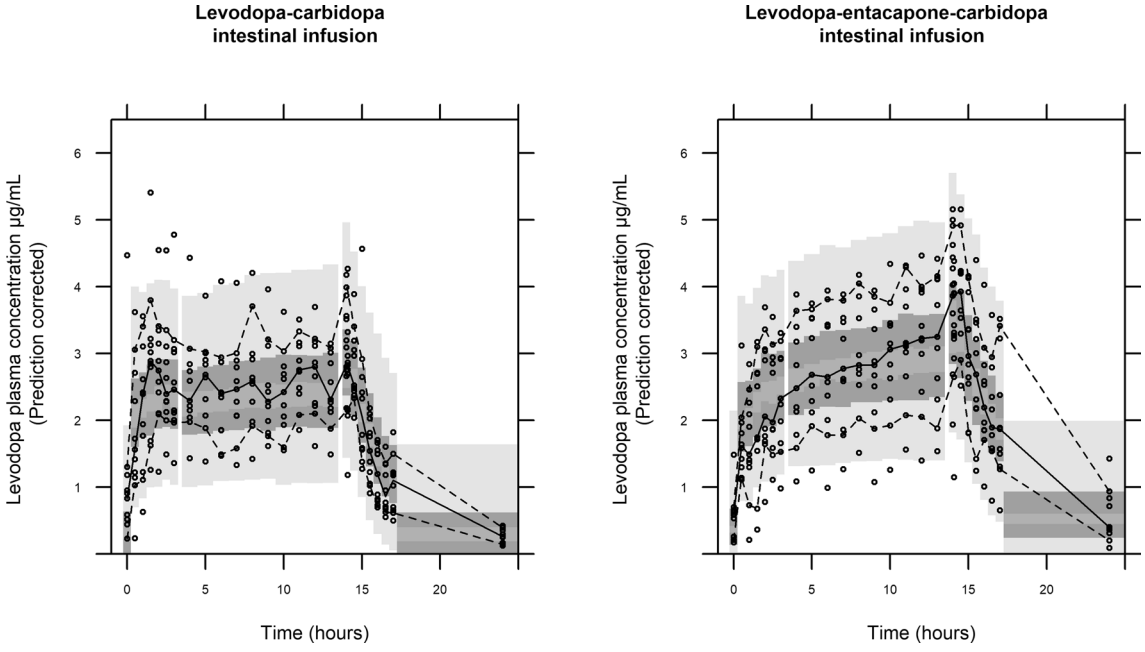

Figure 1. Prediction corrected visual predictive check (1000 samples) of the concentration–time data for LCIG and LECIG. The solid line is the median of the observed data. The dashed lines represent the observed 10th and 90th percentiles of the observations. The top and bottom light grey areas are the 95% confidence intervals for 10th and 90th percentiles of the simulated data. The middle dark grey area is the 95% confidence interval for the median of the simulated data.

90th percentiles. Administration of the same levodopa dose with LECIG as with LCIG, i.e. 0% lower morning and continuous maintenance dose, shows that the predicted plasma concentration increases during the infusion period. In the original study, a 20% lower morning dose and maintenance dose was given, and as previously observed, this results in a slight increase in levodopa plasma concentrations over the 14-h infusion period. A decrease of the continuous maintenance dose by 35% results in similar drug exposure as LCIG, indicating that, on a population level, this would be an appropriate dose adjustment.

The results of estimated individual CL/F, for levodopa with and without entacapone, stratified on genotype are shown in Fig. 3, together with a plot showing the individual shift in CL/F with the addition of entacapone. The results from the $COMT_{SNP}$ (rs4680) genotyping showed that three patients had genotype $COMT^{AA}$ (low), four patients had $COMT^{AG}$ (intermediate) and four patients had $COMT^{GG}$ (high). There is no clear trend observed

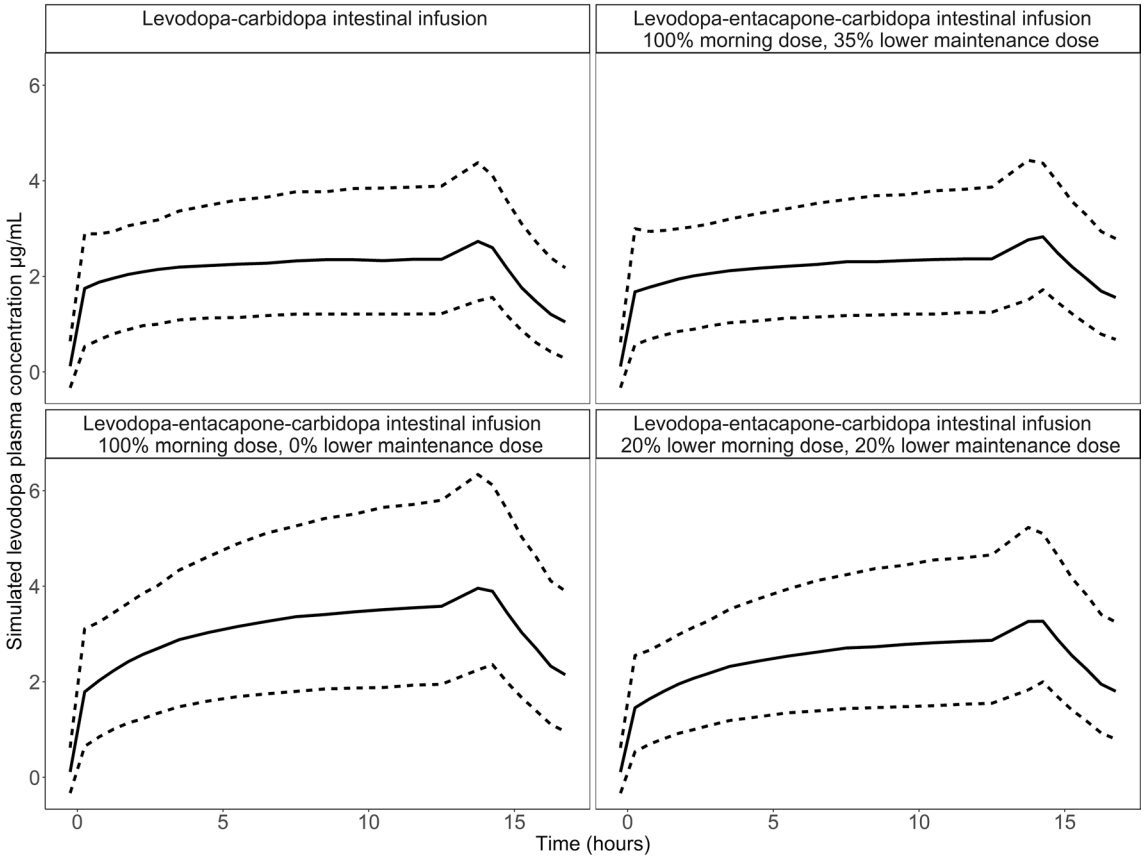

Figure 2. Simulated plasma concentration for the study population, with unchanged patient doses of LCIG as reference (top left plot) and decreased continuous doses for LECIG treatment by: 0% lower morning dose and 35% lower maintenance dose (top right plot), 0% lower morning and maintenance dose (bottom left plot) and 20% lower morning and maintenance dose (bottom right plot). The solid line represents the median of the simulated data, the top and bottom dashed lines represent the 10th and 90th percentiles of the simulated data.

in CL/F between the COMT activity subgroups (Fig. 3A). All $COMT_{SNP}$ genotypes display a decrease in CL/F with the addition of entacapone (Fig. 3B). One patient had been genotyped with DDC_{SNP}^{CC} (low), four patients with DDC_{SNP}^{CT} (intermediate) and six patients with DDC_{SNP}^{TT} (high activity). Patients with high activity of DDC, based on the single nucleotide polymorphism results, showed a tendency to have a higher CL/F (Fig. 3C). However, the one patient with a low activity, had an estimated CL/F that was higher compared with the other two groups. This patient, on the other hand, has a high activity of DDC based on the DDC_{INSDEL} and of COMT according to $COMT_{SNP}$. The DDC_{INSDEL} genotyping (rs3837091), revealed four patients with $DDC_{INSDEL}^{AGAG/-}$ (intermediate), and seven patients with $DDC_{INSDEL}^{AGAG/AGAG}$ (high). Patients with intermediate activity seem to have slightly lower median CL/F, compared with patients with high activity (Fig. 3D).

Discussion

In this analysis, the difference in levodopa pharmacokinetics, administered as an intestinal infusion with and without simultaneous entacapone infusion was investigated using a population modelling approach. Following oral administration and intestinal infusion, levodopa pharmacokinetics has previously been described both with one- and two-compartment models[10,17]. The data following continuous infusion did in our case not allow for an estimation of a second, peripheral compartment. The estimated typical value for levodopa CL/F following treatment with LCIG was 28 L/h/70 kg (95% SIR CI 25–32 L/h). This is in agreement with previous reported values, from population pharmacokinetic studies that included advanced PD patients that received high doses co-administered with carbidopa. Othman et al.[10] reported a CL/F of 25 (95% CI 20–27) L/h for levodopa administered as intestinal infusion, Jorga et al.[18] reported separate levodopa CL/F for a fluctuating and non-fluctuating patient population, of 25 L/h and 29 L/h respectively, and Simon et al.[17] reported a CL/F of 37 (95% CI 31–43) L/h for oral levodopa/carbidopa administration. The previously reported values for V/F vary widely, between 43 and 131 L[10,17,18]. We estimated V/F to 75 L/70 kg (95% SIR CI 63–88 L), which is in line with the previously reported estimates. The wide difference in estimates could be a result from differences in the study population (e.g. disease severity), the doses administered, blood sampling time points, as well as the route of administration. As an example, Jorga et al.[18] estimated different V/F, for the fluctuating and non-fluctuating patient population (99 and 124 L respectively).

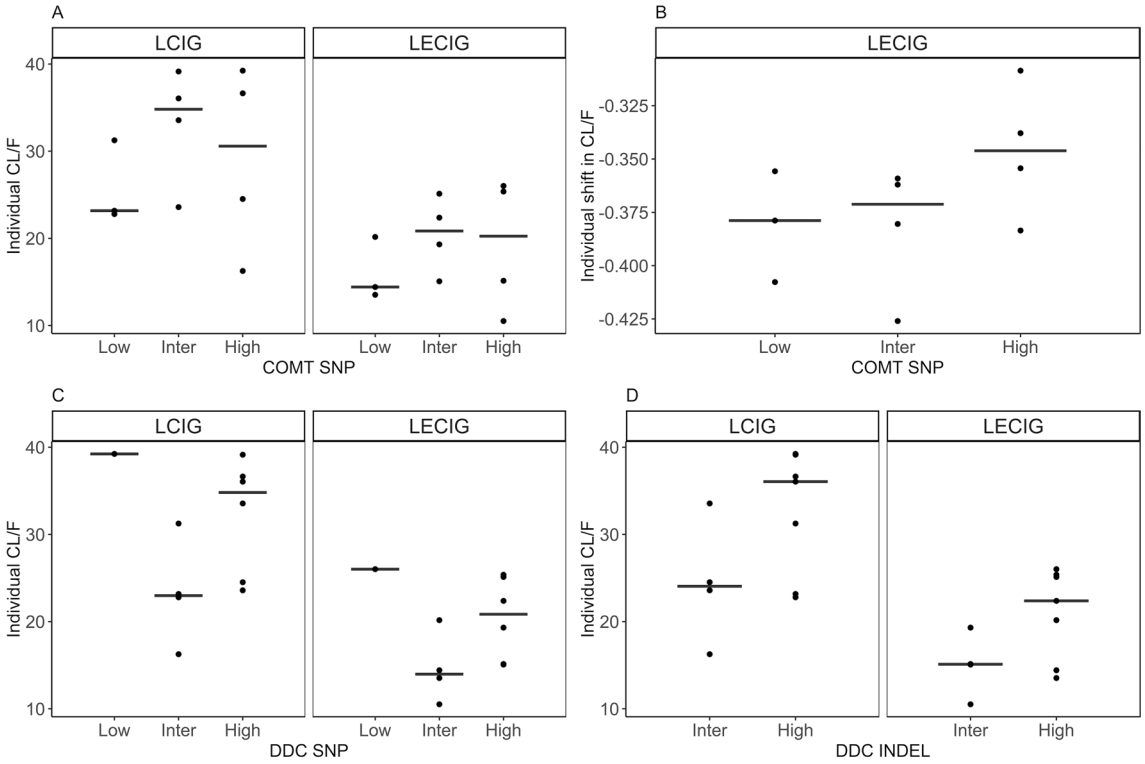

Figure 3. Graphical analysis of genotype results and individual estimated LD CL/F, with LCIG and LECIG treatment. COMT (rs4680, top left, A) and individual shift CL/F (top right, B), DDC_{SNP} (rs321451, bottom left, C), DDC_{INSDEL} (rs3837091, bottom right, D). The middle line represents the median of the data.

The CL/F with entacapone addition was estimated be 37% lower (95% SIR CI 33–39%), i.e. 17.7 L/h/70 kg, compared with LCIG. In the previous non-compartmental analysis, it was observed that the plasma concentration was increasing over time with LECIG, and that the doses had not been adjusted appropriately with the addition of entacapone. The current analysis, using population modelling, has the advantage that extra doses and oral dosing are appropriately taken into account, and that the change in plasma concentration over time can be described. Another advantage is that the variability between individuals, as well as the magnitude of the unexplained variability, can be handled with a model based analysis. The conclusion from this analysis is that the continuous maintenance dose should be reduced by approximately 35%, on a population level, when entacapone is simultaneously infused. This is in contrast to the previously suggested reduction by 20% when an LCIG infusion is administered with oral entacapone[4]. Entacapone undergoes extensive first-pass metabolism. A recently developed model, investigating entacapone pharmacokinetics suggested that 6–11% is lost due to intestinal metabolism[19]. The immediate delivery of entacapone to the small intestine with the infusion, and perhaps a shorter intestinal residence time, may result in a higher bioavailability of entacapone, and thereby higher inhibition of COMT compared with oral administration. An infusion of entacapone results in an even plasma concentration, as opposed to oral administration, where administration every 5 h could result in decreased inhibition before next dose intake and more fluctuations in levodopa plasma concentration, although this was not observed in the infusion study with oral entacapone administration[4]. Maximum inhibition of COMT is probably reached early on during the infusion since entacapone reaches steady state within 1 h and because previous studies indicate that there is no delay between maximum entacapone plasma concentration and COMT inhibition[19].

From the observed plasma concentration–time curve, there is a tendency that the model initially over predicts the plasma concentration following LECIG administration (Fig. 1). The reason for this low initial levodopa concentration is not clear, but has been observed previously with oral multiple-dose administration of levodopa/entacapone/carbidopa[20,21]. One suggested reason for the observed slower absorption was that more levodopa was available with the addition of COMT inhibitor, and that is thereby competing with itself for the saturable large neutral amino acid transporters that transport levodopa across the intestinal membrane. It was also suggested that the delay in absorption could be related to a delayed gastric emptying, caused by higher levodopa concentrations, however this would not be an influencing factor in this study with the infusion treatment, which is bypassing the gastric emptying. Entacapone has molecular similarities to levodopa, and may also compete for transport across the intestinal membrane with levodopa, potentially affecting the rate of levodopa absorption, however, this has been investigated for one of the transporters responsible for levodopa transport and not been found to be the case[22].

The data did not allow for an estimation of a difference in rate of absorption or volume of distribution between the investigated treatments and thus it is difficult to make conclusions regarding any adjustments of the morning

bolus. To investigate this further, administration of bolus doses only, and/or repeated sampling at a longer time period post dosing could be informative.

A formal covariate analysis of the effect of genotype on CL/F was not performed, due to the low number of included subjects, and with relatively high shrinkage in CL/F shift parameter (23.5%), the results are primarily exploratory. The comparison in CL/F based on the different genotypes was only graphically investigated. Corvol et al.[5] found a significant decrease in CL/F for both low (by 25%) and high (by 40%) activity COMT groups (according to $COMT_{SNP}$ rs4680) when oral levodopa/carbidopa was co-administered with 200 mg of oral entacapone. The decrease in the group with high COMT activity was significantly higher compared to the low activity group. Similarly, we found that CL/F decreased for all individuals (95% SIR CI 34–40%), regardless of genotype, with the addition of entacapone. In contrast, we do not see any clear trend in the decrease in CL/F for patients with high COMT activity compared to other $COMT_{SNP}$ subgroups. The results suggest that all patients, irrespective of COMT rs4680 polymorphism, have a high reduction in CL/F with an addition of simultaneously infused entacapone. No clear trend was observed between administered doses of entacapone and the model predicted decrease in CL/F (data not shown), so a dose dependent decrease in CL/F was not explored.

The plasma concentrations were variable within an individual, with trends observed around the time points of food intake. Protein intake was therefore investigated as a covariate on the rate of absorption and relative bioavailability. Protein intake may interact on transporters in the gastro-intestinal tract and across the blood–brain barrier, possibly causing lower levodopa plasma concentration and an absence or delay of effect after dose intake in patients[23]. However, possibly due to high inter-individual variability and few individuals, it was not possible to characterize the food effect in the present model. Further, to study the food effect was not one of the objectives of the study, and the sampling times were not optimized for this investigation. The variability in plasma concentration over time observed in the data could also be due to other effects, such as differences in gastro-intestinal motility, and overall mobility of the patients, which could coincide with food intake.

Conclusion

The CL/F is estimated to be 36.5% lower with simultaneous infusion of entacapone. When switching from LCIG to LECIG, our results suggest that the continuous maintenance dose needs to be decreased by approximately 35% on a population level. An effect from entacapone was identified on all individuals, regardless of $COMT_{SNP}$ polymorphism.

Data availability

The data that support the findings of this study are available from Lobsor Pharmaceuticals AB. Restrictions apply to the availability of these data, which were used under license for this study. Data are available from the authors with the permission of Lobsor Pharmaceuticals AB.

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Author contributions

M.S. wrote the main manuscript text and prepared Figs. 1–3. All authors reviewed the manuscript. Each author (M.S., D.N., E.I.N.) made substantial contributions to the conception of the work, the acquisition, analysis, and interpretation of data. All have approved the submitted version and have agreed both to be personally accountable for their own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the authors were not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Competing interests

Dr Nyholm has received lecture fees from AbbVie and NordicInfu Care. He also has consulted for NeuroDerm and received compensation. Dr Senek and Dr Nielsen declare no potential conflict of interest.

Additional information

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Double-Blind, Double-Dummy, Randomized Study of Continuous Intrajejunal Infusion of Levodopa-Carbidopa Intestinal Gel in Advanced Parkinson's Disease

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CWO interpreted data, wrote the report, and approved the final draft. KK interpreted data, contributed to writing the report, and approved the final draft. PO collected and interpreted data, contributed to writing the report, and approved the final draft. AJE collected and interpreted data, contributed to writing the report, and approved the final draft. DGS collected and interpreted data, contributed to writing the report, and approved the final draft. HHF collected and interpreted data, contributed to writing the report, and approved the final draft. AV collected and interpreted data, contributed to writing the report, and approved the final draft. AAO contributed to study design, contributed to writing the report, analyzed and interpreted data, and approved the final draft. KLW contributed to writing the report, interpreted data, and approved the final draft. WZR contributed to study design, analyzed and interpreted data, contributed to writing the report, and approved the final draft. YP contributed to study design, analyzed and interpreted data, contributed to writing the report, and approved the final draft. KC contributed to study design, interpreted data, contributed to writing the report, and approved the final draft. JB contributed to study design, interpreted data, contributed to writing the report, and approved the final draft. RAL contributed to study design, interpreted data, contributed to writing the report, and approved the final draft. AA interpreted data, contributed to writing the report, and approved the final draft.

Conflicts of interest

CWO has received consultancy fees from Abbott (AbbVie), Novartis/Orion, Impax, Ceregene, and Teva/Lundbeck, AstraZeneca, Biotie/Synosia, CHDI, Civitas, LZ Therapeutics, Medivation, Neuroderm, Pharma2B, Phytopharm, Siena, Synagile, UCB, Otsuka, Upsher Smith, and US World Med, has stock in Clintrex, and is on scientific advisory board of the Michael J Fox Foundation. KK has been a consultant to the National Institutes of Health (NINDS), the US FDA, the USVA, Abbott (AbbVie), Acorda, Aptiv, AstraZeneca, Auspex, Biogen Idec, Biotie, Biovail, Boehringer Ingelheim, Ceregene, CHDI, Civitas, Clintrex, Cynapsus, Endo, EMD Merck Serono, Genzyme, Impax, Intec, Ipsen, Isis, Knopp, Lilly, Link Medicine, Lundbeck, LZ Therapeutics, Medivation, Merck, Merz, Neotope/Elan Pharmaceuticals, Novartis, Orion, Otsuka, Pharm2B, Phytopharm, Roche, Schering-Plough, Siena Biotech, Synosia, Solvay, Synagile, Sofinnova, Teva, UCB Pharma, Upsher-Smith, Vaccinex, Vectura, and Xenoport, has received grant/research support from Medivation, Michael J Fox Foundation, National Institutes of Health (NEI, NINDS, NIA, NICHD), Neurosearch, and Pfizer, and has acted as a legal consultant for Pfizer, Thompson Hine, and the Welding Rod Litigation Defendants. PO has acted as a study investigator in AbbVie-sponsored studies, has received compensation from AbbVie, Boehringer-Ingelheim, Britannia, Cephalon, GSK, Ipsen, Lundbeck, Nordic Infucare, and UCB for serving as a consultant and/or lecturer, and has received honoraria from Movement Disorder Society.

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Abstract

Background—Levodopa is the most effective therapy for Parkinson's disease (PD), but chronic treatment is associated with the development of potentially disabling motor complications. Experimental studies suggest that motor complications are due to non-physiologic, intermittent administration of the drug, and can be reduced with continuous delivery. Levodopa-carbidopa intestinal gel (LCIG) is a form of levodopa that can be delivered continuously through an intrajejunal percutaneous tube.

Methods—We performed a 12-week double-blind, double-dummy, double-titration, multi-center trial to evaluate the efficacy and safety of LCIG compared to optimized, oral, immediate-release levodopa-carbidopa (LC-IR) in advanced PD patients with motor complications. The primary endpoint was change from baseline to final visit in motor “Off” time. Motor “On” time without troublesome dyskinesia was the key secondary endpoint.

Findings—71 patients with advanced PD were randomized to receive continuous LCIG infusion plus placebo LC-IR capsules (n=37) or to receive LC-IR capsules plus continuous placebo LCIG infusion (n=34). Both groups were titrated to optimal effect. 93% of subjects (n=66) completed the trial. In comparison to LC-IR, LCIG significantly reduced “Off” time by a mean (±SE) of 1·91±0·57 hours (P=0·0015) and increased “On” time without troublesome dyskinesia by a mean of 1·86±0·65 hours (P=0·006). Adverse events were primarily related to the surgical procedure and the device, and while potentially serious, were not associated with residual deficit or mortality.

Interpretation—In comparison to standard oral LC-IR, LCIG significantly reduced “Off” time and increased “On” time without troublesome dyskinesia in patients with advanced PD. Adverse events were largely due to the procedure and the device. Benefits are of greater magnitude than have been obtained with medical therapies to date, and represent the first demonstration of the benefit of continuous levodopa delivery in a double-blind controlled study.

Keywords

Parkinson's disease; Levodopa/Carbidopa Intestinal Gel; Motor fluctuations

Introduction

Parkinson's disease (PD) is characterized by degeneration of dopamine neurons in the substantia nigra pars compacta (SNc) with resultant depletion of striatal dopamine leading to

the core motor features of the disease. The mainstay of treatment is levodopa, the amino-acid precursor of dopamine. Virtually all PD patients have a beneficial response, and no present medical or surgical therapy has been shown in controlled trials to provide greater anti-parkinsonian benefit. However, chronic oral levodopa therapy is associated with the development of potentially disabling motor complications (motor fluctuations and dyskinesia) in the majority of patients.[1] Motor fluctuations consist of an initial benefit after a dose of levodopa ("On" period) followed by a return of parkinsonian features ("Off" period) prior to the onset of benefit from the subsequent dose. Dyskinesias are levodopa-induced involuntary movements that typically occur during "On" periods. Higher doses of levodopa can reduce "Off" time but tend to increase dyskinesia, while a reduction in levodopa dose can reduce dyskinesia but tends to worsen "Off" time. In advanced PD patients, it can be difficult to find a dose of levodopa that satisfactorily controls "Off" time without inducing dyskinesia. Multiple classes of medication (dopamine agonists, COMT-inhibitors, MAO-B inhibitors) have been developed to try to reduce "Off" time, but they typically provide only modest benefit and are frequently complicated by worsening dyskinesia.[2] Deep brain stimulation (DBS) is widely employed to improve both "Off" time and dyskinesia, but requires a neurosurgical intervention that is associated with potentially serious complications.[3,4] The development of a levodopa formulation that provides benefits without inducing or worsening motor complications is a major unmet need in PD.

Clinical and laboratory evidence suggests that levodopa-induced motor complications are related to the non-physiologic restoration of brain dopamine with intermittent doses of standard oral levodopa.[5] Striatal dopamine levels are normally maintained at a relatively constant level. This is not the case in PD, where in the absence of nigro-striatal terminals striatal dopamine levels are dependent on the peripheral availability of levodopa. Intermittent dosing with standard oral levodopa formulations provides fluctuating plasma levels due to erratic gastric emptying, variable jejunal absorption, and the short half-life of the drug (60-90 minutes).[6,7] In the dopamine-depleted state, this variability in plasma levodopa concentration is translated into abnormal, fluctuating, striatal dopamine concentrations,[8,9] which in turn are associated with non-physiologic intermittent or pulsatile stimulation of dopamine receptors. This results in gene and molecular changes in striatal neurons, neurophysiologic changes in the firing pattern of pallidal output neurons, and the development of motor complications.[5] It has been hypothesized that continuous delivery of levodopa could restore brain dopamine in a more physiologic manner, and thereby avoid or reduce motor complications associated with traditional levodopa therapy.[5,10] Indeed, continuous levodopa infusion has been reported to reduce both "Off" time and dyskinesia in open-label studies in patients with advanced PD.[11–13] It has, however, proven difficult to develop oral or patch formulations that deliver levodopa in a continuous manner.

Levodopa-carbidopa intestinal gel (LCIG) (AbbVie Inc., North Chicago, IL) is a carboxymethylcellulose aqueous gel that can be delivered continuously to the proximal jejunum via a percutaneous gastrojejunostomy (PEG-J) tube connected to a portable infusion pump (CADD-Legacy® Smiths Medical, MN, USA). Pharmacokinetic studies show LCIG jejunal infusion provides relatively constant plasma levodopa levels with less variability than oral formulations,[14,15] and open label studies report a marked reduction (improvement) in "Off" time without worsening of dyskinesias.[16–19] Despite the lack of double blind trials,

LCIG is approved for use in 43 countries. However, open label interventional studies in advanced PD patients have frequently not been confirmed in double blind trials.[20] We present the results of the first prospective, double-blind, placebo-controlled study evaluating the safety and efficacy of continuous LCIG infusion in patients with advanced PD. This is also the first double-blind controlled trial testing the hypothesis that continuous delivery of levodopa can reduce “Off” time without worsening dyskinesia.

Methods

Study design

The study was a 12-week prospective, multi-center, placebo-controlled, parallel group, double-blind, double-dummy, double-titration study. Candidates were patients with advanced PD complicated by “Off” periods that could not be satisfactorily controlled with “optimized” medical therapy. “Optimized” was defined as an adequate trial in the judgment of the investigator of levodopa-carbidopa, a dopamine agonist, and at least one other class of anti-parkinsonian therapy (COMT inhibitor, MAO-B inhibitor). Following confirmation by an independent Enrolment Steering Committee that the subject was an appropriate candidate, patients signed an informed consent that was approved by the IRB at each participating site. Subjects were then hospitalized for jejunal placement of a PEG-J tube under local anesthesia using endoscopic and/or fluoroscopic guidance and randomly assigned to treatment with either a) over-encapsulated immediate release levodopa-carbidopa 25/100 (LC-IR) plus placebo LCIG gel infusion, or b) LCIG infusion plus over-encapsulated placebo LC-IR.

Subjects

Male and female patients of any race who were at least 30 years of age with a diagnosis of PD consistent with United Kingdom Brain Bank criteria were eligible to participate. Patients had to be receiving stable doses of levodopa for at least four weeks prior to enrollment, and to be experiencing recognizable “On” and “Off” periods with a minimum of three hours of “Off” time per day based on a home diary assessment[21]. Subjects receiving sustained release levodopa-carbidopa, Stalevo®, or other formulations of levodopa were permitted into the study but had to be converted to equivalent doses of LC-IR and to have been on stable doses for at least four weeks prior to entry. Concurrent anti-parkinsonian drugs (except apomorphine) were permitted if patients were on stable doses for four weeks prior to randomization, and the dose was not changed during the study. Exclusion criteria included atypical or secondary parkinsonism, previous neurosurgical treatment for PD, clinically significant medical, psychiatric or laboratory abnormalities in the judgment of the investigator, or any condition that might interfere with absorption, distribution, metabolism, or excretion of study drug or contradict placement of an intrajejunal PEG-J tube.

Randomization and Masking

Eligible subjects who signed an informed consent were randomized to treatment group in a 1:1 ratio according to a central, computer-generated, pre-determined, randomization code. Randomization was stratified by site, with a mixed block size of 2 or 4. An interactive voice response system (IVRS) supported by a contracted vendor generated the randomization

schedule and assigned subjects to treatment groups. Subjects were enrolled by site investigaors. All participants and investigators were masked to group assignment. Those analyzing data were masked until after the database was locked. Simultaneous titration of both active and placebo therapy was performed for patients in both groups in order to maintain the integrity of the blind (see details below), but masking of subjects and investigators was not formally evaluated..

Dosing

Both LCIG and LC-IR were initially administered at the subject's total daily levodopa dose prior to randomization. LCIG was delivered as an aqueous intestinal gel (containing 20 mg/mL levodopa and 5 mg/mL carbidopa monohydrate solution) in 100 gram cassettes or matching placebo gel (sodium carboxymethylcellulose solution alone) administered as a morning bolus (5-10 ml) followed by continuous infusion at a constant rate for the remainder of each patient's waking day (approximately 16 hours). The infusion was stopped overnight. LC-IR capsules containing 25/100 mg of carbidopa/levodopa or matching placebo were initially administered in divided doses over the course of their waking day (approximately 16-hours) beginning at the same time as the infusion and at the same dose and frequency as at baseline. There was a four-week titration period, during which dosing for patients in either group could be adjusted once daily during the first two weeks (during the in-patient hospital stay) and weekly during weeks 3 and 4 (during scheduled outpatient visits). LCIG could be adjusted by changing the infusion rate in 100 mg daily increments; LC-IR could be adjusted by increasing one or more doses by 100 mg but the dosing frequency could not be changed. Changes in dose were made solely based on investigator judgment; subjects could not change the dose or titration rate on their own. Any change in the dosage of an active intervention in a given subject had to be matched by a corresponding change in the placebo treatment, so that both treatments (active and placebo) for each patient were adjusted at the same time. In this way the blind was maintained for patients in both groups. Dosage adjustment could be made for patients in both the LCIG or LC-IR treatment groups so that all patients were titrated to their optimal state. The titration period was followed by an eight-week maintenance period during which patients were maintained on stable doses of their assigned treatment. Open label LC-IR could be used as rescue therapy for persistent “Off” episodes for patients in either group.

Visits and Evaluations

Visits were performed at baseline, and weeks 1, 2, 3, 4, 6, 8, 10, and 12. For three consecutive days prior to baseline visit and each visit beginning at week 2, patients completed a 24 hour home-diary assessment of motor status at 30-minute intervals, recording if they were “Off”, “On” without dyskinesia, “On” with non-troublesome dyskinesia, or “On” with troublesome dyskinesia or asleep.[21] Prior to entry into the study patients were trained in the use of the home diary, and had to have at least 75% concordance with investigator rating and at least 75% compliance in completion of home diary. Additional evaluations at each visit included vital signs, Unified Parkinson Disease Rating Scale (UPDRS; Part II in the “On” state, and Part III in the “On” state approximately 2-4 hours after an oral dose),[22] Parkinson Disease Questionnaire (PDQ-39),[23] EuroQual quality of life-5 Dimensions (EQ-5D),[24] Zarit care-giver burden interview (ZBI),[25] and

investigator-rated clinical global impression (CGI-I). Safety assessments were performed at each visit. Plasma concentrations of levodopa were obtained in the first 20 subjects at weeks 4, and 12 at 12, 16, 17, and 18 hours post-initiation of intestinal gel and the next day prior to infusion and at 1, 1.33, 1.67, 2, 2.33, 2.67, 4, 4.33, 4.67, 8, 8.33 and 8.67 hours after start of infusion. For the remaining subjects, sampling was performed at week 6 prior to initiation of intestinal gel infusion and at 1, 2, 4, and 8 hours after start of infusion,

Outcome measures and Statistical analyses

The primary efficacy endpoint was the change between baseline and final visit (week 12) in the mean number of “Off” hours collected on the home diary during the three days prior to each visit, normalized to a 16 hour waking day. An important secondary outcome was change from baseline to final visit in “On” time without troublesome dyskinesia. Other secondary outcomes measures in hierarchical order of analysis included change from baseline in PDQ-39 summary index, CGI-I, UPDRS part II (Activities of Daily Living subscore), UPDRS part III (Motor subscore), ZBI score, and EQ-5D summary index.

The primary endpoint was analyzed using an analysis of covariance (ANCOVA) model including effects for treatment group and country, with baseline “Off” time, and average daily rescue levodopa dose as covariates. Missing data were imputed using the last observation carried forward. A mixed model repeated measures (MMRM) was performed as a sensitivity analysis which included baseline as a fixed-effect covariate; treatment, country, and time (scheduled assessment visits) as fixed-effect (categorical) factors, and interaction between time and treatment as well as between time and baseline. An unstructured matrix was used for the covariance of the within-subject repeated measures. Pre-specified hierarchical testing and a Gatekeeping procedure were used to maintain the family-wise error rate at 0·05. The hierarchical testing method uses a fixed sequence approach that allows testing of each of the null hypotheses at a significance level of 0.05 without adjustment, as long as the null hypotheses are hierarchically ordered and pre-defined.[26] Claims of statistical significance stop as soon as the first null hypothesis in the testing sequence is not rejected (p-value > 0.05). Inter- and intra-subject coefficients of variation for levodopa plasma concentrations were estimated using a linear mixed-effects model. For safety data, the incidence of adverse events (AEs) and serious AEs (SAEs) were summarized. The Full Analysis data set, consisting of all randomized subjects with data for baseline and at least 1 post-baseline assessment was used for all efficacy analyses. The Safety dataset consisted of all randomized patients who underwent the PEG-J procedure.

Sample size was estimated based on previous open label trials and indicated that 31 subjects per group would provide 90% power to detect a difference between the LCIG and LC-IR groups of 2·5±2·85 hours in “Off” time with alpha=0·05 and a dropout rate of 5%. Two identical studies were originally planned and initiated. After discussion with regulatory authorities, the protocols and statistical analysis plan were amended to combine the studies while they were ongoing, prior to database lock and analysis of any data.

Role of Sponsor

The study was registered at ClinicalTrials.gov (NCT00357994 and NCT00660387). AbbVie Inc. funded the study and was responsible for data collection, monitoring, and statistical analysis. The authors were responsible for study design, interpretation of data, and writing the manuscript. Authors had full access to all data in the study. AbbVie participated in the study design, reviewed the manuscript and provided comments for author consideration, and approved the submission of the manuscript; however, the authors made the final decision on the content. The corresponding author had the final responsibility for the decision to submit for publication.

Results

Twenty-six centers in the United States, New Zealand, and Germany participated in the study. Seventy-one patients met entry criteria, were approved by the enrollment steering committee, signed an IRB-approved informed consent, and were randomly assigned to a treatment group (LCIG=37, LC-IR=34). The mean number of patients per Center was 2.8, and 34 patients were enrolled in the 5 largest sites. A total of 66 patients (LCIG=35; LC-IR=31) completed the trial. A CONSORT diagram is provided in Figure 1. Baseline characteristics are summarized in Table 1; there were no significant differences between treatment groups. Titration to stable dose was achieved in a mean of 7 days for LCIG subjects and 8 days for LC-IR subjects; 90% were titrated to stable doses in ≤ 9 days.

The efficacy analyses performed in hierarchical order demonstrated statistically significant results for “Off” time, “On” time without troublesome dyskinesia, PDQ-39 summary index, CGI-I score, and UPDRS Part II score. Efficacy results are summarized in Table 2. In comparison to LC-IR, LCIG treatment provided significantly greater reduction (improvement) in “Off” time between baseline and final visit, the primary endpoint (difference between groups was −1·91±0·57 hours; P=0·0015). LCIG treatment was also associated with significantly greater improvement than LC-IR in “On” time without troublesome dyskinesia, the important secondary endpoint (difference between groups 1·86±0·65 hours; P=0·0059), as well as in “On” time without any dyskinesia (difference between groups 2·28±0·90hrs; P=0·0142; Figure 2a). Results at each time point are provided in Figure 2b. We utilized a large number of sites to facilitate enrollment in this complex study, but there was no Center effect in the analysis. The results of the primary analysis were confirmed by the MMRM sensitivity analysis. The benefits of LCIG compared with standard LC-IR were reflected by significant improvement in the activities of daily living subscale of the UPDRS (Part II), and measures of quality of life (Table 2). No significant difference between treatment groups was detected for UPDRS Part III (motor subscale). Levodopa doses are shown in Table 2; the change from baseline levodopa dose and the amount of rescue levodopa employed were greater in the LC-IR group. Intra-subject variability in plasma levodopa concentration was less for LCIG-treated (21%) than LC-IR-treated (67%) subjects.

AEs were reported in 35 (94·6%) LCIG patients and 34 (100%) LC-IR patients; it should be noted that patients in both groups received PEG-J placement. SAEs occurred in 13.5% and 20.6% respectively (Table 3). Three AEs resulted in study termination; one LCIG-treated

patient had psychosis, one LC-IR patient had peritonitis and pneumonia, and one LC-IR subject had a post procedural discharge. Most AEs were related to the surgical procedure or the device, were mild to moderate in severity, occurred almost exclusively within the first week, and resolved in all cases; there were no deaths (see details in Table 3 and in Figure 3). It should be noted, however, that 2/71 (2.8%) patients discontinued from the study due to complications of surgery and that 63 (88.7%) experienced device-related complications including tube dislocations 17 (23.9%), PEG-J insertion complications 15 (21.1%), stoma insertion complications 7/71, pump malfunctions, 6/71 (8.5%), and pneumoperitoneum 5/71 (7.0%). Symptoms consistent with the possibility of polyneuropathy were recorded in four patients (LCIG-1, LC-IR-3); no cases of Guillain-Barre syndrome were reported. There were no clinically significant laboratory abnormalities.

Discussion

We demonstrate in a prospective double-blind, double-dummy, double-titration study that, in comparison with intermittent doses of immediate release oral levodopa (LC-IR), continuous intrajejunal infusion of levodopa gel (LCIG) provides a significant reduction in “Off” time in patients with advanced PD. Importantly, this benefit of LCIG is also associated with a significant increase in “On” time without troublesome dyskinesia. “Off” time in LCIG-treated patients was reduced by 1·91 hours in comparison to standard oral levodopa, and by 4 hours in comparison to baseline. This magnitude of benefit is greater than has been achieved with medical therapies evaluated in double-blind studies in which there was no increase in troublesome dyskinesia,[2] and is of similar magnitude to that reported with DBS in open label studies.[3]

Treatment was optimized for patients in both treatment groups. Thus, it is unlikely that the greater reduction in “Off” time seen in the LCIG group was due to disproportionate levodopa dosing in the LCIG group. Indeed, there was a greater increase from baseline in total daily levodopa dose in the LC-IR group, and there was no difference between the groups in UPDRS motor scores. A summary of the study rationale and results is provided in the panel on “Research in Context”.

Research in Context

Background—Chronic treatment with standard levodopa/carbidopa is associated with motor complications in the majority of patients with Parkinson's disease (PD). These can be a source of disability, and represent the major reason for surgical therapy in PD patients. Laboratory studies suggest that motor complications are related to fluctuating plasma levels of levodopa and might be avoided with continuous delivery of the drug[5]. However, it has proven difficult to accomplish this with long-acting oral or patch formulations. Levodopa-carbidopa intestinal gel (LCIG) is a novel formulation of levodopa that is administered by continuous intra-intestinal infusion (duodopa®) to provide relatively constant plasma levodopa levels.

Systematic Review

We performed a Pubmed search and an extensive literature review on August 15, 2013 under the search terms of “duodopa”, “levodopa carbidopa intestinal gel” “continuous levodopa infusion”, “continuous levodopa delivery” and “continuous dopamine stimulation” with no restriction on date or language. There were no double-blind, placebo-controlled parallel group trials assessing the safety and efficacy of LCIG or any other form of continuous levodopa delivery in patients with Parkinson's disease and motor complications.

Interpretation—We performed a 12-week double-blind, double-dummy, placebo-controlled, double-titration parallel group trial comparing continuous infusion of LCIG to optimized treatment with standard LC-IR. In comparison to optimized LC-IR, continuous intraintestinal LCIG infusion provided a significant reduction in “Off” time, significant increase in “On” time without troublesome dyskinesia, and significant improvement in measures of quality of life. Benefits were of a greater magnitude than have been achieved in placebo-controlled trials with available medical therapies for “the treatment of Off” time, and in a similar range as reported with Deep Brain Stimulation[3]. The study provides the first double-blind data evaluating the safety and efficacy of continuous levodopa delivery as a treatment strategy for Parkinson's disease. These results are consistent with the concept of continuous dopaminergic stimulation as a therapy for PD; future longer-term studies are required to test the potential for LCIG to reverse established dyskinesia.

Great efforts were employed to maintain the integrity of the blind. Patients were randomized, all investigators and subjects were blinded as to treatment group, titration was performed simultaneously for both active and placebo treatments such that any change in dose of one form of drug delivery had to be matched by a comparable change in the other during the titration period, no change in dosage was permitted during the maintenance phase, and the pump was locked so that the dose couldn't be modified by the patient. We did not perform formal evaluations to assess masking of subjects or investigators; there were no reports of unblinding during the study.

Evidence in dopamine-lesioned rodents and primates indicates that intermittent oral levodopa dosing induces molecular changes in striatal neurons, and physiologic changes in pallidal neurons that are associated with the development of motor complications.[5] These can be avoided with more continuous or long-acting dopaminergic therapies. We believe that the significant reduction in “Off” time and significant increase in “On” time without worsening of troublesome dyskinesia observed in the LCIG group in patients with advanced PD was due to restoration of brain dopamine in a more physiologic manner than can currently be achieved with intermittent oral administration of the drug. The possibility that benefits were simply due to bypassing gastric emptying has been considered, but LCIG and LC-IR have comparable bioavailability in pharmacokinetic studies[14], and we believe that the continuous levodopa delivery is a more reasonable explanation.

Continuous levodopa delivery has been reported to reduce dyskinesia as well as off time in open label studies.[12] Indeed, LCIG subjects in the present study had a significant improvement in both “off” time and “On” time without dyskinesia (Figure 2). However, the present study was designed to assess the effect of LCIG on “Off” time. Accordingly,

subjects were selected based on having > 3 hours “Off” time per day, and had very low baseline levels of dyskinesia. This precluded determining if LCIG also provides a benefit with respect to established dyskinesia. Further studies to assess the effect of LCIG on dyskinesia are required.

AEs were primarily related to the surgical procedure or the device and included pneumoperitoneum, peritonitis, pump malfunction, obstruction of catheter, tube displacement, and the need for additional procedures to repair or replace the catheter. These primarily occurred within the first two weeks and were not associated with residual deficit. Further, serious device-related AEs were fewer than have been reported in the literature[27] which may reflect a benefit of increased experience with the procedure. Polyneuropathy and Guillain-Barre syndrome have been reported with LCIG infusion,[28] but neuropathy has also been reported in association with oral levodopa,[29] and a specific relationship to LCIG treatment has not been established. In the present study, Guillain-Barre syndrome was not encountered in any patient, and symptoms potentially related to neuropathy were only reported in one LCIG subject compared to three LC-IR subjects. An open-label, long-term safety study is currently underway.

LCIG represents a potentially important therapeutic advance in the management of PD patients with motor complications, and represents an alternative to DBS that avoids the need for a neurosurgical procedure, although LCIG does require an intervention that is associated with potentially serious complications. The study was 12 weeks in duration, and longer-term studies are required to better assess safety, to evaluate the effect of LCIG on dyskinesia, and to determine what level of expertise is required to manage patients who have this procedure. Similar reductions in “Off” time have been reported in open label studies with continuous subcutaneous delivery of apomorphine,[30,31] but this procedure has not been evaluated in a double-blind trial and it is associated with troublesome skin nodules as well as the side effects of dopamine agonists. There are presently no trials directly comparing LCIG infusion with DBS and apomorphine infusion, and randomized studies are awaited.

There are several limitations to the study. Because of the complexity of the study we utilized a large number of sites which only had limited numbers of subjects, but statistical analyses showed no center effect. We did not conduct a formal evaluation of the blind, and as with all effective therapies there is the possibility that a beneficial response could cause unblinding however, there were no reports of unblinding and the study was designed so as to minimize this risk. The study was only 12 weeks in duration, which precludes an evaluation of the complications associated with LCIG infusion and the J-tube that might develop after this time period. The relatively short duration of the study and the patient population that was studied also prevent an evaluation of the potential of LCIG treatment to reduce established dyskinesia. Finally, it should be noted that the procedure can be associated with potentially serious adverse events and is a rather complex procedure that likely will need to be performed in specialty centers.

In summary, this study demonstrates that LCIG provides a therapeutic option for patients with advanced PD who suffer “Off” episodes that cannot be satisfactorily controlled with standard medical therapies. The present study also represents the first double-blind study to

provide data consistent with the concept of continuous dopaminergic stimulation as a treatment for the motor complications of PD. Longer term studies to determine if continuous levodopa infusion reduces dyskinesia in addition to off time are required to prove this hypothesis. In the final analysis, the value of LCIG as a treatment for PD patients with motor complications will ultimately be determined by trials that provide a full assessment of its relative safety, efficacy, and cost in comparison to other available therapies such as DBS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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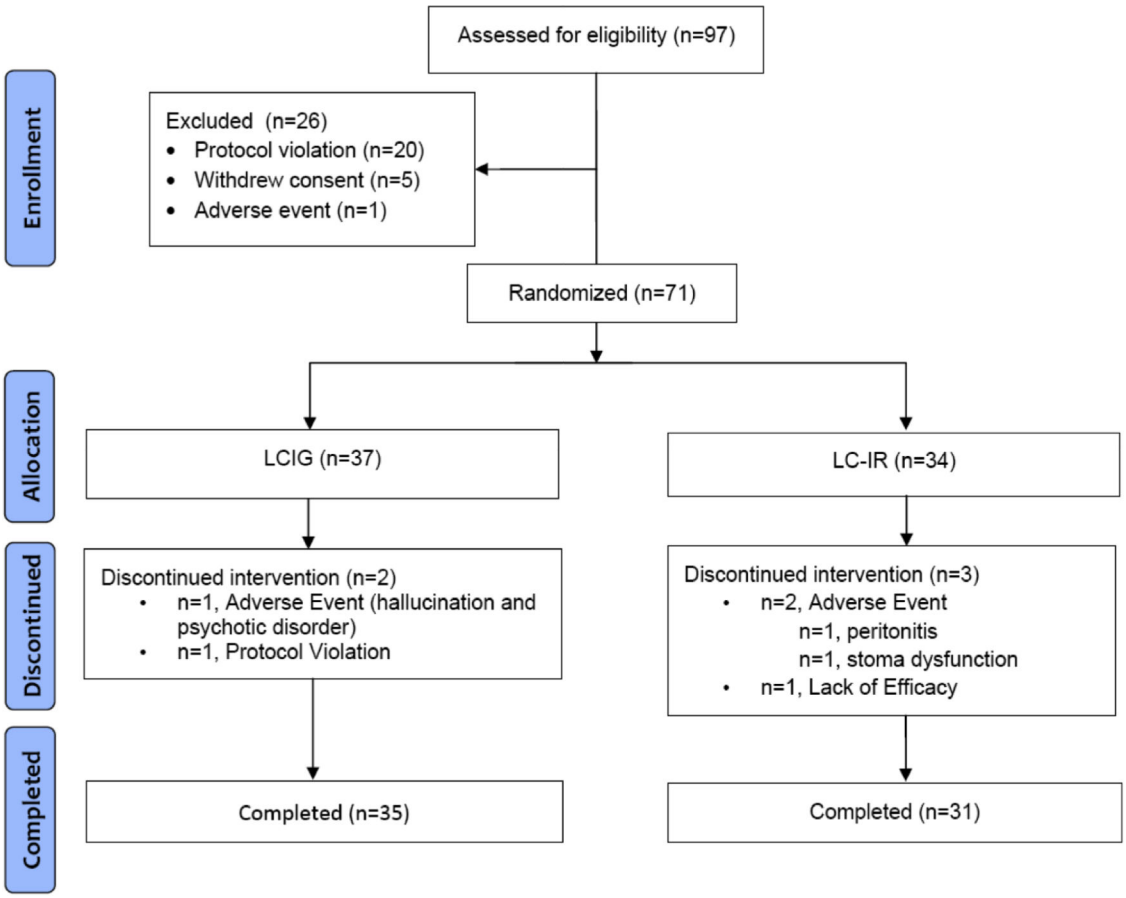

Figure 1.
CONSORT Diagram

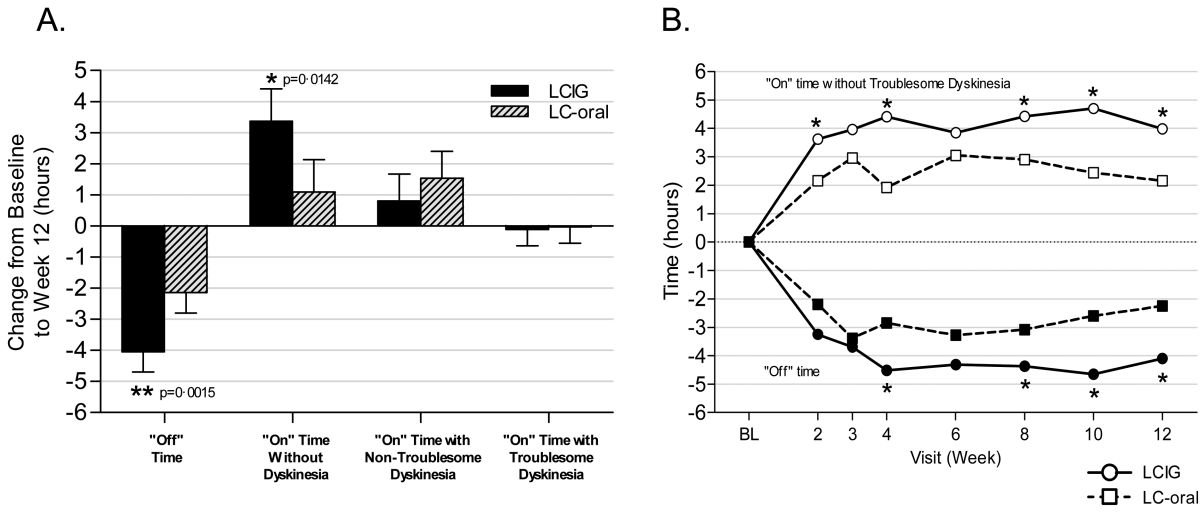

Figure 2. Diary measures

A. Home Diary Results: Change between baseline and Week 12 in the various PD motor states. B. Home Diary Results: PD motor states at each visit. For each variable, data shown are the average from the symptom diary for the 3 consecutive days prior to the clinic visit, normalized to a 16-hour waking day. “On” time without Troublesome Dyskinesia = “On” time without dyskinesia + “On” time with non-troublesome dyskinesia. N = 35 (LCIG), 31 (LC-IR). *P<0·05 between treatment groups.

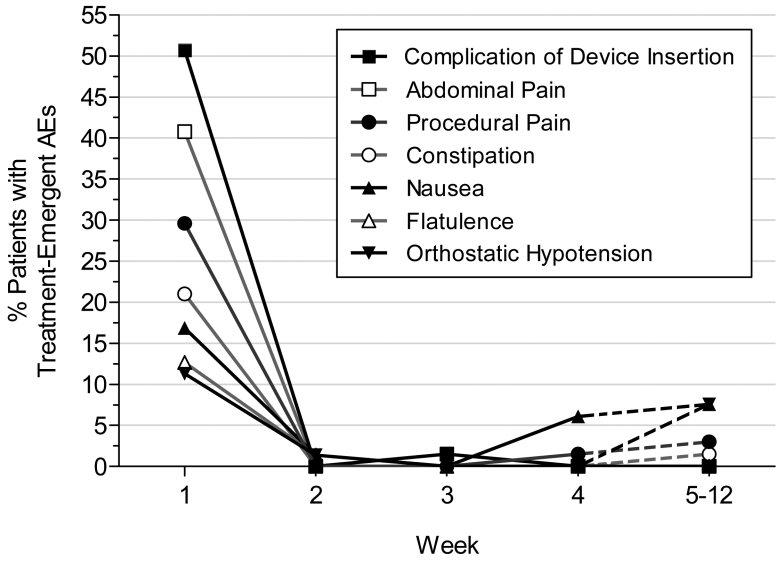

Figure 3. Incidence of treatment-emergent adverse events (AEs) reported by >10% of patients during any time interval

Week 5-12 time point summarizes AEs initiating over multiple weeks.

Table 1

Baseline Characteristics

Baseline Characteristic	LCIG (N=37)	LC-IR (N=34)
Mean age, years (SD)	63·7 (9·5)	65·1 (6·8)
Male, n (%)	24 (64·9)	22 (64·7)
White, n (%)	35 (94·6)	31 (91·2)
Mean duration of PD, years (SD)	10·0 (4·6)	11·8 (5·6)
Mean “Off” time, h/d (SD)[a]	6·3 (1·7)	7·0 (2·1)
Mean “On” time without dyskinesia, h/d (SD)[a]	6·3 (2·7)	5·6 (3·2)
Mean “On” time with non-troublesome dyskinesia, h/d (SD)[a]	2·4 (1·8)	2·2 (2·2)
Mean “On” time without troublesome dyskinesia, h/d (SD)	8·7 (2·0)	7 ·8 (2·5)
Mean “On” time with troublesome dyskinesia, h/d (SD)[a]	1·0 (1·6)	1·2 (1·7)
UPDRS, mean (SD)[a]		
Part I	1·8 (1·7)	1·8 (1·8)
Part II	11·6 (6·9)	11·8 (7·0)
Part III	18·1 (9·9)	22·5 (11·7)
Total	31·5 (15·6)	35·8 (18·9)
PDQ-39[a]	35·1 (18·0)	38·6 (17·9)
Mean Mini-Mental State Exam (SD)	28·7 (1·4)	28·9 (1·4)
Mean daily levodopa dose, mg (SD)	1005·4 (373·6)	1123·5 (477·9)
Anti-Parkinsonian Medication Use, n (%)		
Dopamine agonist	22 (59.5)	26 (76.5)
COMT inhibitor	18 (48.6)	15 (44.1)
MAOB inhibitor	15 (40.5)	6 (17.6)

[a]Full Analysis data set: N=36 for LCIG, N=33 for LC-IR; “On” time without troublesome dyskinesia = “On” time without dyskinesia + “On” time with non-troublesome dyskinesia.

Table 2

Summary of Efficacy Findings

Assessment	LCIG N = 35	LC-Oral N = 31	Treatment Difference
Primary Efficacy Measure			
"Off" time, hrs/day			
Mean change from baseline (SE)	−4·04 (0·65)	−2·14 (0·66)	−1·91 (0·57)**
Important Secondary Efficacy Measure			
"On" time without troublesome dyskinesia, hrs/day			
Mean change from baseline (SE)	+4·11 (0·75)	+2·24 (0·76)	+1·86 (0·65)**
Other Endpoints			
"On" time without dyskinesia, hrs/day[a]			
Mean change from baseline (SE)	+3·37 (1·04)	+1·09 (1·05)	+2·28 (0·90)*
"On" time with non-troublesome dyskinesia, hrs/day[a]	+0·81 (0·86)	+1·54 (0·86)	−0·73 (0·74)
Mean change from baseline (SE)			
"On" time with troublesome dyskinesia, hrs/day[a]	−0·11 (0·52)	−0·03 (0·52)	−0·08 (0·45)
Mean change from baseline (SE)			
PDQ-39 Summary Index			
Mean change from baseline (SE)	−10·9 (3·3)	−3·9 (3·2)	−7·0 (2·8)*
CGI-I[b]			
Mean score at final (SE)	2·3 (0·4)	3·0 (0·4)	−0·7 (0·3)*
UPDRS Part II[c]			
Mean change from baseline (SE)	−1·8 (1·3)	+1·3 (1·3)	−3·0 (1·1)**
UPDRS Part III[c]			
Mean change from baseline (SE)	−1·5 (2·4)	−2·9 (2·4)	+1·4 (2·1)
EQ-5D			
Mean change from baseline (SE)	+0·05 (0·04)	−0·02 (0·04)	+0·07 (0·04)
Zarit Burden Interview			
Mean change from baseline (SE)	−2·8 (3·7)	+1·7 (3·3)	−4·5 (3·1)
Levodopa total daily dose			
Mean change from baseline (SE)	+91·7 (96·6)	+249·7 (94·9)	−158·0 (83·3)
Levodopa rescue dose			
Overall mean, mg (SD)	139.8 (81./3)	180.6 (156.2)	

"On" time without troublesome dyskinesia = "On" time without dyskinesia + "On" time with non-troublesome dyskinesia.

+ = increase in score, − = reduction in score

[a] Measure not part of hierarchical analysis

[b] CGI-I, 1= very much improved, 2=much improved, 3=minimally improved, 4=no change, 5=minimally worse, 6=much worse, 7=very much worse.

[c] UPDRS was completed in the "On" state

*P<0 05

**P<001

Table 3

Summary of Adverse Events (AEs) and Device Complications N (%)

Overall, N (%)	**LCIG N = 37**	**LC-Oral N = 34**	**Total N = 71**
Any AE	**35 (94·6)**	**34 (100·0)**	**69 (97·2)**
Serious AE *	**5 (13·5)** *a*	**7 (20·6)** *b*	**12 (16·9)**
Abdominal pain	19 (51·4)	11 (32·4)	30 (42·3)
Nausea	11 (29·7)	7 (20·6)	18 (25·4)
Procedural Pain	11 (29·7)	12 (35·3)	23 (32·4)
Constipation	8 (21·6)	7 (20·6)	15 (21·1)
Incision Site Erythema	7 (18·9)	4 (11·8)	11 (15·5)
Flatulence	6 (16·2)	4 (11·8)	10 (14·1)
Dyskinesia	5 (13·5)	4 (11·8)	9 (12·7)
Orthostatic Hypotension	5 (13·5)	8 (23·5)	13 (18·3)
Depression	4 (10·8)	1 (2·9)	5 (7·0)
Fall	4 (10·8)	4 (11·8)	8 (11·3)
Insomnia	4 (10·8)	4 (11·8)	8 (11·3)
Pneumoperitoneum	4 (10·8)	1 (2·9)	5 (7·0)
Post-procedure Discharge	4 (10·8)	3 (8·8)	7 (9·9)
Wound Infection	4 (10·8)	8 (23·5)	12 (16·9)
Device complication	**34 (91·9)**	**29 (85·3)**	**63 (88·7)**
Intestinal tube comp	14 (37·8)	12 (35·3)	26 (36·6)
Leakage	2	1	3
Insertion complication	3	1	4
Dislocation	8	9	17
Occlusion	5	4	9
Unintentional removal	0	1	1
PEG-J comp	11(29·7)	12 (35·3)	23 (32·4)
Breakage	1	0	1
Insertion complication	8	7	15
Dislocation	2	3	5
Occlusion	0	1	1
Connection issue	1	3	4
Unintentional removal	0	1	1
Pump comp	5 (13·5)	8 (23·5)	13 (18·3)
Breakage	1	0	1
Malfunction	3	3	6
Occlusion	1	2	3
Stoma comp	15 (40·5)	15 (44·1)	30 (42·3)
Leakage	2	1	3
Insertion complication	2	5	7
Dislocation	0	1	1
Connection issue	0	1	1

[*] SAEs included:

[a] 2 events of confusional state, and 1 event each of pneumoperitoneum, complication of device insertion, catheter site cellulitis, hypersomnia, delusions, hallucinations, mutism, and psychotic disorder

[b] 2 events of pheumonia, and 1 event each of neutropenia, abdominal pain, peritonitis, postprocedural complication, elevated body temperature, depressed level of consciousness, mental status change, psychosis, and orthostatic hypotension. More than 1 could be in the same individual.

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Review Article

Treatment of Advanced Parkinson's Disease

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Patients at late stage Parkinson's disease (PD) develop several motor and nonmotor complications, which dramatically impair their quality of life. These complications include motor fluctuations, dyskinesia, unpredictable or absent response to medications, falls, dysautonomia, dementia, hallucinations, sleep disorders, depression, and psychosis. The therapeutic management should be driven by the attempt to create a balance between benefit and side effects of the pharmacological treatments available. Supportive care, including physical and rehabilitative interventions, speech therapy, occupational therapy, and nursing care, has a key role in the late stage of disease. In this review we discuss the several complications experienced by advance PD patients and their management. The importance of an integrative approach, including both pharmacological and supportive interventions, is emphasized.

1. Introduction

Advanced Parkinson's disease (PD), stage 4 or 5 of the Hoehn and Yahr Scale [1], is characterized by very limited mobility without assistance, severe motor deficits, risk of falls, and cognitive and psychotic problems. The mean time from disease onset to wheelchair-dependence is estimated at 14 years [2], although about a third of patients seem to have a relatively milder disease and remain stable for many years [3]. With the advent of the L-Dopa and other dopaminergic treatments, the progression of PD has become markedly slower. However, over the years treatment loses its efficacy, while a number of complications, such as motor fluctuations and dyskinesia, develop, probably due to the progressive loss of dopaminergic neurons and their striatal and cortical connections [4]. These complications are observed in 50% of patients after 5 years of disease and in 80% of patients after 10 years of treatment [5, 6]. However, the response to L-Dopa therapy predictability decreases over the years.

While worsening of motor function and the drug-induced motor complications represents a major challenge in patients with mid to advanced disease, in the advanced stage of PD the most troublesome and distressful complications are usually in the area of non-motor symptoms, including psychiatric and cognitive disorders, autonomic disturbances, and sleep disorders that significantly increase the need for supportive. These symptoms are frequently neglected in clinical practice due to limited consultation time, perception of the patient and caregivers that their symptoms are unrelated to the disease, or insufficient awareness of the clinicians who generally focus the consultation towards motor symptoms [7].

Psychosis and dementia are frequent and share a common pathophysiology in a significant proportion of patients [8, 9], where the impact on patients and family is variable. Dementia is associated with reduction in quality of life [10] and patient lifespan [11], psychosis is a risk factor for nursing home placement [12], and both are important sources of caregiver distress [13].

Management of motor and non-motor complications should be tailored to the individual patient. This implies a careful assessment of whether the symptom is a side effect of the medications or is related to the progression of the disease. In advanced disease, patients may also experience an enhanced sensitivity to small changes in L-Dopa or become more prone to adverse reactions to antiparkinsonian drugs.

Proper supporting care becomes increasingly important in advanced PD. Rehabilitative and support services for

patients and family also become key interventions as the disease reaches its more debilitating stages and pharmacological or surgical treatment becomes less relevant.

In this article we discuss the spectrum of the motor and non-motor complications seen in advanced stage PD and present an evidence-based review of current therapeutic options in the management of these complications.

2. Motor Disability

PD is defined as advanced when the patient is severely disabled. As per Hoehn and Yahr classification, patients in stage 4 are still able to walk and stand unassisted, but they are markedly incapacitated in their ability to perform activities of daily living (ADL). Patients in stage 5 are confined to bed or wheelchair unless aided.

Many patients in advanced stage range from stage 4 to 5 during the day because of the inconsistent and limited response to their medications.

Even when patients are still able to ambulate without assistance, limited motor ability due to marked bradykinesia and inability to perform fine and alternate movements lead them to dependency in ADLs, being unable to provide for basic personal care like dressing, bathing, and often feeding.

Advanced patients are frail individuals exposed to high risk of several unfavorable circumstances during daily activities, like falls.

The incidence of falls in advanced PD is high (40–70%) [14], even when patients are optimally medicated. Falls in advanced PD occur because of very unstable gait, loss of center of gravity, poor balance, orthostatic hypotension, side effects of medications like antidepressants and benzodiazepines, and disturbances of posture like camptocormia or retropulsion. Falls lead to injuries and fracture that further reduce patient independence and increase the risk of nursing home admission. Patients with previous falls often develop fear of falling which further limits their mobility, contributing to increased weakness and deterioration.

Because of the devastating consequences, an assessment of falls risk should be taken in all advanced PD patients. A combination of both disease-specific and balance- and mobility-related measures is necessary to accurately predict falls in patients with PD [15].

Treatment of falls implies a complex approach aimed at reducing all the potential risk factors, muscle strengthening, range of motion exercise and balance, and postural control training.

Although there is still insufficient evidence for effective prevention of falls, exercise interventions have shown to be effective at improving physical functioning, leg strength, balance, and walking [16]. Thus, physical interventions should be emphasized in advanced stages of disease, particularly as falls are currently not well addressed either by pharmacotherapy nor by subthalamic nucleus deep-brain stimulation (DBS) surgery.

The neuroanatomical substrates of posture and gait are poorly understood but a number of important observations suggest a major role for the pedunculopontine nucleus and adjacent areas in the brainstem. A recent double-blinded study reported a significant reduction in falls in the on and off medication states both at 3 and 12 months after pedunculopontine nucleus DBS as captured in the Unified Parkinson's Disease Rating Scale part II scores in six advanced Parkinson's disease patients with significant gait and postural abnormalities [17].

It has to be noted, however, that advanced patients are at high risk of short- and long-term complications from the DBS procedure, and surgical treatment is generally contraindicated in these patients. Furthermore, literature on pedunculopontine nucleus DBS is still limited, and long-term follow-up studies investigating safety and efficacy are unavailable.

3. Motor Complications

Long-term motor complications of PD are due to duration of disease and treatment, and to cumulative intake of L-Dopa, with several central and peripheral mechanisms involved. The progressive degeneration of the nigrostriatal dopaminergic transmission results in fewer and fewer terminals capable of taking up exogenously administered L-Dopa and converting it to dopamine for subsequent storage and release [6]. Unlike early and mid-stage PD patients advanced- and end-stage patients experience an enhanced sensitivity to small changes in plasma L-dopa levels [18, 19], that narrow the therapeutic window and negatively impact motor function.

3.1. Wearing-Off, On-Off Fluctuations, and Management Strategies. "Wearing-off" refers to the recurrence of motor and non-motor symptoms preceding the scheduled dose of L-Dopa, while the on-off fluctuations are sudden unpredictable shifts between "well-" or "over-" treated status (on) and an undertreated state with severe Parkinsonism symptoms (off).

"Wearing-off" and on-off fluctuations overlap in advanced patients.

"Wearing-off" is a direct consequence of the nonphysiological, pulsatile dopaminergic stimulation, and its occurrence is generally predictable following the L-Dopa administration with progressive therapeutic window progressively narrowing over the years.

A plethora of sensory, psychiatric, and autonomic symptoms may be associated with the motor fluctuation. Patients, indeed, may present with paresthesia, pain, anxiety, shortness of breath, sweating, and other symptoms that may not be recognized as part of the L-Dopa response pattern [20].

Management strategy for "wearing-off" phenomena is focused on prolonging the effect of individual L-Dopa doses without increasing the pulsatile dopaminergic stimulation.

Strategies include fragmentation of dosing, with more frequent administration of lower doses, and use of COMT inhibitor (entacapone and tolcapone), MAO inhibitor (selegiline and rasagiline), and use of dopamine agonists.

Adjunctive therapy with a COMT inhibitor extends the duration of the L-Dopa effect, hence ameliorating wearing

off, by blocking the COMT enzyme in the peripheral catabolism of L-Dopa. Potential adverse event, however, may arise from the COMT inhibitors. Increasing synaptic dopamine levels may also be associated with dyskinesia and increased L-Dopa toxicity leading to worsening of dementia and psychosis.

Fragmentation of oral therapy, with L-Dopa administered up to 6-7 times a day at about 3-hour intervals, is a commonly used and effective strategy [21]. However, lowering individual doses of L-Dopa may increase the risk of occasional drug failure or delayed response.

Substitution of regular with controlled-release L-dopa preparations may be particularly reasonable in end-stage patients [22], but the available extended release formulations are not always affective and reliable.

The use of dopamine-agonists (DAs), although theoretically useful in regulating fluctuations by direct stimulation of the postsynaptic receptors, is generally contraindicated in late-stage disease in order to avoid hallucinations and psychosis, and worsening of autonomic dysfunction.

The main challenge in controlling the on-off response is to improve the "on" time without increasing the dyskinesia.

In very late-stage PD this can be achieved using liquid formulations of L-Dopa [23], which can be prepared by dissolving ten 25/100 mg standard-release carbidopa/levodopa tablets and 2 g of ascorbic acid in 1 L of tap water [24].

Gastrointestinal dysfunction, with erratic gastric emptying worsening over the years, is a common cause of poor absorption of L-Dopa in PD. There is no gastric absorption of L-Dopa, indeed; so gastric emptying and transit via the pyloric sphincter are critical factors for regular intestinal absorption [25].

The liquid effervescent levodopa formulation of melevodopa (methyl-ester levodopa) plus carbidopa is a prodrug with a high solubility (about 250 times more than L-Dopa) in small volume of water, and it is able to reach quickly the small intestine where it is absorbed in a more regular and rapid way compared to solid formulations [26]. One clinical advantage of this formulation is that it avoids erratic absorption and the related unpredictability in the plasma L-Dopa concentration curve [27]. The drug is approved in certain European countries and currently under phase II investigation in the US.

Continuous infusion of levodopa/carbidopa gel through portable duodenal systems (Duodopa) using percutaneous endoscopic gastrostomy (PEG) can be a practical alternative [28, 29]. The infusion provides constant plasma levodopa concentration and continuous dopamine availability and receptor stimulation. This solution may be particularly reasonable in very advance patients with severe dysphagia, as the PEG may also be used for nutrition. Intrajejunal L-dopa/carbidopa gel infusion is effective in reducing off time, severity and duration of dyskinesia in advanced PD [30, 31]. Most importantly, a recent multicenter study demonstrated that intrajejunal L-dopa/carbidopa infusion provides a beneficial effect on several nonmotor complications, including cardiovascular, gastrointestinal, and urinary symptoms, sleep/fatigue, attention/memory, and pain [32]. Adverse event can occur, however, from the procedure or from the dislocation or occlusion of the intestinal tube. Advanced patients may also experience local complications at the site of entry, particularly inflammation and infections.

Apomorphine subcutaneous infusion is also an effective option for patients with severe fluctuations poorly controlled by oral treatment [33]. Apomorphine infusion is often limited by the development of skin reaction at the site of injections after few years of treatment.

3.2. Dyskinesias. Dyskinesias are involuntary choreiform, twisting and turning movements invariably occurring in patients undergoing long-term L-Dopa treatment. Dyskinesias usually occur in "on" state, as chorea, myoclonus or dystonic movement. In end-stage patients dyskinesia may appear in off state as dystonic posture, especially in the lower limbs. Off dystonia is generally most troublesome upon morning awakening but in advanced disease may also develop complex twisting dystonic movements during the day. Because of the narrow therapeutic window at this stage it is also not uncommon for patients to experience diphasic dyskinesia. These are usually repetitive alternating movements occurring at the beginning as well as at the end of the interval between two L-Dopa doses [34].

Management of dyskinesias implies detailed understanding of the L-Dopa cycle.

The most common approach is to lower the single L-Dopa dose. Controlled-release levodopa may worsen dyskinesias, especially later in the day due to cumulative effect. Amantadine in doses between 100 mg and 400 mg can be effective, but side effects are frequent in more advanced patients and should be carefully monitored. These include edema, livedo reticularis, and confusional state or hallucinations and psychosis.

Clozapine, an atypical dopamine receptor antagonist, has been found to be effective in reducing dyskinesia in advanced patients [35, 36], and it may be particularly useful when hallucinations are also present. Advanced patients, however, are particularly prone to develop agranulocitosis, with high risks of infections, and thus the white cell count should be regularly monitored.

Recent evidence suggests that memantine is also effective in reducing dyskinesia when other options are contraindicated [37, 38].

Despite limited evidence-based data high-frequency subtalamic DBS (DBS-HFS) has been shown by several reports to be surgically safe and able to produce improvements in dopaminergic drug-sensitive symptoms and reductions in subsequent drug dose and dyskinesias are well documented. However, the procedure is associated with adverse effects, mainly neurocognitive, with side-effects created by spread of stimulation to surrounding structures, depending on the precise location of electrodes. The occurrence of cognitive complications limits the motor improvements induced by STN-HFS to a short period of time, because patients' quality of life is greatly impaired by the progressing cognitive disorder. In late stage of disease the rate of patients eligible for surgical treatment of PD is extremely low, due to age and general debilitation that significantly increase the risks of short- and long-term complications.

3.3. Drug Failure Response. As the disease progresses, the efficacy of L-Dopa progressively decreases and patients may not respond at all to administered doses. This phenomenon is more pronounced later during the day and may be related to poor gastric emptying and insufficient intestinal absorption. Domperidone is an effective option, where available. The neutral aromatic amino acids contained in dietary proteins may compete with L-Dopa for intestinal absorption and transport across the blood-brain barrier, thus limiting its efficacy and being responsible for the occurrence of motor fluctuations. Low-protein dietary regimens with protein redistribution by shifting protein intake to the evening are an effective strategy to ameliorate the response to L-Dopa. Low-protein products designed for chronic renal failure patients are also a safe, well-tolerated, and useful option for end-stage patients [39].

4. Nonmotor Complications

The neuroanatomical and neurochemical substrates of the majority of non-motor symptoms are still unclear, although the concept of Parkinson's disease as a six-stage pathological process introduced by Braak and collegues [40] provided critical information to understand the physiopathology of several nonmotor symptoms, such as sleep disorder, autonomic dysfunction, and visual hallucinations.

Several studies have shown that non-motor symptoms impact significantly on quality of life and institutionalization is greater than for the motor symptoms [41, 42]; so in recent year attention was focused on the development of measures specifically designed to recognize and quantify these symptoms in advanced patients, and they are now also widely used in the clinical trials.

The development of clinical measures useful in recognizing and quantifying these symptoms deeply improved the clinical care as well as the clinical trials.

The Non-Motor Symptoms Scale, for instance, is a 30-item scale for assessment of nine dimensions (cardiovascular, sleep/fatigue, mood/cognition, perceptual problems, attention/memory, gastrointestinal, urinary, sexual function, and miscellany), that has proven to be a valid, reproducible, and accurate tool in rating severity and frequency of non-motor symptoms in PD [43, 44].

4.1. Dementia. Community-based studies of dementia in patients with PD have reported a prevalence between 28% and 44%, with longitudinal studies estimating that dementia occurs in up to 75% of patients [45]. The pattern of deficits is similar to dementia with Lewy bodies and differs from that in Alzheimer's disease for the predominant involvement of executive, visuospatial, and attention dysfunction and for the presence of cognitive fluctuations [46–49].

The cognitive symptoms are a consequence of dopaminergic depletion [50] in the corticostriatal loop and of dysfunction of the cholinergic system [51]. Serotoninergic and noradrenergic mechanisms may also be involved, though their role is not well defined.

Dopaminergic replacement does not lead to cognitive improvement or may even worsen it, but cholinergic enhancement can instead be helpful. Cholinesterase inhibitors, in fact, may be effective in ameliorating cognition, but their tolerability seems variable due to peripheral cholinergic adverse effects and in some case can worsen motor functions. Rivastigmine seems the most useful agent [52], while more controversial is the benefit produced by donepezil [53, 54].

Avoiding the medications that can possibly worsen dementia, like anticholinergics and DA-agonists, as well as maintaining L-Dopa at the lowest effective doses, is certainly a key strategy to contain confusion, hallucinations, and psychosis in advanced patients [55].

4.2. Hallucinations and Psychosis. Behavioral disorders, and especially hallucinations, illusions, and other psychotic symptoms, are also frequent in advanced PD with frequency rates ranging from 25 to 30%. Resembling very closely those seen in dementia with Lewy bodies, psychotic symptoms in PD are represented by delusions (false and fixed beliefs maintained despite evidence to the contrary) and, particular, hallucinations (abnormal perceptions that can involve any sensory modality in the absence of a physical stimulus). Visual hallucinations, simple or complex in form, are the most common psychotic symptom in advanced PD patients, typically occurring in dim surrounding, but often occurring through the entire day in late-stage patients [56].

A range of factors contributes to the development of hallucinations and psychosis in PD, including intrinsic pathology and dopaminergic replacement therapy.

In the treatment of these complications the first step should always be to evaluate the role of drugs that can potentially induce or worsen psychosis, such as amantadine, anticholinergics, COMT-inhibitors, and DA-agonists. These drugs should be tapered off, balancing the effect on psychosis with worsening of motor function.

All precipitating events, like urinary and pulmonary infections, cerebrovascular events, and metabolic dysfunctions, should be also carefully investigated and treated if possible, as even mild metabolic imbalance or infection can profoundly affect the development of psychotic symptoms.

Decreasing the dose of L-Dopa should also be considered when severe psychosis persists, even though this action could worsen parkinsonim.

All traditional antipsychotic drugs, such as haloperidol, aripriprazole, and chlorpromazine, should be avoided because of the high sensitivity of PD patients to the motor adverse effects induced through potent antagonisms of D_2receptors.

Clozapine and quetiapine are the only two newest antipsychotic that should be considered atypical, thus safe in PD, due to their predominant affinity for D_1 and D_4receptor and low affinity for D_2 receptors.

There is a wealth of evidence demonstrating the efficacy and tolerability of clozapine in PD, but its use is limited by the need of weekly blood testing for the initial 6 months of treatment [57]. A more practical alternative is represented by quetiapine. Unlike clozapine, quetiapine does not require monitoring of blood cell counts and it is effective in suppressing hallucinations and psychosis in the majority

of patients at relatively low doses, ranging from 12.5 mg to 100 mg.

Main side effects of quetiapine and clozapine are sedation and postural hypotension.

4.3. Depression and Anxiety. Depression affects 40–60% of patients with PD and appears to be a major determinant of health-related quality of life in PD [58].

In some cases depression occurs during off periods; thus controlling the on-off fluctuation can improve depression.

Sedating antidepressants, like tricyclic (TCA), and more activating antidepressants, like selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), are useful but significantly limited in advanced patients by the ancticholinergic and orthostatic negative effects. SSRIs are also contraindicated in patients receiving slelegiline, because of the potential drug-drug interation leading to "serotonin syndrome".

S-Adenosyl-methionine (SAMe), a natural molecule present in all eucaryotic cells that participates as methyl group donor to a number of metabolic events, is reported to have an effective antidepressant effects [59], without worsening of Parkinsonism [60].

Anxiety often occurs during "off" periods and improves with better control of motor symptoms but can be a major source of distress for patients even during "on" state. Low doses of benzodiazepines are effective when anxiety is persistent and debilitating but may cause amnesia and confusion in advanced patients and are a risk factor for falls.

4.4. Sleep Disorders. Sleep disorders occur in almost all patients with advanced PD, and they consist of sleep fragmentation, REM sleep behavior disorders (RBDs), excessive daytime sleepiness, and altered sleep-wake cycle.

Sleep fragmentation can be caused by difficulty turning in bed or nocturnal dystonia and can be ameliorated with controlled-release levodopa. Increased nocturnal urinary frequency can also affect sleep and can be controlled by reducing the amount of liquids in the evening, when anticholinergic drugs are contraindicated.

RBD is a disruption of the normal REM sleep cycle, in which the paralysis that normally occurs during REM sleep is incomplete or absent, making the patient "act out" their dreams, that usually are vivid, intense, and violent. Dream-enacting behaviors can be complex, including talking, yelling, punching, kicking, jumping from bed, and grabbing, with great distress for the patient and bed partner. RBD also prevents physiological nocturnal restoration of dopamine reseverve in cells, with worsening of parkinsonian symptoms. RBD improves when dopaminergic medications are reduced at bedtime. When RBD persists, low doses of clonazepam are effective and should be considered.

Modafinil, a wake promoting agent approved for narcolepsy, is effective in ameliorating daytime sleepiness induced by dopamine-agonists without significant side effects [61] and can be helpful in ameliorating alertness in advanced PD.

4.5. Autonomic Dysfunction

4.5.1. Orthostatic Hypothension (OH). OH is defined as a fall in systolic blood pressure below 20 mmHg and I diastolic pressure below 10 mmHg within 3 minutes of standing. Orthostatic intolerance related to OH results from a reduction of cerebral perfusion when upright and presents in severe cases with lightheadedness or syncope, exposing the patient to high risk of fall.

Careful education of patients and caregivers on factors that can trigger the OH symptoms, like avoiding rapid changes of position or straining during micturition or defecation, is essential in the management of OH.

Fluid intake, particularly in the morning, should be maintained at around 2 L of water daily and at least 8 g of sodium chloride is recommended to ensure adequate hydration [62].

Antihypertensive therapy, when present, should be reconsidered and eventually discontinued. Thromboembolic elastic stocking and abdominal binders can be helpful and should be encouraged.

When OH becomes more severe, it is necessary to start pharamchological agents such as plasma volume expander, like fludrocortison, and vasoactive agents, like midodrine.

4.5.2. Dysphagia, Nutrition, and Hydration. Severe dysphagia occurs frequently at late stage of disease causing weight loss, malnutrition, dehydration, and significantly increasing the risk of inducing aspiration pneumonia and death.

In order to make the swallow safer and more effective swallowing maneuvers, like the supraglottic swallow maneuver, the super supraglottic swallow maneuver, the Mendelsohn maneuver, and the effortful swallow maneuver, should be taught to patients.

Dysphagia for fluid can be controlled adding thickening agents, or thickeners, to liquids, increasing their viscosity without substantially modifying their other properties, such as taste. They provide body, increase stability, and improve suspension of added ingredients. Some thickening agents are gelling agents, forming a gel that can be swallowed by patients significantly reducing the risk of chocking.

When dysphagia becomes more severe, PEG should be considered. In this phase PEG could be a useful solution to guarantee to patients' adequate food and fluid intake as well as dopaminergic therapy through infusion.

4.5.3. Genitourinary and Elimination. Constipation is a common and early manifestation of PD but in late stage can become particularly severe due to the combination of anti-PD medications, slowed intestinal motility, immobility, and dehydration. Constipation should be well managed in order to avoid bowel occlusion and in order to ensure proper absorption of L-dopa and other medications. Dietary supplementation of fibers that stimulate intestinal motility should be encouraged as well as increased fluid intake. A conservative therapeutic option is administration of macrogol (polyethylene glycol), which can lead to marked improvement [63].

Many late-stage PD patients face urinary problems such as urgency or frequency or stress incontinence, which can cause anxiety and feelings of social isolation. Overactive bladder is the result of loss of normal inhibition by the basal ganglia and the frontal cortex to the sacral spinal cord [64]. Anticholinergics are commonly used to inhibit the overactive bladder, although their use should be discouraged in late-stage patients due to cognitive and other central anticholinergic adverse effects [65]. Newer generation of peripheral anticholinergics, like trospium, is better tolerated and can be used sometimes even in advanced patients. Recently, botulinum toxin injections in the detrusor muscle have demonstrated marked efficacy in reducing the urinary frequency with no side effects [66].

Reduced mobility and difficulty toileting often lead to the use of urinary pads or catheters at end stage of disease, exposing the patients to high risk of urinary dangerous infections when hygienic measures are not appropriate.

5. Supportive Care

Supportive care in advanced PD patients should include physical and rehabilitative therapy, occupational therapy, speech therapy, social work, and nursing care. These care services could greatly benefit late-stage patients by prolonging independency in the ADL and reducing complications like pain, decubiti, and falls.

5.1. Mobility. Full mobility should be encouraged and maintained as long as possible. Occupational and physical therapy should be encouraged whenever possible. Individual rehabilitative therapy sessions should be encouraged two to three times weekly for 30- to 40-minute durations even at late-stage when the patient is able to safely ambulate. Falls are perhaps the greatest concern for late stage PD patients who are still mobile, and patients should be discouraged to stand or walk without assistance at very late stage of disease. If patients are bedridden, residual mobility should be maintained through active and passive movement exercises, frequent position changes, and breathing exercises to prevent complications associated with being bedridden, such as decubitus, contracture, pain, and pneumonia [67].

5.2. Nutrition, Hydration, and Genitourinary Care. Malnutrition is a common problem in advanced PD patients. It is caused by difficulty feeding, altered satiety mechanism, diminished gastric and intestinal motility, inactivity, lack of appetite, dysphagia, and metabolic syndrome. In patients still able to eat independently, meal and portion sizes should be monitored in order to provide sufficient nutrition. Any effort, including compensatory strategies, should be considered to delay the PEG placement. Adequate hydration is another concern for late-stage PD patients, since even mild temperature change can lead to relative dehydration and exacerbate confusion and OH and cause syncope. Many patients become embarrassed when eating or drinking, and nursing assistance, can assure adequate nutrition and hydration through a nonjudgmental caregiver that assist patients with the administration of meals.

5.3. Communication. Difficulty with speech with severe dysarthria, hypophonia, tachylalia, and freezing of speech is another problem associated with late-stage PD and leads to significant source of frustration for patients and families. Speech therapy should be encouraged whenever possible. The Lee Silverman Voice treatment has been shown, clinically and scientifically, to be a powerful method of improving speech and related functions such as swallowing and facial expression in PD, with documented Improvement in vocal loudness, voice quality, prosody, and speech articulation, sustained at 1-year and 2-year follow-ups [68]. Simplified and codified communications (like asking yes/no questions, or by using alphabet boards or speaking dictionaries) can become the only way of effective communication [69] and should be considered.

6. End of Life Care

When patients with advanced PD encounter a medical illness requiring an extended rehabilitation stay, they are often transferred to subacute rehabilitation facilities with no expertise in treating Parkinson's disease. These transfers often lead to an inevitable decline due to worsening of dementia, psychosis, and social withdrawal. Nursing home placement should be delayed as long as possible, because of the well-known risk of reduced survival. As death approaches for late-stage PD patients, it is important to provide them with the best care possible in a passionate environment. Many patients choose to do this through hospice care. Support to families, through social work and psychological counseling, should be offered at this time.

7. Conclusion

The management of end-stage PD challenges clinicians, patients, and families in many ways.

The main goal should be to maintain acceptable levels of functioning through careful balance not limited to drug management, but including strong and supportive services.

Many patients with advanced PD, in fact, benefit from a more intensive intervention to address the complexity of the disease. Medication management can become arduous with on/off fluctuations and dyskinesias, frequent falls, constipation, blood pressure instability, cardiac problems, and other medical complications of PD developing and becoming more severe as the disease progresses. The process is further complicated when speech is affected, and swallowing becomes difficult with malnutrition and risk of developing aspiration pneumonia. Psychological problems often accompany these later stages of the disease, including anxiety, depression, and insomnia. Cognitive problems and hallucinations also are prominent.

There comes a time when it becomes too difficult to manage all these complexities at home. Patients and caregivers become overwhelmed, often with unnecessary

catastrophic consequences. Institutionalization typically follows the dramatic period of declining health and diminished ability to cope. For most persons with advanced PD the quality and dignity of a life at home are much superior to what they can ever expect in a nursing home. A well-designed interdisciplinary intervention can, in most cases, resolve many problems and render the care of patients much more manageable at home. Unfortunately, medical facilities are unprepared to accommodate the needs of the neurologically frail and complex PD patients.

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REVIEW ARTICLE

Catechol-*O*-Methyltransferase Inhibitors in Parkinson's Disease

Thomas Müller

Abstract Inhibitors of catechol-*O*-methyltransferase (COMT) are commonly used as an adjunct to levodopa in patients with Parkinson's disease (PD) for the amelioration of wearing-off symptoms. This narrative review aims to discuss the role of COMT inhibitors on peripheral levodopa metabolism and continuous brain delivery of levodopa, and to describe their metabolic properties. Oral application of levodopa formulations with a dopa decarboxylase inhibitor (DDI) results in fluctuating levodopa plasma concentrations, predominantly due to the short half-life of levodopa and its slowing of gastric emptying. Following transport across the blood–brain barrier and its metabolic conversion to dopamine, these peripheral 'ups and downs' of levodopa are reflected in fluctuating dopamine levels in the synaptic cleft between presynaptic and postsynaptic dopaminergic neurons of the nigrostriatal system. As a result, pulsatile postsynaptic dopaminergic stimulation takes place and results in the occurrence of motor complications, such as wearing-off and dyskinesia. More continuous plasma behaviour was observed after the combination of levodopa/DDI formulations with COMT inhibitors. These compounds also weaken a levodopa/DDI-related homocysteine increase, as biomarker for an impaired methylation capacity, which is involved in an elevated oxidative stress exposure. These findings favour the concept of chronic levodopa/DDI application with concomitant inhibition of COMT and monoamine oxidase, since deamination of dopamine via this enzyme also generates free radicals. This triple combination is suggested as standard levodopa application in patients with PD who need levodopa, if they will tolerate it.

Key Points

Catechol-*O*-methyltransferase (COMT) inhibitors are well established for the treatment of wearing-off phenomena in patients with Parkinson's disease (PD).

Inhibition of COMT supports a more continuous brain delivery of levodopa. Inhibition of monoamine oxidase enables more continuous central dopamine levels. Both therapeutic approaches have synergistic effects for the principle of continuous dopamine substitution, which improves motor complications in patients with PD, as shown in clinical trials.

Therefore, chronic levodopa/dopa decarboxylase application with concomitant inhibition of COMT and monoamine oxidase is suggested as standard levodopa application in patients with PD who need levodopa, if they will tolerate it.

1 Introduction

Parkinson's disease (PD) is the most common chronic neurodegenerative disease that affects movement behaviour. Approximately 2 % of individuals over the age of 65 years and up to 5 % of those aged over 85 years suffer from the disorder [1]. PD is mainly pathologically

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characterised by dopaminergic neuronal loss in the substantia nigra and consecutively by striatal dopamine loss with the accumulation of the protein α-synuclein [2–4].

However, chronic neuronal death also affects other neurotransmitter systems both in the periphery and in the brain. In the periphery, orthostatic hypotension is a result of sympathetic neurocirculatory failure, characterized by cardiac sympathetic failure. Both are in line with a reduced generation of biogenic amines in the adrenal medulla of patients with PD [5]. In the brain, the neurodegenerative process also takes place in the predominant norepinephrinergic locus ceruleus, the serotonergic Raphe nuclei and the cholinergic nucleus basalis Meynert. All of them induce dysfunction of cortical and limbic projections and disturbances of vegetative nervous system function in the region of the dorsal nucleus of the vagus nerve or the sympathetic ganglia [2–4].

Additionally, serious cytoskeletal damage is found in glutamatergic, gamma aminobutyric acid-ergic, cholinergic, noradrenergic, serotonergic and peptidergic neurons [6]. As a result, an individually pronounced and different expression of symptoms occurs in each patient with PD.

Tremor at rest, bradykinesia, rigidity and postural instability are the main features of motor impairment. These symptoms are accompanied by the onset of a wide variety of non-motor symptoms. Non-motor symptoms have gained increasing attention in previous years as further characteristic clinical features of the disease [7–9]. The term PD actually reflects a superordinate concept for a variety of different kinds of diseases. They resemble each other and do not always share the concept of the neuropathological manifestation of Lewy bodies as an essential initial step to the onset of the disease process [4, 10, 11].

1.1 The Cause of Parkinson's Disease (PD) is Still Not Known

Various hypotheses exist as to the cause of PD, including genetic defects or gene mutations, impaired detoxification capacity, exposure to acute and chronic endogenous and exogenous toxins such as pesticides, deficiencies of mitochondrial function, infection by prion-like proteins, protein misfolding, inflammation, and decrease of neurotransmitter capacity, including monoamine storage vesicles and glutamate metabolism [12–14]. In the cascade of events leading to neuronal death, all of these hypotheses share one common step, which is an increased synthesis of free radicals. All of these mechanisms contribute to the onset of the heterogeneous forms of PD as a result of the predominant death of dopamine-synthesizing presynaptic nigrostriatal neurons [6, 12–14].

2 Objectives

This narrative review aims to discuss the role of catechol-*O*-methyltransferase (COMT) inhibition on peripheral levodopa metabolism, continuous brain delivery of levodopa and the metabolic advantages of COMT inhibition.

3 The Role of Dopamine in PD

3.1 Dopamine Supplementation as Therapeutic Principle

Substitution of the dopamine loss is the most essential treatment approach in PD for the alleviation of motor symptoms and of certain non-motor symptoms, for instance apathy and/or cognitive slowing. Both result from a decline in dopaminergic stimulation of the mesolimbic system and frontal brain structures. Non-motor and motor features of PD respond to dopaminergic therapy in an individually pronounced fashion. The compensation of this dopamine deficiency with the various available therapeutic modes of dopamine substitution preponderantly ameliorates, for instance, the motor symptoms akinesia and rigidity and, to a lesser extent, tremor. However, disturbances of postural reflexes do not respond to dopamine substitution [15]. This adjustment of the impaired nigrostriatal dopamine neurotransmission also prevents an adaptation of the patient with PD to the disease process itself. This is, for instance, the case for symptoms like walking with small steps only or the manifestation of bound posture. This altered movement behaviour partially results from an unconscious learning process to balance the PD-related deficits of emotion and motion execution [16]. Therefore, evidence is emerging that treatment in PD should start as early and as effectively as possible. This concept is supported by results from various long-term trials on the effects of early optimum direct or indirect adjustment of the nigrostriatal dopamine loss [17]. Direct dopamine application as the best theoretical physiological way is not possible, since dopamine itself may not pass the blood–brain barrier (BBB), in contrast to its direct metabolic precursor, levodopa. In the brain, levodopa is converted by the enzyme dopa decarboxylase to dopamine in dopaminergic and serotonergic neurons [15].

3.2 Levodopa: The Double-Edged Sword in the Treatment of PD

The most efficacious and best-tolerated drug for the treatment of PD is levodopa. The introduction of levodopa was a therapeutic breakthrough, although it is worth mentioning that levodopa as a drug for PD patients would probably not be approved in the contemporary clinical research world

with its demands for safety and tolerability by the approving authorities due to too frequent onset of electrocardiographic changes, nausea and gastrointestinal disturbances [15, 18].

The effect of levodopa in PD was first reported by Birkmayer and Hornykiewicz [19], who had the courage to ask adventurous patients to take levodopa as an infusion in an adequate dosage. At that time, they tested a new treatment paradigm in a clinical research world with distinctly fewer administrative hurdles and bureaucratic overload [18]. Nowadays, the optimal use of levodopa is still under debate, predominantly due to the clinical observation of the onset of fluctuations of movement in the course of PD. These so-called motor complications are predominantly associated with levodopa due to its short plasma half-life [15].

3.3 Levodopa-Related Fluctuations of Movement

Motor complications may be roughly subdivided into OFF-phenomena, which describe the reappearance of a reduced motor performance after an ON-interval of good response to adequate dopaminergic neurotransmission, and into dyskinesia, which are involuntary movements that mostly result from an over-stimulation of the dopaminergic system.

Dyskinesia can occur during both ON and OFF periods. Classification of dyskinesia is generally performed in relation to the timing of levodopa administration. ON dyskinesia appear either (1) during the period when patients experience maximal relief from their motor symptoms, in which case they are classified as peak-dose dyskinesia [20–22], or (2) in a biphasic fashion, soon after intake of levodopa, when the patient starts to turn ON; they reappear again when the levodopa effect is wearing off and the patient begins to turns OFF. The threshold concentration for dyskinesia onset and the concentration necessary to get patients out of the OFF state increasingly converge with the progression of PD [23–25]. Therefore, the maximum levodopa plasma level after drug intake often causes peak-dose dyskinesia as the most common form of these involuntary movements [20]. As the disease progresses, patients may develop dyskinesia throughout the whole ON time, spreading over the whole body in an individually pronounced fashion [26]. Generally, PD patients better tolerate and accept mild dyskinesia more than OFF periods [27].

The risk of developing dyskinesia has been associated with a number of clinical factors. The severity of PD and the dosage of levodopa therapy, and a younger age of the patient are currently believed to be among the variables that best predict the development of dyskinesia [20, 28]. Dose, dosing strategy, and the timing of meals are further essential determining factors for the development of dyskinesia and motor complications [23, 26].

3.3.1 Motor Complications as an Essential Feature for the Progression of PD

Generally, the onset of motor fluctuations is regarded as one essential clinical milestone in the progression of PD. Peaks and troughs of plasma levodopa levels are, to a certain extent, transferred into ups and downs of dopamine concentrations [24]. This results in a pulsatile stimulation of postsynaptic dopamine receptors, which in turn supports onset of movement fluctuations [29]. Initially they are predictable and thus in relation to prior drug intake. Later they become unpredictable and show no relation to previous drug intake. Generally motor fluctuations can be brief or long term, lasting for minutes, or even hours. They cause patient disability, embarrassment and frustration and caregiver burden [20].

3.3.2 Treatment of Motor Complications: Still an Unmet Need

Therapy or even prevention of motor complications, particularly wearing-off phenomena, is still a major problem. These motor side effects of long-term levodopa therapy initiated a long debate with a focus on various hypothetical models of basal ganglia interaction and dysfunction [23, 24]. The long-term side effects of levodopa application in relation to the blocking of its metabolizing enzymes were only considered to a certain extent.

3.4 Levodopa: Modes of Oral Application

Levodopa was initially administered as an infusion, followed by an oral form without inhibition of the essential levodopa-degrading enzymes dopa decarboxylase and COMT. Treatment with oral levodopa was subsequently improved with the combination of oral levodopa formulations and dopa decarboxylase inhibitors (DDIs). The two commonly used dopa decarboxylase inhibitors are carbidopa and benserazide, both of which only act in the periphery. The next step was the introduction of COMT inhibitors [15, 19, 30].

3.5 Blocking of Levodopa Metabolism

The basic pharmacological principle underlying many of the approved drugs for PD involves enzymatic inhibition of levodopa degradation, leading to a reduction in the peripheral conversion of levodopa to dopamine. Therefore, the plasma bioavailability of each orally administered levodopa compound rises due to the extension of the plasma half-life of levodopa. Accordingly, the clinical benefit of levodopa on motor behaviour improves in patients with PD.

The addition of a DDI to levodopa allows a four- to fivefold reduction of the oral levodopa dose. As a result, the frequency of levodopa-related peripheral side effects,

such as nausea and vomiting, declines. DDI shifts the peripheral levodopa turnover to the COMT enzyme.

COMT is a major catabolic regulator of synaptic catecholamine neurotransmitters. COMT catalyzes the transfer of a methyl group to catecholamines and degrades dopamine, norepinephrine and epinephrine [31]. COMT is densely expressed throughout both the prefrontal cortex and the limbic system [32]. High COMT activity is also found in the liver, kidney and gut wall. The enzyme activity is controlled by the *COMT* gene.

The two forms of COMT (soluble COMT [S-COMT] and membrane-bound COMT [MB-COMT]) are coded by a single gene. This gene is located on the chromosome band 22q11.2 [31, 33]. S-COMT contains 221 amino acids. MB-COMT has an additional amino terminal extension of 43 (rat) or 50 (human) amino acids. The hydrophobic 17 and 24 amino acid residues in rats and humans, respectively, form an alpha-helical transmembrane domain, which is the membrane anchor. MB-COMT is not a precursor of S-COMT [33]. Constraint of COMT enzyme activity further diminishes peripheral levodopa metabolism.

This adjunct prolonging of the levodopa plasma half-life elevates its plasma appearance and, accordingly, its brain delivery. Experimental and clinical study results underline the efficacy of peripheral dual inhibition of the main levodopa-metabolizing enzymes, which reduce peripheral dopamine generation and accumulation of the levodopa metabolite 3-*O*-methyldopa (3-OMD) [34, 35].

3.6 3-OMD

The plasma half-life of the *O*-methylated levodopa derivative 3-OMD is between 15 and 24 h and depends on renal excretion [34]. 3-OMD competes with levodopa at the large neutral amino acid transport carriers of the gastrointestinal tract and of the BBB. Elevation of peripheral 3-OMD concentrations may interact with the absorption, plasma bioavailability and brain delivery of levodopa. COMT inhibition reduces 3-OMD synthesis [36–38]. Hypotheses suggest that this 3-OMD reduction contributes to a better absorption and BBB transfer of levodopa [34, 35]. This issue is still under debate. It was also suggested that at clinical concentrations 3-OMD makes a small contribution to the large total neutral amino acid pool competing with levodopa for brain entry [36, 39].

3.7 Oral Levodopa Administration and Continuous Dopaminergic Stimulation

The first therapy interval with levodopa is generally described as the honeymoon period, since it is associated with good tolerability and motor response to oral levodopa intake. The onset of predominantly levodopa-related motor complications may occur after an interval of several months or years [23]. The plasma levodopa half-life of approximately 60–90 min following oral intake determines its pharmacokinetic behaviour to a considerable extent, which is characterised by ups and downs of peripheral levodopa plasma levels [40–43].

This variability in levodopa levels is further promoted by gastrointestinal transport and absorption mechanisms. Following the swallowing process, levodopa-containing tablets must pass the stomach and reach the jejunal structures, where levodopa absorption predominantly takes place [44]. Pharmacokinetic investigations comparing oral levodopa/carbidopa application in a standardized fashion, with and without intake of the COMT inhibitor entacapone on two different days, described nearly identical concentration–time curves [40, 41, 43, 45]. However, there were differences between subjects. One possible reason is the influence of the gastric emptying rate. Slowed or delayed gastric emptying decreases plasma levodopa occurrence in general and delays peak levodopa concentrations. Thus, the gastric emptying velocity is one further essential determinant for the onset of levodopa effect on motor symptoms in patients with PD [41–43, 46].

All these peripheral mechanisms of gastrointestinal transport, absorption and pharmacokinetic behavior of levodopa predispose for occurrence of motor complications [44]. Accordingly, continuous, duodenal levodopa/carbidopa infusion pump systems reduce these peripheral levodopa plasma fluctuations. They circumvent the impact of gastric emptying. Frequency and intensity of motor complications in patients with very advanced PD considerably improve [47]. An additional further essential component of levodopa absorption may be the impact of food. High protein content, for instance in meat, eggs or fish, interacts with the gastrointestinal transporter system and limits levodopa uptake. Proteins or fat may also slow the gastric emptying velocity. These nutritional factors further facilitate an inter- and intra-individual variability of the peripheral levodopa metabolism behaviour in patients with PD [44]. Therefore motor complications may vary from day to day in terms of frequency and severity.

3.8 Central Prerequisites in the Brain for Manifestations of Motor Complications

After its transport across the BBB, levodopa is transformed and stored in vesicles of presynaptic dopamine-generating neurons or, as an alternative, in serotonin-generating neuronal cells. Progression of PD increases presynaptic neuronal degeneration and thus reduces the capacity of presynaptic dopamine storage. Moreover, control of synaptic dopamine concentrations via the presynaptic dopaminergic autoreceptors, and thus regulation of presynaptic endogenous

dopamine synthesis, gets increasingly lost [25, 48]. Both mechanisms of neuronal degeneration ease the release of abnormally high dopamine concentrations into the synaptic cleft. They enable non-physiological fluctuations of striatal dopamine levels, which complement with the ups and downs of levodopa plasma levels. These processes finally support a pulsatile, irregular stimulation of postsynaptic dopamine receptors and neurons.

Further downstream, intracellular changes take place [49]. They limit adequate physiological neuronal function, which is normally based on the principle of a continuous neurotransmission of dopamine [50]. Therefore, motor complications occur in the long term. The duodenal levodopa/carbidopa gel infusion by a pump system allows a more direct, external fine tuning of levodopa and indirectly more continuous dopamine supply in nigrostriatal structures. However, this method is expensive, complex and may cause dangerous, severe gastrointestinal infections and neuropathy [51–54]. Therefore, an optimisation of the oral levodopa drug supply is warranted by routes circumventing gastrointestinal absorption via transdermal or subcutaneous application routes or the additional use of modulators of peripheral and central levodopa and dopamine metabolism [55, 56]. These drugs are inhibitors of COMT and monoamine oxidase B (MAO-B).

4 The Development of COMT Inhibitors

4.1 The Failed Retarded Release Concept

Various approaches were undertaken to prolong the efficacy of each levodopa dose and to smooth out the fluctuations of levodopa plasma levels. The aim was to enable a more continuous levodopa brain delivery. First, oral levodopa/DDI formulations were developed with an extended release profile. These tablets showed a declined clinical efficacy in comparison with the conventional levodopa/DDI tablets, when the same oral L-dopa dosage was given. No delay of onset of motor complications according to clinical study outcomes was observed. However, these clinical investigations were not designed to assess or determine OFF phenomena and dyskinesia in detail [57, 58]. There is some evidence that retarded-release levodopa formulations show some additional benefit when applied with COMT inhibitors [59]. However, this concept was not further developed in randomized clinical trials.

4.2 Pharmacokinetic Behaviour, Dosing Intervals, Levodopa and COMT Inhibition: A Complex Issue

The principle of dual enzyme inhibition of dopa decarboxylase and COMT during oral levodopa/DDI therapy was further investigated in view of an experimental animal study outcome. It described less frequent and less intense dyskinesia during treatment with levodopa/DDI combined with the COMT inhibitor entacapone, given four times daily, when the COMT inhibitor was started right from the beginning of treatment [29, 60].

One clinical attempt to translate this concept into clinical practice was undertaken with the STRIDE-PD (STalevo Reduction In Dyskinesia Evaluation) study [61]. This investigation aimed to initiate levodopa therapy with levodopa/carbidopa or levodopa/carbidopa/entacapone, given four times daily at 3.5 h intervals. The primary endpoint was defined as the interval to onset of dyskinesia. Levodopa was applied with a fixed dosing regimen. No adaption of the dosing interval or dosage was allowed with an initial appearance of motor complications. However, this is common in clinical practice [62]. The study design did not consider pharmacokinetic findings on levodopa metabolism in patients with PD. They demonstrated that repeat additional dosing of entacapone, for instance every 3 h, or of tolcapone, increased the maximum and minimum plasma levodopa concentrations [40, 41, 43, 63, 64]. This result was not found after a single application of COMT inhibitors to a levodopa formulation [43, 63–65]. Elevation of bioavailability and an increase in the peak concentrations of levodopa after repeated levodopa intake support the risk for onset of peak-dose dyskinesia.

4.3 Reasons for the Performance of STRIDE-PD

Earlier clinical studies circumvented the problem noted in the STRIDE-PD trial [66, 67]. They were performed in levodopa-naïve patients. According to the study protocols, subjects received levodopa only three times daily (tid), with a distinctly longer dosing interval between drug administration than was used in STRIDE-PD.

4.3.1 FIRST-STEP-Study

The FIRST-STEP (*F*avorability of *I*mmediate-*R*elease Levodopa/Carbidopa vs *ST*alevo *S*hort-*T*erm comparison in *E*arly *P*arkinson's disease) study compared the efficacy of two different modes of levodopa application in patients with early PD who needed to initiate levodopa therapy. One study arm received conventional levodopa/carbidopa tablets. The other group of patients received levodopa/carbidopa together with the COMT inhibitor entacapone in one tablet. This multicentre, double-blind, randomized, parallel-group study administered a fixed oral levodopa dose of 300 mg/day, administered as 100 mg levodopa formulations tid at approximately 5 h intervals to 424 patients with PD. In the 39-week study, patients in the levodopa/carbidopa/entacapone arm performed

significantly better than those in the levodopa/carbidopa-treated cohort, both after week 4 and throughout the remaining course of the study according to the computed sum scores of the Unified Parkinson's Disease Rating Scale (UPDRS) part II (activities of daily living) and UPDRS part III (motor examination) as main primary outcome at the remaining study visits.

Thus, the FIRST-STEP trial only demonstrated that levodopa/carbidopa was inferior to levodopa/carbidopa/entacapone treatment, probably due to the more continuous peripheral levodopa plasma occurrence as a result of the COMT inhibition [63, 66]. The known additional levodopa/DDI efficacy-enhancing effects of entacapone, given as an extra tablet, to an existing levodopa/DDI regimen in treated patients was confirmed.

4.3.2 The ELLDOPA Study

Levodopa/carbidopa was given in different dosages (50 mg, 100 mg and 200 mg levodopa tid) in comparison with placebo tid in the ELLDOPA (Earlier versus Later LevoDOPA) study. Therefore, a plasma accumulation of levodopa was also unlikely and, if at all, occurred only after administration of the higher levodopa dosages with more pronounced ups and downs of levodopa plasma concentrations. Accordingly, dyskinesia rarely appeared and, if at all, mostly in higher dosages, for instance in the 200 mg tid arm after 39 weeks [67].

4.3.3 Interpretation of Both Trials

Both FIRST-STEP and ELLDOPA were designed as short-term follow-up studies. They did not assess the rate of motor complications during chronic therapy of patients with PD as a primary objective. Nevertheless, both trials showed some interesting findings regarding the onset and frequency of wearing off in patients with PD. In the FIRST-STEP trial, the number of monitored wearing-off phenomena was higher in the levodopa/carbidopa arm than in the levodopa/carbidopa/entacapone-treated patients. The frequency of noted wearing-off phenomena was rather low in relation to the size of the study population and the short observation interval. Therefore, this difference was not significant in the patients with early PD. Nevertheless, the results of these earlier studies allowed the conclusion at that time that entacapone supplementation may help to prevent the onset of wearing off due to a more continuous brain delivery of levodopa. In the ELLDOPA trial, the number of patients experiencing wearing off increased with higher levodopa/carbidopa dosing, probably due to more pronounced fluctuations of levodopa plasma levels in comparison with less pronounced levodopa fluctuations in plasma during the application of lower levodopa dosages.

4.3.4 The Failure of STRIDE-PD

STRIDE-PD confirmed that COMT inhibition improves wearing off. The interplay between pharmacokinetic plasma behaviour, shorter dosing intervals, COMT inhibition and the demands of the design with the missing possibility to adapt the levodopa dosage after initial onset of probable—mostly peak dose—dyskinesia were essential reasons for the premature onset of dyskinesia in the levodopa/carbidopa/entacapone arm in the STRIDE-PD trial [63, 68]. A more pronounced levodopa plasma accumulation took place, particularly where the higher oral levodopa dose was administered every 3.5 h [61]. This was indirectly confirmed in a further analysis of the failed STRIDE-PD trial, which described the oral levodopa dosage and thus more pronounced fluctuations of levodopa plasma levels as the main prerequisite for the onset of wearing off and dyskinesia in PD [28, 69].

4.4 The Importance of Pharmacokinetics for the Pharmacodynamics of Levodopa

It is noteworthy that certain levodopa-equivalent calculations should be scrutinized in randomized clinical trials. These so-called evidence-based medicine reviews suggest that patients receive levodopa 100 mg with one levodopa/carbidopa 100 mg formulation, levodopa 133 mg with one levodopa/25 mg carbidopa/200 mg entacapone tablet and levodopa 150 mg with one levodopa/carbidopa 100 mg combined tablet administered with tolcapone 100 mg. Therefore, one may assume a better efficacy of levodopa on motor behaviour based on higher levodopa plasma occurrence only [70]. However, this concept of equivalent dosage calculations is misleading, since the pharmacokinetic behaviour of levodopa during repeated intake and the dosing intervals are not considered in terms of pharmacodynamic effects of levodopa [40, 62]. The importance of these functional aspects of levodopa behaviour in plasma and delivery to the brain were shown in a pharmacokinetic trial. Within a standardised design, patients received the following via oral administration: (1) levodopa 100 mg plus carbidopa 25 mg in the morning and 4.5 h later again; (2) 1 week later, they received the same oral administration of levodopa plus entacapone 200 mg each; and (3) 1 week later the same oral levodopa dosage with tolcapone 100 mg each. Interestingly, the plasma bioavailability did not significantly differ between all three conditions. More continuous levodopa plasma behaviour was observed during additional entacapone/tolcapone intake. Thus, a less pronounced fall of levodopa, higher minimum levodopa concentrations and a lower fluctuations index appeared during additional COMT inhibition. All caused a beneficial effect on motor response during COMT inhibition [63].

Therefore, individually adapted dosing intervals during levodopa fractionation are essential in clinical practice to minimize levodopa plasma fluctuations and to optimise the motor response to levodopa [41, 63, 71].

5 COMT Inhibition and Gastrointestinal Absorption of Levodopa

A further difference exists between the levodopa/DDI administration with and without COMT inhibition. Generally, levodopa uptake depends on gastric emptying time, gastrointestinal absorption and transport via the gastrointestinal amino acid transporter system, as mentioned previously. Patients with PD often receive combination therapy involving multiple daily dosing of a particular compound and additional supplementation with other drugs, which share modes of action. Efficacy of all administered compounds depends on patient compliance, the nature of the delivery system, physicochemical properties of the drug and physiological considerations. Therefore, interactions between these compounds are likely. They may affect the rate at which the drug is absorbed throughout the gastrointestinal tract, then its bioavailability and metabolism [42, 44, 70].

Consistent COMT inhibition promotes the synthesis of more basic levodopa metabolites, i.e. the tyrosine aminotransferase-dependent substrates dihydroxyphenylpyruvate acetate and trihydroxyphenylacetate. Therefore, COMT inhibition may model the environmental pH and thus intestinal conditions for the duodenal absorption rate of levodopa. COMT appears in higher concentrations in the cells of the gastrointestinal tract. The physicochemical properties of a drug also affect its absorption through the gastrointestinal tract. Compounds, including levodopa, are weak bases or weak acids or are the salts of them and, as such, demonstrate pH-dependent solubility. The pH partition hypothesis asserts that the passage rate of a drug through a membrane depends on the environmental pH and the acid-base dissociation constant (p*K*a) of the drug. Drugs with low p*K*a are not ionized in the stomach and subsequently are rapidly absorbed. On passage to the small intestine, with its comparatively increased pH, the rate of ionisation changes and absorption subsequently slows.

The converse is true for drugs with a higher p*K*a value. This influences the bioavailability of hydrophilic drug formulations. They have a narrow window of absorption, limited predominantly to the stomach or the upper intestine.

Absorption is also limited by low p*K*a values and/or the site of active transport absorption mechanism, for instance in the case of levodopa [72]. Additionally, the absorption behaviour of oral levodopa/DDI tablets also depends on gastrointestinal transit rates, since uptake of levodopa occurs mainly in the proximal third of the small intestine (duodenum/jejunum) but not in the stomach. Intestinal levodopa absorption is rapid, but the plasma bioavailability of levodopa is only 30 % as a result of prior degradation to dopamine by DDI and to a lesser extent to 3-OMD by COMT, i.e. in the gut cells. The longer levodopa remains in the stomach and the small intestine, the more extensively it is metabolized and becomes less available for absorption [34]. A formulation sharing the peripheral absorption site profile of levodopa, is sodium octanoate. It is used as [^{13}C] marked substrate in breath tests, which are non-invasive, feasible, alternative methods without ionizing radiation to assess the gastric emptying velocity of solids and liquids. After intake, [^{13}C]-sodium octanoate is rapidly absorbed from the proximal intestine and carried to the liver via the portal venous system. There it is oxidized and eliminated as CO_2 in the breath, reflecting gastric emptying as the rate-limiting step of the process. Significant relations between levodopa plasma concentrations and the outcomes of the [^{13}C]-octanoic acid breath test were shown. There was no impact of COMT inhibition on gastric emptying time. However, the COMT inhibitor increased the recovery rate of the salt [^{13}C]-sodium-octanoate [72]. Therefore, one may assume that levodopa is better absorbed during COMT inhibition due to a more basic environment, which improves the absorption of the acid levodopa. This may further enhance the absorption and the bioavailability of levodopa due to COMT inhibition. The COMT inhibitors tolcapone ($C_{14}H_{11}NO_5$) and entacapone ($C_{14}H_{15}N_3O_5$) are both weak acids and have low aqueous solubility at acidic pH, which increases considerably in basic pH conditions. In turn, this supports absorption of both COMT inhibitors themselves in a basic environment. Tolcapone is significantly more lipophilic than entacapone at physiological pH values, therefore it passes the BBB as precondition for its central actions [63, 73, 74]. Further additional metabolic aspects of COMT inhibition exist.

6 Metabolic Aspects of COMT Inhibition

COMT is a widespread enzyme in the human body. One of its main tasks is the adding of methyl groups to a wide variety of compounds [34].

6.1 Chronic Methylation of Toxins and Drugs

Generally, metabolism also generates harmful substances during reduction, oxidation, conjugation and excretion. If substances such as drugs are present in higher concentrations than normal or are not expected to be present or produced, they will be metabolised [75]. Important

enzymes for the hepatic microsomal detoxification degradation are in the cytochrome P450 system. Transferases, i.e. UDP-glucuronosyltransferase, glutathione *S*-transferase, *N*- and *O*-methyltransferase are further examples [75, 76]. These enzymes are responsible for the turnover of certain drugs and endogenous and exogenous toxins. Toxins often lose their dangerous effect if a methyl group is added [34, 77]. In case of overstraining this kind of detoxification by chronic exposure or excessive occurrence of too high concentrations of the methylated substrate, this chronic reaction leads to an up-regulation of homocysteine synthesis as a biomarker for an elevated methyl group consumption [34, 76].

6.2 Examples for Drug-Induced Limitations of Methylation Capacity

The degradation by methylation of many anticonvulsive and centrally acting drugs, such as valproic acid or levodopa, also consumes methyl groups. Accordingly, chronic therapy with these compounds induces a homocysteine increase [78, 79]. Subsequently, a decline of methyl group-donating vitamins, such as folic acid or vitamins B_6 or B_{12}, occurs, as these vitamins promote the conversion of homocysteine to the methyl group donor methionine again. In the long run, a drug-induced deficiency of methyl group-donating vitamins occurs [75]. As a consequence of these metabolic changes during long-term administration of levodopa, an acceleration of ageing-associated brain atrophy, small vessel disease, cognition deterioration and peripheral nerve dysfunction may hypothetically appear [46, 80]. These symptoms are found in the course of PD, particularly when patients are receiving a high-dose levodopa/DDI regimen [16, 81].

6.3 The Role of Levodopa Turnover

In the presence of a DDI, the degradation of levodopa is predominantly shifted to *O*-methylation of levodopa to 3-OMD by COMT. COMT transfers a methyl group from the donor methionine. The resulting derivative *S*-adenosylmethionine is transformed into the short-living *S*-adenosyl-homocysteine and then to homocysteine [16, 81].

6.4 Homocysteine as a Marker for the Capacity of Methylation Processes

The up-regulation of homocysteine production reflects an inappropriate or reduced capacity for metabolism of other methylation processes. *N*-methyltransferase and *O*-methyltransferase, for instance COMT, have a broad detoxification potential that is regulated by a limited availability of methyl groups. If chronic drug degradation via COMT, like in the case of levodopa, consumes methyl groups, endogenous or exogenous toxins will no longer be detoxified by methylation processes in an adequate manner. Accordingly, the vulnerability for exposition against endogenous xenobiotics or exogenous substances, such as rural toxins and pesticides, increases [16, 81]. Against this background, it is interesting that chronic pesticide exposure is under investigation as a PD onset and progression-supporting phenomenon. However, researchers do not yet consider a possible impact of chronic levodopa/DDI exposure in PD patients on their findings [82].

6.5 The Methylation Potential and the Reversible Homocysteine Degradation to Methionine

A homocysteine increase also changes the ratio between the methyl group donor methionine, its metabolic intermediates *S*-adenosylmethionine and *S*-adenosyl-L-homocysteine, and finally homocysteine. This ratio is defined as methylation potential. The methylation potential also describes the flow of methyl groups between cells. Chronic homocysteine elevation is associated with higher levels of *S*-adenosyl-L-homocysteine and a low methylation potential. Thus, the re-methylation capacity declines. An increase of *S*-adenosyl-L-homocysteine also supports a further feedback inhibition of the *S*-adenosylmethionine-dependent methyltransferases, including the DNA methyltransferases. Thus, a low methylation potential is related to a decreased DNA methylation capacity, which may also weaken methylation-dependent gene regulation [75]. For instance, hyperhomocysteinemia exerts highly selective inhibitory effects on cyclin A transcription through a hypomethylation-related mechanism, which blocks cell cycle progression and regeneration [75]. In addition to remethylation of homocysteine, a further pathway for homocysteine decrease is an irreversible turnover of homocysteine to cysteine (Fig. 1) [16, 76, 83, 84].

6.6 Homocysteine Transformation to Cysteine and Synthesis of Antioxidants

This transsulfuration reaction metabolizes homocysteine to cystathionine by the enzyme cystathionine β-synthase [84]. Normal cystathionine β-synthase activity is essential for the generation of the cystathionine metabolite cysteine. Levels of this amino acid increase following the irreversible vitamin B_6-dependent degradation of homocysteine. Accordingly, a cysteine elevation occurs in levodopa-treated patients [85]. Cysteine, L-glycine and glutamine acid are the essential parts of the antioxidant glutathione, which is also known as the tripeptide γ-glutamyl-cysteine-glycine. Its levels reflect the thiol redox state, which is a fundamental mediator of numerous cell processes.

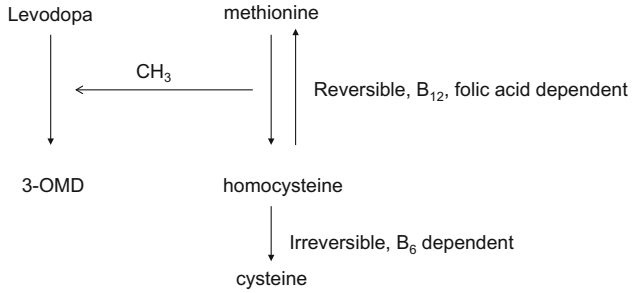

Fig. 1 Simplified schema of homocysteine turnover. *3-OMD* 3-*O*-methyldopa, *CH_3* methyl group

Glutathione is available in reduced monomeric and in oxidized dimeric forms, named GSSG, when thiol groups were reduced [86, 87]. Glutathione is abundant in the cytosol, nuclei and mitochondria, which are essential determinants of neuronal excitability and viability.

6.7 Glutathione Metabolism and Free Radical Generation

If glutathione scavenges free radicals, it will not be transformed to its metabolite cysteine-glycine. As consequence of this, the fall of cysteine-glycine following the application of a compound may be looked upon as an indirect biomarker for the induction of oxidative stress by this agent [88]. This was found following levodopa application with and without COMT inhibition. It is known that free radicals consume antioxidants like glutathione, which is subsequently converted to the derivative GSSG, the dimer of glutathione [88, 89]. An up-regulation of glutathione production may additionally encounter increased free radical appearance. Accordingly, a cysteine decay following a drug intake also indirectly reflects antioxidant consumption due to free radical scavenging. Unphysiological and too high free radical concentrations are involved in irregular harmful cellular metabolism, altered communication between cells and progression of neuronal degeneration [88, 89]. Generally, the activity of certain enzymes involved in glutathione metabolism may be associated with oxidative stress reduction, but other pathways also cause free radicals.

7 Monoamine Oxidase and Oxidative Stress

One of the pathways that causes free radical production is the mitochondrial monoamine oxidase, which is important for glial and mitochondrial dopamine degradation. Two types exist. Preponderantly, the subtype MAO-B is responsible for the oxidative deamination of dopamine. This reaction is supplemented by a reduction of molecular oxygen to hydrogen peroxide, a reactive oxygen species. If either the synthesis of reactive oxygen species is increased or the levels of antioxidants are reduced, oxidative stress will increase [13, 14, 86, 87].

7.1 Findings in Patients with PD

Chronic and high dosing of levodopa elevated homocysteine concentrations in levodopa-treated PD patients, but not in levodopa-naïve patients and healthy controls. These findings further confirm that levodopa, rather than the disease per se, induces hyperhomocysteinemia [90]. In addition to the levodopa dose, the treatment duration and disease severity and duration may also contribute to the elevation of homocysteine levels. This homocysteine rise was particularly found during high-dosage levodopa treatment with duodenal—or chronic oral—levodopa intake in PD patients [91]. This concomitant homocysteine increase during levodopa treatment is not only under suspicion as being associated with onset of neuropsychiatric symptoms but also of accelerating the ensuing peripheral axonal and central neurodegeneration, predominantly in the nigrostriatal, dopaminergic system [52, 91–97]. It may also contribute to elevated mortality rates from arteriosclerotic diseases and small vessel disease identified post-mortem in PD brains [80, 98].

7.2 COMT Inhibitors Lower or Even Prevent Homocysteine Rise

One further approach for homocysteine reduction is COMT inhibition on a regular basis, when levodopa/DDI treatment is performed and patients tolerate the COMT inhibitor. Since the combination of levodopa/DDI and COMT inhibitors reduces *O*-methylation of levodopa, it also decreases homocysteine levels [99]. A small prospective pivotal trial showed that addition of tolcapone to a stable anti-parkinsonian drug regime reduced homocysteine and its precursor *S*-adenosyl-L-homocysteine [100]. American prospective investigations with the COMT inhibitor entacapone failed, probably due to folate supplementation in the American diet, leading to a milder increase in homocysteine than expected [101, 102]. European observational non-prospective studies showed lower homocysteine in entacapone-treated patients. Thus, folate supplementation in the North American diet may also explain the heterogeneity of results [99, 100, 102–109].

8 Consequences of the Functional and Metabolic Advantages of COMT Inhibition for Levodopa Therapy in PD Patients

All these considerations and findings would hypothetically favour the concept of chronic levodopa/DDI application

with immediate concomitant COMT and MAO inhibition once levodopa has to be introduced in the therapy of PD as the most efficacious therapeutic compound [17, 18, 110] (Fig. 2). This combination is suggested as standard for levodopa application in PD therapy.

8.1 Levodopa/Dopa Decarboxylase Inhibitor (DDI) plus Inhibition of COMT and MAO: Reasons

There are two essential reasons for the implementation of levodopa therapy with DDI, COMT inhibitors and MAO-B inhibitors, which actually inhibit also MAO-A during repeated dosing [110]. A precondition is that this combination is safe and tolerable for the patient, which mostly is a result of individual exposure of the patient to this combination in clinical practice.

8.2 Continuous Dopamine Substitution

The first reason is that this concept is supported by a more continuous levodopa brain delivery on the basis of less fluctuating levodopa plasma levels with COMT inhibition and a more stable dopamine concentration in the synaptic cleft provided by MAO-B inhibition, with subsequent sparing of levodopa in the long term [111]. Whether the probably fluctuating COMT inhibition over the day, which requires intake of the available COMT inhibitors, is a certain drawback in terms of continuous levodopa pharmacokinetic behaviour is not yet known. However, both pharmacologic principles complement each other in terms of more continuous dopaminergic stimulation [112, 113]. A central-acting, safe COMT inhibitor without application restrictions would be of further advantage but is not currently available.

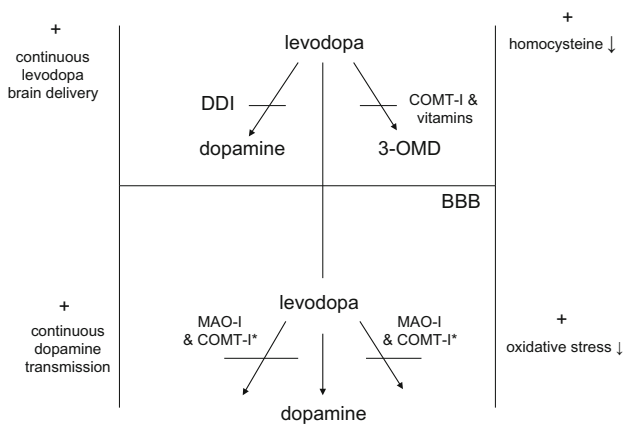

Fig. 2 Suggestion for an optimised dopamine substitution with levodopa. *BBB* blood–brain barrier, *COMT-I* catechol-*O*-methyltransferase inhibitor, *COMT-I** not available central-acting catechol-*O*-methyltransferase inhibitor without use restrictions, *DDI* dopa decarboxylase inhibitor, *MAO-I* monoamine oxidase inhibitor, *vitamins* methyl group donating vitamins, *plus* indicates advantage

8.2.1 *Less Oxidative Stress*

The second reason is that the combination of both MAO-B inhibition and COMT inhibition also decreases oxidative stress induced via the metabolism of dopamine via MAO-B and the decrease of free radical generation triggered by homocysteine elevation at least in the periphery (Fig. 1) [112, 113]. A supplemental intake of methyl group-donating vitamins is recommended.

9 Available COMT Inhibitors

In the late 1980s, several compounds with a nitrocatechol structure were developed as potent, selective and reversible COMT inhibitors. They were considered to be second-generation COMT inhibitors, namely tolcapone and entacapone. Following positive results from large clinical trials in patients with fluctuating PD symptoms, both were introduced into clinical practice for PD in the late 1990s for the treatment of wearing off. Their pharmacological properties have been reviewed in detail [35]. From the pharmacological point of view, tolcapone appears to be more efficacious than entacapone, with higher inhibition of COMT activity, central action and a longer duration of action after oral administration to rats and humans [73, 74, 114]. The most common observed side effect of the available COMT inhibitors is harmless discoloration of the urine. An additional clinically relevant adverse event of COMT inhibition is diarrhoea, sometimes occurring even up to 2–4 months following treatment initiation [115].

9.1 Entacapone

The only peripherally acting COMT inhibitor, entacapone, was initially given as an extra tablet with each levodopa/DDI dose [115]. It improved the efficacy of levodopa on motor impairment with the focus on reduction of OFF-time in patients with fluctuating PD [116–124].

9.1.1 *Safety and Tolerability*

Phase III studies and post-marketing surveillance showed the safety, tolerability and efficacy of levodopa in combination with the DDIs, carbidopa or benserazide, and entacapone even with co-administration of selegiline, dopamine agonists and antidepressants such as imipramine [116–124].

9.1.2 *Regulatory Affairs*

One entacapone 200 mg tablet is taken with each levodopa/DDI dose. The maximum recommended dose in Europe is

200 mg ten times daily, i.e. 2,000 mg of entacapone. This increased number of tablets may reduce compliance [115]. This disadvantage of entacapone therapy was improved with the introduction of the triple fixed-dose combination of levodopa/carbidopa/entacapone (Stalevo®) [45].

9.1.3 Stalevo®

Patients with advanced PD must frequently take levodopa, sometimes up to every 2 h. This levodopa 'fractionation' reduces temporary loss of efficacy, which is associated with reappearance of motor symptoms. The introduction of Stalevo® was an essential step forward from the older levodopa/DDI plus an extra entacapone tablet regimen, as the frequency of tablet intake was reduced. The pill size was also distinctly smaller, which further eased swallowing and improved patients' acceptance of the drug. Dysphagia is a well recognized symptom of PD. Patients experiencing this symptom are not expected to comply with oral administration or to obtain optimal bioavailability of levodopa [45]. One drawback was that only few levodopa strengths were initially available, which, from the treating physician's perspective, limited the ability to individually adapt titration form the original levodopa/DDI. This situation improved with the introduction of additional Stalevo® formulations, which enabled individual adaptation of levodopa in a range between 50 and 200 mg in 25 mg levodopa equivalents [45, 125–129]. Switching from levodopa plus entacapone coadministration to Stalevo® in patients with advanced PD provides additional benefit in clinical practice, and compliance problems may be reduced. Hypothetically, the improved timing of enzyme blockade with synchronous ingestion of both enzyme inhibitors is better for the pharmacokinetic levodopa behaviour; however, this has not yet been proven in a pharmacokinetic trial.

9.2 Tolcapone

Tolcapone was introduced before entacapone and, similarly, may also induce dyskinesia to a considerable extent, dependent on the design of the trial. Repeated dosing of levodopa is known to result in an increase of maximum concentration and bioavailability in plasma. Tolcapone also possesses centrally acting properties, but a tolcapone trial in levodopa-naive patients failed [130]. The hypothesis was that striatal dopamine is metabolized by COMT and MAO-B and thus central COMT inhibition with tolcapone alone or in combination with MAO-B inhibition might provide a symptomatic benefit for patients not receiving levodopa. This pilot study investigated the tolerability, safety and efficacy of tolcapone alone and in combination with oral selegiline in untreated patients with early PD. Patients were randomized to receive tolcapone 200 mg tid or placebo for the 8 weeks of the study. Open-label oral selegiline (5 mg in the morning and midday) was administered to all patients during the second 4 weeks of the study. There was no difference between treatment groups according to the investigator's assessment of tolerability at week 4. During the initial 4 weeks, 95 % of patients treated with tolcapone and 98 % of those receiving placebo experienced good tolerability. A decrease in tolerability occurred in the tolcapone group during the second 4 weeks of the study following the addition of selegiline. No symptomatic benefit was associated with tolcapone alone or in combination with oral selegiline in these otherwise untreated patients. However, this trial points out that even central COMT inhibitors are only efficacious on motor behaviour in combination with levodopa. In patients with more advanced PD, switch-over studies with the competitor entacapone, which acts only in the periphery, showed that tolcapone with its additional central effects on dopaminergic neurotransmission is more efficacious in terms of reduction of PD symptoms. However, the outcomes of the best randomized controlled trial available were not conclusive. In this study, the primary outcome was number of patients (proportion) with ≥1 h ON time response. The results were entacapone 32 (43 %) and tolcapone 40 (53 %) ($p = 0.191$). Although the results were statistically not significant, it was suggested that the tendency favouring tolcapone was consistent and probably not a result of chance [131]. The addition of tolcapone also reduced motor symptoms to a similar extent in comparison with the dopamine agonists, bromocriptine and pergolide, in open-label trials. Quality of life scores were significant better under tolcapone due to fewer dopamine agonist-related side effects. Moreover, titration was not necessary with tolcapone but was with the dopamine agonists, which additionally biased this outcome. These trials have limited value, since they were under-powered to detect clinically relevant differences between tolcapone and the dopamine agonist [132–135].

9.2.1 Safety Issues

One hypothesizes that mutations in the *UDP-glucuronosyltransferase 1A9* gene, which leads to defective glucuronidation activity, predispose for COMT inhibitor-induced hepatotoxicity [136]. After three fatal cases of hepatotoxicity, tolcapone was temporarily withdrawn nearly all over the world from November 1998 until April 2004. It was approved again; however, its prescription now demands a strict control of liver enzyme activity on a regular basis, both in Europe and to a lesser extent in the USA. For instance, the administration of tolcapone is now restricted to prescription and supervision by physicians

experienced in the management of advanced PD. Additionally, liver function tests must be carried out on a regular basis. If the dose of tolcapone is raised to 200 mg tid, liver enzymes should be checked prior to initiating the higher dose, and the monitoring scheme should be reset from the beginning. A further criterion is that patients with PD must fail to respond or be intolerant of other COMT inhibitors before starting tolcapone. Contraindications include severe dyskinesia, previous history of non-traumatic rhabdomyolysis, hyperthermia or the neuroleptic malignant syndrome symptom complex. Side effects of tolcapone are similar to those of other dopaminergic compounds. Onset and or aggravation of dyskinesia, nausea, vomiting, anorexia, insomnia, orthostatic symptoms and hallucinations were the most common adverse events in clinical trials. The most frequent non-dopaminergic adverse event was diarrhoea, occurring sometimes even 2–4 months after following treatment initiation. This may be due to the hypothetical inhibition of serotonin 5-HT metabolism in the gastrointestinal tract in some patients [137]. Headache, increased sweating and associated xerostomia, probably due to aggravation of dyskinesia in clinical trials, abdominal pain and—similar to entacapone—harmless urine discoloration due to the yellow colour of tolcapone were further side effects, which were more frequently reported than in the placebo arm in clinical trials.

9.3 Tolcapone or Entacapone in Clinical Practice

The discussion on the liver toxicity of tolcapone and the need for a previous failed response or intolerance of entacapone intake still bias prescribing preference towards entacapone. Tolcapone only requires an additional intake of three tablets to an existing levodopa/DDI regime. Tolcapone is superior to entacapone in combination with the Duodopa® pump system [138].

9.4 Unmet Needs in the Context of Therapy with COMT Inhibitors

There need for the development of further COMT inhibitors with novel and better pharmacodynamic profiles is still unmet. These compounds should lead to more sustained levodopa levels in patients with PD and should have a lower frequency of drug administration. One example is opicapone.

9.5 Opicapone

Opicapone (also known as BIA 9-1067, manufactured by BIAL-Portela & C[a]) is a long-acting, purely peripheral third-generation nitrocatechol COMT inhibitor. The compound possesses a high binding affinity to the enzyme with a slow dissociation constant. Opicapone produces a stronger and more prolonged inhibitory effect upon erythrocyte S-COMT than that reported for tolcapone and entacapone [139–146]. At 6 h after levodopa/benserazide administration, the 3-OMD concentration was still 25 % of the concentration in the controls. A dose of opicapone (100 mg/kg) used in monkeys corresponded to the disposition observed in humans for a dose of 100 mg. Opicapone in the cynomolgus monkey was shown to double the systemic exposure of levodopa, with a shift in time to maximum concentration (t_{max}) to a later time, but without significantly affecting maximum concentration of levodopa (see also Table 1).

9.5.1 Opicapone in Healthy Subjects

Opicapone provides a sustained COMT inhibition in erythrocytes [145, 147–149]. A single-centre, randomized, double-blind, gender-balanced, placebo-controlled study in healthy subjects administered with once-daily opicapone 25, 50 or 75 mg/day or placebo for 11 days and levodopa/carbidopa 100/25 mg, entacapone 200 mg or placebo tid showed that mean levodopa area under the concentration–time curve (AUC) plasma values were higher when levodopa/carbidopa was administered with any opicapone dose group than when administered concomitantly with entacapone. Maximum S-COMT inhibition was higher with all opicapone doses than with entacapone [149]. Single rising oral doses of opicapone 10–1,200 mg were studied under a double-blind, randomised, placebo-controlled design. Eight sequential groups of eight subjects were enrolled. Within each group, six subjects were randomised to receive opicapone and two subjects to receive placebo. Opicapone/placebo was administered after a 10 h overnight fast. The extent and rate of systemic exposure (AUC and maximum plasma concentration) to opicapone increased in an approximately dose-proportional manner. Despite the relatively short half-life of opicapone (1–4 h), inhibition of S-COMT activity in erythrocytes was long-lasting, ranging from 6 % (10 mg) to 55 % (1,200 mg) at 72 h post-dose. Maximum S-COMT inhibition occurred between 1 and 6 h post-dose and was 34.5, 71.7, 93.8, 96.3, 100, 100, 100 and 100 with the doses of 10, 25, 50, 100, 200, 400, 800 and 1,200 mg, respectively. Urine levels of opicapone and its metabolites usually remained below the limit of quantification, showing that the kidney is not the primary route of excretion. Opicapone was well tolerated at all doses tested. Similar effects were found during repeated dosing [149–151].

9.5.2 Opicapone in Patients with PD

The efficacy of opicapone has been demonstrated in patients with PD who were taking levodopa/carbidopa or

Table 1 Characteristics of available and experimental catechol-*O*-methyltransferase inhibitors

Property	Opicapone	Entacapone	Tolcapone
Route	Oral	Oral	Oral
Frequency of administration per day	1	≤10	3
In vivo maximal inhibition after (hours)	3	0.5	1
COMT inhibition	Periphery	Periphery	Periphery and brain
3 h post-dose ED_{50} (mg/kg)	1.05 ± 0.04	1.77 ± 0.1	7.8 ± 0.7

COMT catechol-*O*-methyltransferease, ED_{50} the amount of a substance required to produce a specific effect in half of an animal population comprising a test sample

levodopa/benserazide, as well as in patients with fluctuating PD symptoms.

Once-daily 5, 15 and 30 mg doses were applied to PD patients with motor fluctuations treated with standard-release 100/25 mg levodopa/carbidopa or levodopa/benserazide in a multicentre, double-blind, randomised, placebo-controlled study in four parallel groups. Subjects were sequentially and randomly assigned to be administered, once daily, during the 21- to 28-day maintenance phase with placebo or opicapone 5, 15 and 30 mg. They performed two levodopa challenge tests, one on the morning of the day after admission and another following the maintenance phase.

They also completed a diary to record their ON/OFF periods. In comparison with placebo, levodopa plasma exposure increased 24.73, 53.93 and 65.61 % following opicapone 5, 15 or 30 mg, respectively. Maximum S-COMT inhibition ranged from 52 % (5 mg) to 80 % (30 mg opicapone). The exploratory analysis showed improvement of various motor outcomes, including a dose-dependent change in absolute OFF time corresponding to a percentage decrease of 0.77, 4.16, 29.55 and 32.71 % with placebo and opicapone 5, 15 and 30 mg, respectively [152].

Another trial investigated the efficacy and safety of opicapone 25 and 50 mg administered once daily in comparison with placebo, in PD patients receiving levodopa treatment and with wearing-off motor fluctuations. This pivotal phase III, multinational, multicentre, double-blind, placebo-controlled and parallel-group study randomized patients to placebo (N [number of participants in each arm] = 135) to opicapone 25 mg ($N = 125$) or opicapone 50 mg ($N = 147$). The double-blind phase lasted 14–15 weeks. The primary efficacy endpoint was the change from baseline in absolute OFF-time, based on patient diaries. Mean reduction in absolute OFF-time in both opicapone 25 and 50 mg groups was greater than in the placebo group (1.7, 2.0 and 1.1 h, respectively). There was a high placebo response; nevertheless, opicapone 50 mg but not 25 mg was significantly better than placebo ($p = 0.0084$). Opicapone once daily was safe and well tolerated [153] (see also Table 1).

9.5.3 Future Advantages of Opicapone in Clinical Practice

Despite that latter negative outcome in the so-called BIPARK II study in the opicapone 25 mg arm, this compound has one essential advantage over the available COMT inhibitors. It only requires the additional intake of one tablet in a group of patients with considerable compliance problems [153–155]. Due to its pharmacologic profile, one may speculate that opicapone may provide a more sustained and thus less fluctuating COMT inhibition, which will probably further enhance the metabolic advantages of COMT inhibition during chronic levodopa/DDI therapy in PD patients.

10 Conclusions

Concomitant COMT inhibition during chronic levodopa/DDI therapy in patients with PD improves the efficacy of levodopa, reduces fluctuating levodopa plasma levels and ameliorates motor complications, particularly wearing-off phenomena. Entacapone and tolcapone are established drugs for COMT inhibition, whereas opicapone, with its once-daily application, is still in clinical trials.

COMT inhibition counteracts levodopa-associated homocysteine increase, which is a biomarker for a limited methylation capacity and supports oxidative stress generation.

Disclosures Professor Dr. Thomas Müller has performed studies, served on advisory boards and held lectures for Roche, Novartis, Orion and MEDA in the past 20 years. This manuscript was not funded by any of these companies.

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Short communication

Levodopa and neuropathy risk in patients with Parkinson disease: Effect of COMT inhibition

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ABSTRACT

Objective: Our purpose was to determine whether the use of catechol-*O*-methyltransferase-inhibitors (ICOMT) can reduce the risk of developing levodopa (LD)-induced neuropathy in Parkinson's disease (PD) patients.
Methods: A multicentre study of 197 PD patients was performed. 144 were exposed to LD for more than three years (LELD group); 53 simultaneously assumed Entacapone for at least eighteen months (LELD_ICOMT group).
Results: The prevalence of neuropathy in LELD patients was 19.4% whereas it was 5.7% in LELD_ICOMT group with a significant difference ($p = 0.025$). In LELD_ICOMT cohort the daily LD dose and serum VB12 levels were significantly higher ($p < 0.0001$), the serum Hcy levels were significantly lower ($p = 0.001$) compared to LELD group.
Conclusion: Our results suggest that ICOMT could have a protective effect on the development of LD-induced neuropathy. Their action probably occurs through the metabolic rebalancing of the one-carbon-pathway cycle and is independent of the PD duration and severity and the duration of LD intake.

In the last decade several clinical-pathological papers [1–5], including our previous large multicentric study [4], showed an increased prevalence of peripheral neuropathy (PN) in patients with Parkinson Disease (PD) and long history of Levodopa (LD) exposure, thus supporting the role of LD as a main risk factor.

It has been reported that LD could favour nerve damage by interacting with the one-carbon-pathway cycle [1–6]. LD-treated PD patients could exhibit increased levels of homocysteine (Hcy) as a consequence of LD methylation by catechol-*O*-methyltransferase (COMT). Catabolism of Hcy depends on folate, vitamin B12 (VB12), and vitamin B6 (VB6): both folate and VB12 play a role in the Hcy re-methylation to methionine as co-factors of the catalysing enzyme methionine-synthase. Inhibition of LD methylation by the concomitant use of COMT inhibitors (ICOMT) was able to reduce or prevent plasmatic Hcy peaks and restore VB12 and folate levels, impaired by LD administration [7–10].

Since elevate Hcy and low VB12 and folate serum levels have been related to peripheral nerve damage in patients with long LD exposure [1–6], as the next step of our former study [4], we compared patients with stable combined ICOMT (Entacapone)-LD therapy to patients with an equivalent LD exposure without simultaneous ICOMT intake in order to assess a possible protective ICOMT effect on the development of LD-induced neuropathy.

1. Methods

Between September 2014 and September 2015, we conducted a

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cross-sectional study involving three Italian tertiary referral PD centres (Cagliari, Pisa and Turin) by enrolling consecutive patients who met the inclusion criteria of the study. The study protocol received approval from each centre's Ethics Committee. A written informed consent form was obtained from each patient.

2. Patients

All patients fulfilled the UK Brain Bank criteria for the clinical PD diagnosis. We included subjects who were exposed to LD for more than three years and simultaneously assumed Entacapone, at a minimum maintenance daily dose of 400 mg, for a period not shorter than 50% of that of LD treatment (LELD_ICOMT group). The enrolment of patients with LD exposure of at least three years allowed us to compare the LELD_ICOMT group with the LELD group of our previous study [4], in which patients had a similar long exposure to LD (>3 years) without concomitant ICOMT intake.

These criteria were arbitrarily decided. However, they were driven by the fact that, while experimental data demonstrate that Entacapone may quickly reduce hyperHcy induced by subacute LD administration [7], longer duration of Entacapone treatment was associated with greater Hcy reduction than short-term one [8]. Therefore, it is likely that patients with long-lasting LD treatment had a more profound metabolic changes requiring a prolonged ICOMT co-administration to be counterbalanced.

We excluded patients who had a history of systemic illnesses, such as chronic infectious diseases, diabetes, or other metabolic, endocrine, or autoimmune illnesses; cancer; chronic alcohol consumption; toxic exposure, and any family history of neuropathy. The daily dose and duration of LD, ICOMT and dopamine agonists were determined for each patient by a retrospective chart review. We defined the daily LD dose as the average daily dosage of LD in the last 6 months.

Assessment using the motor subscale from the Unified Parkinson's Disease Rating Scale (UPDRS III) and a complete neurological examination were carried out in all patients. None of the patients was taking any vitamin supplementation.

3. Procedures

Nerve function was evaluated using a validated neuropathy composite score, the reduced version of the total neuropathy score (TNSr), which is the version previously validated by Cornblath et al. [11] and modified by Cavaletti et al. [12] (i.e. evaluation of sensory symptoms, pin sensibility, vibration sensibility, strength, and deep tendon reflex, without the quantitative determination of vibration threshold but with the neurophysiological investigation of sensory sural and motor peroneal peripheral nerves). As required for TNS design, the presence of sensory, motor, or autonomic symptoms was first assessed by interviewing the patients. The neurological examination was based on the standard evaluation of strength, deep tendon reflexes, and examination of pin sensibility using a sterile disposable needle; and disturbance of vibration sensibility was demonstrated by decreased perception of the 128-Hz diapason vibration, as described by Cavaletti et al. [12].

Nerve conduction studies were performed using standard laboratory techniques. Right sural sensory antidromic and peroneal motor nerve conduction was studied using standardized techniques and fixed distances. The temperature was >32 °C.

To calculate the TNSr, the amplitude of the antidromic sensory potential (SAP) in one sural nerve and the compound muscle action potential (CMAP) in the ipsilateral common peroneal nerve were used. The neurophysiological normal reference values necessary for TNS calculation were previously determined in each neurological department in age-matched individuals. According to the results from the clinical and neurophysiological examinations, the TNSr score was calculated as previously described [11]. For each item, the possible score ranged from 0 (normal) to 4 (worst possible results), so that the score ranged from 0 to 28. According to previously published criteria [13], only patients who presented a combination of neuropathic symptoms or signs with at least an abnormal parameter in one of the explored nerves were considered as patients with neuropathy.

We determined serum VB12 and Hcy levels in all patients involved in the study. Fasting blood tests were carried out 12 h after the last dose of LD. For those with neuropathy, we performed further investigations, including complete blood count, urea, creatinine, liver enzymes, liver function tests, glucose and hemoglobin A1C (HbA1C), electrolytes, thyroid function, erythrocyte sedimentation rate, C-reactive protein, antinuclear antibody, extracted nuclear antibody testing, rheumatoid factor, and serum protein electrophoresis.

4. Statistical analysis

Our study was carried out on a sample of 197 patients, 144 with LELD and 53 with LELD_ICOMT.

The eventual difference, in cases of neuropathy, between patients with LELD_ICOMT and LELD was assessed using Fisher's exact test, and 95% confidence intervals were calculated according to the Poisson distribution.

The Mann-Whitney test was used to evaluate differences in serum VB12, Hcy, folate levels and LD daily dose, between patients with LELD_ICOMT and patients with LELD. The same test was also used and to assess differences in TNSr score between patients with and without neuropathy; a p value < 0.05 was considered statistically significant.

Data from the survey were analysed using the Statistical Package for the Social Sciences (version 20; SPSS Inc., Chicago, IL, USA).

5. Results

Our LELD_ICOMT group consisted at first of 54 PD patients. One was subsequently excluded because hematological findings disclosed previously unrecognized diabetes (high glucose and HbA1C levels).

The personal and clinical data of the 53 LELD_ICOMT patients (24 women) finally included were: median age 70 years; median disease duration 12 years; median UPDRS III score 26. Median LD exposure was 9 years while median duration of ICOMT therapy was 6 years. Median LD dose (650 mg/day) was also calculated for each patient.

We compared our results with those of the LELD cohort reported in our previous study: 144 patients (56 women) with median age 70 years; median disease duration 10 years; median UPDRS III score 23.5, median LD dose 475 mg/day, median LD exposure was 7.5 years.

We report in Table 1 median and percentiles in LELD_ICOMT and LELD patients for LD dose, Serum VB12, Serum Hcy, Folate levels.

The prevalence of neuropathy was significantly lower in LELD_ICOMT patients (5.7% C.I. 1.9%–17.0%) compared to LELD patients (19.4% C.I. 13.2%–27.8%), $p = 0.025$.

The Mann-Whitney test indicated that in LELD_ICOMT patients the daily LD dose was significantly higher ($p < 0.0001$), serum VB12 levels were significantly higher ($p < 0.0001$), serum Hcy levels were significantly lower ($p = 0.001$) and folate levels were significantly higher ($p = 0.038$).

Median disease duration was 12 in patients with LELD_ICOMT and 10 in patients with LELD with a significant difference $p = 0.002$ (Mann-Whitney test).

Table 1
Median and percentiles in LELD_ICOMT and LELD patients for LD dose, Serum VB12, Serum Hcy, Folate levels.

	25° percentile	Median	75° percentile
LD dose			
LELD_ICOMT patients	550	650	1000
LELD patients	400	475	700
Serum VB12			
LELD_ICOMT patients	344	455	632
LELD patients	292	362	456
Serum Hcy			
LELD_ICOMT patients	8	10	12
LELD patients	10	15	20
Serum Folate			
LELD_ICOMT patients	6	8	9
LELD patients	5	6	9

leads to a sequence of events (Hcy accumulation and VB6, VB12 and folate depletion) which alters peripheral nerve homeostasis.

Hcy can cause neurotoxicity by increasing vulnerability to mitochondrial toxins and rising free radicals, by inducing inflammatory reactions and also by impairing DNA repair mechanisms [16]. LD-related increased Hcy was associated with signs of axonal neurodegeneration in an electrophysiological study of patients with PD and healthy controls [1]. Furthermore, the role of high Hcy levels in inducing peripheric damage has been confirmed in diabetic neuropathy, in patients with 5,10-methylenetetrahydrofolate reductase deficiency and in a longitudinal clinical and electrophysiological study conducted on a large group of elderly individuals [17].

VB12 and folate are a well-known cause of reversible peripheral neuropathy in older adults [17,18]. It may occur in chronic exposure to elevated LD doses whose metabolism causes high COMT activity. This, in turn, requires an excessive activity of VB12 and folate and results in their relative functional failure. It makes critical metabolic processes essential for the axon homeostasis, causing the peripheral nerves damage. The ICOMT can rebalance this abnormal metabolic loop, therefore preventing the onset of neuropathy.

Table 2
Electrophysiologic data, TNSr, UPDRS III and UPDRS item 30 scores of patients with and without neuropathy.

		TNS score			Amp. SAP sural (μV)			Amp. CMAP SPE (mV)			UPDRS III			UPDRS item 30		
	N	25° per	Med	75° per	25° per	Med	75° per	25° per	Med	75° per	25° per	Med	75° per	25° per	Med	75° per
LELD without PN	116	0	1	3	7	9	12	4	5	7	14.5	22	31	0	1	1
LELD with PN	28	10	12	14	0	2	4	0.5	3	4	21	28	37	1	2	2
LELD ICOMT without PN	50	0	0	2	7	9	11.5	3.25	5	7.75	15.75	26.5	34.25	0	1	2
LELD ICOMT with PN	3	6	8	–	1.29	1.95	2.83	–	–	–	18.25	18.5	–	1	2	–

Electrophysiologic data, TNSr, UPDRS III and UPDRS item 30 scores are reported in Table 2.

6. Discussion

Several studies reported the frequent occurrence of PN during long-term LD treatment [1–6]. In 2013 our previous large multicenter study, conducted on about 500 pts, we stratified the risk of neuropathy based on the length of LD exposure showing a significant PN prevalence in patients taking LD for a longer period (almost 20% in patients with LD exposure > 3 years); neuropathy was also associated with the cumulative dose of LD, high Hcy and reduced VB12 serum levels [4].

Interestingly, COMT inhibition, which is an effective approach to prolong LD activity widely used in the management of fluctuating patients, was shown to decrease Hcy levels and restore VB12 and folate levels, reduced because of LD administration [7–10]. In addition, pre-clinical findings [14,15] have demonstrated a protective effect of ICOMT on the development of neuropathies also regardless of dopamine metabolism.

Therefore, according to such evidences, we have planned to evaluate PD patients on stable LD-Entacapone combination therapy to assess the putative protective role of ICOMT on the PN development.

Our findings provide clear evidence that the LELD_ICOMT cohort has a reduced prevalence of neuropathy when compared to patients with similar prolonged LD exposure but without concomitant COMT inhibition. This protective ICOMT effect was totally independent of the PD duration and severity and the duration of LD intake. In our LELD_ICOMT patients VB12 and folate levels were significantly higher and Hcy values were significantly lower than those observed in LELD patients, indicating a specific drug effect in the metabolic rebalancing of the one-carbon-pathway cycle.

Conversion of LD to dopamine requires a methyl-group donation that is provided by *S*-adenosylmethionine. Such reaction leads to Hcy formation. Subsequent Hcy remethylation needs VB12 as cofactor and alternative ways of degradation require methylenetetrahydrofolate and VB6. The final result is that chronic LD intake

These data seemingly disagree with those of Mancini et al. [5] who screened for neuropathies a heterogeneous group of PD patients under different therapeutic regimens, 19 of the 50 patients treated with oral LD taking ICOMT. The authors found no difference in the PN prevalence, and VB12, folate, and homocysteine levels between the subgroups. We believe that this discrepancy with our results may be related to the low sample size of the patients enrolled in the Mancini study, but, above all, to the fact that the therapy duration with ICOMT is unknown.

This ICOMT PN-sparing effect we observed could be of particular clinical relevance when we taking into account the high prevalence of neuropathy (up to over 50%) [2] and its effects in worsening the balance of PD patients with prolonged LD assumption [4]. Because the postural imbalance due to neuropathy-related sensory disturbances can be treated by specific rehabilitative protocols, which may differ from those classically adopted for PD, strategies for prevention of LD treatment-related neuropathy might have great clinical relevance.

Based on these results we suggest that the ICOMT supplement could be recommended in patients with PD who, due to their long history of exposure to LD, appear at risk of developing PN or, then again, with clinical/neurophysiological signs of neuropathy that might be worsened by treatment with LD alone.

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PLASMA HOMOCYSTEINE AS A RISK FACTOR FOR DEMENTIA AND ALZHEIMER'S DISEASE

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ABSTRACT

Background In cross-sectional studies, elevated plasma homocysteine levels have been associated with poor cognition and dementia. Studies of newly diagnosed dementia are required in order to establish whether the elevated homocysteine levels precede the onset of dementia or result from dementia-related nutritional and vitamin deficiencies.

Methods A total of 1092 subjects without dementia (667 women and 425 men; mean age, 76 years) from the Framingham Study constituted our study sample. We examined the relation of the plasma total homocysteine level measured at base line and that measured eight years earlier to the risk of newly diagnosed dementia on follow-up. We used multivariable proportional-hazards regression to adjust for age, sex, apolipoprotein E genotype, vascular risk factors other than homocysteine, and plasma levels of folate and vitamins B_{12} and B_6.

Results Over a median follow-up period of eight years, dementia developed in 111 subjects, including 83 given a diagnosis of Alzheimer's disease. The multivariable-adjusted relative risk of dementia was 1.4 (95 percent confidence interval, 1.1 to 1.9) for each increase of 1 SD in the log-transformed homocysteine value either at base line or eight years earlier. The relative risk of Alzheimer's disease was 1.8 (95 percent confidence interval, 1.3 to 2.5) per increase of 1 SD at base line and 1.6 (95 percent confidence interval, 1.2 to 2.1) per increase of 1 SD eight years before base line. With a plasma homocysteine level greater than 14 μmol per liter, the risk of Alzheimer's disease nearly doubled.

Conclusions An increased plasma homocysteine level is a strong, independent risk factor for the development of dementia and Alzheimer's disease. (N Engl J Med 2002;346:476-83.)

ALZHEIMER'S disease accounts for more than 70 percent of all cases of dementia, so it is important to identify modifiable risk factors for the disease.[1] During the past decade, there has been growing interest in vascular factors that may underlie Alzheimer's disease. It is now recognized that subjects with cardiovascular risk factors and a history of stroke have an increased risk of both vascular dementia and Alzheimer's disease.[2-4] Plasma total homocysteine has recently emerged as a major vascular risk factor. Elevated total homocysteine levels have been associated with an increased risk of atherosclerotic sequelae, including death from cardiovascular causes,[5,6] coronary heart disease,[6,7] carotid atherosclerosis,[8] and clinical stroke.[9,10] These observations led to the hypothesis that elevated plasma homocysteine may be a risk factor for dementia and Alzheimer's disease. If this hypothesis is valid, it points to a modifiable risk factor, since plasma homocysteine levels can be lowered by supplementation with folic acid.[11]

Previous studies have reported an inverse association between plasma total homocysteine levels and simultaneously assessed cognitive function.[12-16] Two case–control studies have found higher plasma homocysteine levels in persons with Alzheimer's disease.[17,18] However, in a prospective study plasma homocysteine levels were not related to cognitive decline during follow-up in a community-based sample.[19] Elevated plasma homocysteine levels in subjects with cognitive impairment or dementia might be the result of poor nutrition and vitamin deficiencies.[20] A prospective study should be able to show whether elevated plasma homocysteine in cognitively intact adults is associated with an increased risk of dementia and Alzheimer's disease on follow-up. We therefore examined plasma total homocysteine in relation to newly diagnosed dementia and Alzheimer's disease in the elderly, population-based cohort of Framingham Study participants.

METHODS

Subjects

The Framingham Study cohort has been evaluated biennially since 1948. Between 1976 and 1978, a total of 2611 subjects were enrolled in a dementia-free cohort.[21,22] At the 20th biennial examination (between 1986 and 1990), 1592 subjects from this cohort were alive and free of dementia and had follow-up data for at least one year. Of these subjects, 1229 (77 percent) underwent the 20th examination, and in 1092 participants (89 percent of those examined), plasma total homocysteine levels were measured. These 1092 subjects constituted our study sample. There were

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667 women and 425 men, and their mean (±SD) age was 76±6 years (range, 68 to 97). Informed consent was obtained from all study subjects with the use of a consent form approved by the institutional review board for human research at the Boston University School of Medicine.

Diagnosis of New Cases of Dementia and Alzheimer's Disease

Subjects in the cohort that was free of dementia at inception have been monitored with published surveillance techniques since 1978 for the development of stroke or dementia.[21,22] Methods have included a screening Folstein Mini–Mental State Examination[23] at each biennial evaluation, followed by annual neurologic and neuropsychological assessment of subjects with suspected cognitive impairment.

The final diagnosis of dementia was made by a committee, comprising at least two neurologists and a neuropsychologist, that determined the type of dementia and the date of diagnosis. All available information was used to evaluate participants with suspected dementia, including serial neurologic and neuropsychological assessments, a telephone interview with a family member or care giver, medical records, imaging studies, and autopsy data when available. The review committee was unaware of the subjects' plasma homocysteine levels. The diagnosis of dementia was made according to the criteria of the *Diagnostic and Statistical Manual of Mental Disorders,* fourth edition[24]; our definition also required a duration of symptoms greater than six months, and a score for severity of dementia of 1 or higher on the Clinical Dementia Rating scale.[25] Alzheimer's disease was diagnosed when subjects met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association for definite, probable, or possible Alzheimer's disease.[26]

Plasma Homocysteine

Plasma total homocysteine levels were measured in all subjects at the 20th biennial examination (base line). An earlier measure from the 16th biennial examination (performed between 1979 and 1982, approximately eight years before base line) was also available for 935 of the subjects (86 percent). All plasma specimens were stored at or below −20°C. Homocysteine levels were determined with the use of high-performance liquid chromatography with fluorometric detection.[27] The coefficient of variation for this assay was 9 percent.[28]

Apolipoprotein E Genotypes

Data on the apolipoprotein E (*APOE*) genotype were available for 1012 of the subjects (93 percent). The presence of particular alleles was determined by means of isoelectric focusing of the plasma and confirmed by DNA genotyping.[29,30] Participants were divided into two groups, one comprising persons with an *APOE* ε4 allele (ε2/ε4, ε3/ε4, or ε4/ε4 genotype) and another comprising those without an *APOE* ε4 allele.

Vitamin Levels

Plasma concentrations of folate, cyanocobalamin (vitamin B_{12}), and pyridoxal-5′-phosphate (the coenzyme form of vitamin B_6) were estimated at the 20th biennial examination. Plasma folate was measured by a microbial (*Lactobacillus casei*) assay with a 96-well plate and manganese supplementation[31]; plasma vitamin B_{12} levels were estimated with the use of a radioassay kit (Magic, Ciba–Corning, Medfield, Mass.); and pyridoxal-5′-phosphate was measured by the tyrosine decarboxylase apoenzyme method.[32] Coefficients of variation for these assays were 13 percent for plasma folate, 7 percent for cyanocobalamin, and 16 percent for pyridoxal-5′-phosphate.[28] Because of insufficient plasma samples, the vitamin levels were not determined for all patients. Of the subjects with measurements of plasma homocysteine, 85 percent had measurements of vitamin B_{12}, 92 percent had measurements of vitamin B_6, and 98 percent had measurements of folate.

Definition of Additional Risk Factors

Risk factors that could potentially confound the relation between plasma homocysteine and dementia or Alzheimer's disease were defined with the use of data collected at the 20th biennial examination. When appropriate, data from earlier biennial examinations were also used. Educational status was dichotomized at the level of high-school completion. We adjusted the analyses for cigarette smoking using two variables: current smoking status (smoker or nonsmoker) and lifetime exposure to cigarette smoke (<5.0 pack-years, 5.0 to 29.9 pack-years, or ≥30.0 pack-years). Alcohol intake was categorized in terms of the number of drinks per day: zero, less than one, one to two, or more than two.[33] Diabetes mellitus was defined by a recorded casual blood glucose level of at least 200 mg per deciliter (11.1 mmol per liter), a previous diagnosis of diabetes mellitus, or the use of a hypoglycemic agent or insulin. Systolic blood pressure and body-mass index (the weight in kilograms divided by the square of the height in meters) were treated as continuous variables.

Statistical Analysis

The distribution of plasma homocysteine levels in the population was positively skewed. The use of natural-log–transformed values provided the best-fitting model for analyses in which the plasma homocysteine level was treated as a continuous variable. Plasma homocysteine levels were also evaluated with a quartile-based analysis. Since homocysteine levels increase markedly with age,[28,34,35] the quartiles were defined in an age-specific manner for each of several five-year age categories.

Cox proportional-hazards regression models[36] were used to examine the relation between the homocysteine level and the incidence of dementia and Alzheimer's disease during follow-up, after adjustment for age (in one-year increments), sex, and *APOE* genotype (with or without an *APOE* ε4 allele).[37] In supplementary analyses, we also adjusted for vitamin levels and other covariates. Subjects were followed for new cases of dementia from the date of their 20th biennial examination until December 31, 2000. For the analysis of new cases of Alzheimer's disease, data for subjects in whom other types of dementia developed were censored at the date of the diagnosis of dementia, since the diagnostic categories were mutually exclusive. Subjects who had a stroke during the study period were not excluded, since such an event could be part of the causal chain between an elevated plasma homocysteine level and the development of dementia. All statistical analyses were performed with the use of SAS software (SAS Institute, Cary, N.C.).

RESULTS

Base-Line Characteristics

The base-line characteristics of the subjects are presented in Table 1 (further information may be found in Supplementary Appendix 1, available with the full text of this article at http://www.nejm.org). Mild-to-moderate elevation of the plasma homocysteine level (>14 μmol per liter) was present in 30 percent of the subjects. None of the subjects had severe hyperhomocysteinemia (plasma homocysteine, >100 μmol per liter). The mean plasma homocysteine level within each of the five-year age groups is shown in Table 2. The correlation between the base-line plasma homocysteine level in a given subject and the level measured eight years earlier was calculated for the 935 subjects (571 women and 364

men) for whom both measurements were available (Pearson r=0.47, P<0.001).

Dementia, Alzheimer's Disease, and Plasma Homocysteine

Over a median follow-up period of 8 years (range, 1 to 13), dementia developed in 111 subjects (10.2 percent; 74 women and 37 men), and 83 of these subjects (62 women and 21 men) were given a diagnosis of Alzheimer's disease. In five subjects, the clinical diagnosis of Alzheimer's disease was confirmed at autopsy (definite Alzheimer's disease). The diagnosis was probable Alzheimer's disease for 67 subjects and possible Alzheimer's disease for 11 subjects. Other types of dementia diagnosed in the study population included vascular dementia in 11 subjects, non-Alzheimer's degenerative dementias in 11 subjects, and other types of dementia in 6 subjects. The absence of Alzheimer's disease was confirmed at autopsy in 14 subjects.

The overall results relating the plasma homocysteine level to the development of any dementia and to the development of Alzheimer's disease are shown in Tables 3 and 4 and in Figure 1. After adjustment for the age, sex, and *APOE* genotype, the relative risks of dementia and Alzheimer's disease, for each increase of 1 SD in log-transformed base-line homocysteine value, were 1.3 (95 percent confidence interval, 1.1 to 1.6) and 1.4 (95 percent confidence interval, 1.2 to 1.7), respectively. Hyperhomocysteinemia (plasma homocysteine, >14 μmol per liter)[8,18] was correspondingly associated with an increased risk of dementia (relative risk, 1.9; 95 percent confidence interval, 1.3 to 2.8) and Alzheimer's disease (relative risk, 1.9; 95 percent confidence interval, 1.2 to 3.0). An increase in the plasma homocysteine level of 5 μmol per liter increased the multivariable-adjusted risk of Alzheimer's disease by 40 percent (P<0.001). We did not find evidence of modification of this effect by age or sex.

Effect of Vitamin Levels

Low serum levels of certain B vitamins (folate and vitamins B_{12} and B_6) have been associated with elevated plasma homocysteine levels in several studies and with an increased risk of dementia in a few investigations.[38-42] In our study, the observed association between plasma homocysteine and risk of dementia was not significantly altered by adjustment for the plasma levels of these vitamins (Table 3). Furthermore, after adjustment for age, sex, and *APOE* genotype, none of these vitamin levels were independently related to the risk of dementia or Alzheimer's disease (data not shown).

Additional Covariates

The observed association between the plasma homocysteine level and dementia or Alzheimer's disease was not diminished by adjustment for educational status, systolic blood pressure, smoking status, alcohol intake, presence or absence of diabetes, body-mass index, or presence or absence of a history of stroke (Table 3). Serum creatinine was measured at the 15th biennial examination, and cholesterol and

TABLE 1. BASE-LINE CHARACTERISTICS OF STUDY SUBJECTS AT THE 20TH BIENNIAL EXAMINATION.*

CHARACTERISTIC	MEN (N=425)	WOMEN (N=667)
Age (yr)	76±5	77±6
Plasma homocysteine		
Level (μmol/liter)	13.1±6.3	13.0±7.0
Log-transformed value	2.5±0.4	2.5±0.4
>14 μmol/liter (%)†	30	30
>9 μmol/liter (%)‡	81	76
Other plasma levels§		
Folate (ng/ml)	5.9±7.5	6.9±7.1
Vitamin B_{12} level (pg/ml)	416±209	461±233
Pyridoxal-5′-phosphate level (nmol/liter)	74.7±89.0	79.9±94.8
Body-mass index¶	27.0±4.0	26.5±5.0
Systolic blood pressure (mm Hg)	146±22	147±23
Apolipoprotein E genotype (%)		
ε2/ε2 or ε2/ε3	11.0	10.9
ε3/ε3	68.0	68.0
ε2/ε4, ε3/ε4, or ε4/ε4	21.0	21.1
High-school graduate (%)‖	66.1	67.8
History of stroke (%)	7.3	5.1
Current cigarette smoker (%)	10	11
Lifetime smoking (%)**		
<5.0 pack-years	35	63
5.0–29.9 pack-years	22	20
≥30.0 pack-years	43	17
Diabetes (%)	14.4	8.6
Alcohol intake (%)††		
0 drinks/day	37.5	52.7
<1 drink/day	25.9	29.9
1–2 drinks/day	13.7	8.0
>2 drinks/day	22.9	9.5

*Plus-minus values are means ±SD.

†This threshold represents the accepted cutoff point for hyperhomocysteinemia.

‡This threshold represents the mean plasma homocysteine level in the general population.

§To convert values for plasma folate to nanomoles per liter, multiply by 2.266; to convert values for plasma vitamin B_{12} to picomoles per liter, multiply by 0.7378. Data on plasma folate levels were available for 419 men and 657 women; data on plasma B_{12} levels were available for 375 men and 557 women; and data on plasma pyridoxal-5′-phosphate levels were available for 398 men and 611 women.

¶The body-mass index is the weight in kilograms divided by the square of the height in meters; data were available for 411 men and 630 women.

‖Data were available for 413 men and 653 women.

**Data were available for 374 men and 611 women for whom the age when smoking began could be reliably ascertained.

††One drink is defined (according to the criteria established by the National Institute on Alcohol Abuse and Alcoholism) as 12 oz (360 ml) of beer, 5 oz (150 ml) of wine, or 1.5 oz (45 ml) of distilled spirits, each containing approximately 0.5 oz (15 ml) of pure alcohol. Data on alcohol intake were available for 424 men and 666 women.

TABLE 2. DISTRIBUTION OF BASE-LINE PLASMA HOMOCYSTEINE LEVELS WITHIN FIVE-YEAR AGE GROUPS.*

AGE	NO. OF SUBJECTS	PLASMA HOMOCYSTEINE LEVEL		
		MEAN ±SD	RANGE	75TH PERCENTILE
		μmol per liter		
65–69 yr	46	11.5±3.9	5.4–25.5	13.2
70–74 yr	457	12.1±5.9	4.1–66.7	13.8
75–79 yr	315	12.6±5.9	3.5–66.9	14.5
80–84 yr	179	14.2±7.3	4.5–56.1	16.5
85–89 yr	66	15.3±8.0	5.5–59.6	19.3
90–94 yr	29	22.3±12.6	5.4–61.6	26.6

*The difference in mean values between men and women was not significant.

thyrotropin were measured at the 20th biennial examination. Adjustment for these additional variables did not alter our results (data not shown).

Varying the Diagnostic Criteria for Alzheimer's Disease

Higher plasma homocysteine levels have been related to an increased risk of stroke.[8,10] To address the possibility that the association we observed between plasma homocysteine and Alzheimer's disease resulted from the inclusion of subjects who might have vascular dementia rather than Alzheimer's disease, we evaluated separately the association between base-line plasma homocysteine levels and a diagnosis of definite or probable Alzheimer's disease after excluding subjects with a diagnosis of possible Alzheimer's disease. The relative risk per increment of 1 SD in the log-transformed base-line homocysteine value remained essentially unchanged at 1.4 (95 percent confidence interval, 1.2 to 1.7).

Association with Earlier Homocysteine Levels

Unlike stroke or myocardial infarction, clinical dementia begins insidiously. It may therefore be difficult to exclude subjects in whom the disease is incipient at base line. However, subjects who were free of clinical dementia at base line were most likely free of even incipient disease eight years earlier, at the examination from which we derived the previous plasma homocysteine measurement. We examined the relation between the plasma homocysteine level eight years before base line and the risk of newly diagnosed dementia or Alzheimer's disease during the follow-up period between the base-line examination and December 31, 2000. Again, we found a strong association (Table 3), indicating that the elevation of the plasma homocysteine level occurred well before the onset of clinical manifestations.

Quartile-Specific Analysis

Examination of the risks of dementia and Alzheimer's disease in age-specific quartiles of plasma homocysteine levels suggested that subjects with levels in the highest quartile (according to the cutoff points in Table 2) had the highest risk of dementia and Alzheimer's disease. When both measurements of plasma homocysteine were considered, this subgroup had about twice the risk of all other subjects (Table 4 and Fig. 1). Although the effect of the homocysteine level was smaller in the second and third quartiles, we did not find evidence of a specific threshold. When the subjects whose base-line levels were in the lowest age-specific quartile were used as the reference group, the relative risk of Alzheimer's disease was 1.2 (95 percent confidence interval, 0.6 to 2.2) for subjects in the second quartile, 1.3 (95 percent confidence interval, 0.6 to 2.5) for subjects in the third quartile, and 2.2 (95 percent confidence interval, 1.2 to 4.1) for subjects in the fourth quartile. Subjects whose plasma homocysteine levels were consistently high (in the fourth quartile at both the 16th and 20th examinations) had the highest risk.

Population Attributable Risk

In our population, the risk of Alzheimer's disease attributable to a plasma homocysteine level in the highest age-specific quartile was estimated, with the use of standard techniques,[43] at 16 percent. In the same population, 21 percent of subjects had at least one *APOE* ε4 allele, and the age- and sex-adjusted relative risk of Alzheimer's disease associated with the presence of this allele was 2.3 (95 percent confidence interval, 1.5 to 3.7); thus, there was a 21 percent risk of Alzheimer's disease attributable to the presence of an *APOE* ε4 genotype.

DISCUSSION

The results of our prospective, observational study indicate that there is a strong, graded association between plasma total homocysteine levels and the risk of dementia and Alzheimer's disease. An increment in the plasma homocysteine level of 5 μmol per liter increased the risk of Alzheimer's disease by 40 percent. A plasma homocysteine level in the highest age-specific quartile doubled the risk of dementia or Alzheimer's disease. A similar result was found when the single criterion of hyperhomocysteinemia (base-line plasma homocysteine, >14 μmol per liter) was used. The magnitude of this effect is similar to the

TABLE 3. MULTIVARIABLE COX PROPORTIONAL-HAZARDS REGRESSION MODELS EXAMINING THE RELATION BETWEEN THE PLASMA TOTAL HOMOCYSTEINE LEVEL AND THE RISK OF DEMENTIA AND ALZHEIMER'S DISEASE.*

PLASMA HOMOCYSTEINE MEASUREMENT	VARIABLES ADJUSTED FOR	ANY DEMENTIA			ALZHEIMER'S DISEASE		
		NO. OF CASES/NO. OF SUBJECTS	RR (95% CI)	P VALUE	NO. OF CASES/NO. OF SUBJECTS	RR (95% CI)	P VALUE
Base line	Age and sex	111/1092	1.3 (1.1–1.5)	0.007	83/1092	1.4 (1.1–1.7)	0.002
	Age, sex, and *APOE* genotype	105/1012	1.3 (1.1–1.6)	0.003	79/1012	1.4 (1.2–1.7)	<0.001
	Age, sex, *APOE* genotype, and plasma levels of folate and vitamins B_{12} and B_6	77/789	1.4 (1.1–1.8)	0.002	54/789	1.6 (1.2–2.1)	<0.001
	Age, sex, *APOE* genotype, plasma levels of B vitamins, and additional covariates	60/680	1.4 (1.1–1.9)	0.009	44/680	1.8 (1.3–2.5)	<0.001
Eight years before base line	Age and sex	88/935	1.4 (1.1–1.7)	0.02	67/935	1.4 (1.1–1.9)	0.01
	Age, sex, and *APOE* genotype	82/864	1.3 (1.0–1.7)	0.03	63/684	1.4 (1.0–1.8)	0.02
	Age, sex, *APOE* genotype, and additional covariates	72/771	1.4 (1.1–1.9)	0.01	56/771	1.6 (1.2–2.1)	0.004

*The plasma total homocysteine level was analyzed as a continuous variable. The relative risks (RRs) are per increment of 1 SD (0.4) in the log-transformed homocysteine value. The base-line homocysteine level was estimated on the basis of plasma samples collected from nonfasting subjects at the 20th biennial examination (between 1986 and 1990); the level eight years before base line was estimated on the basis of plasma samples collected from nonfasting subjects at the 16th biennial examination (between 1979 and 1982). Log-transformed values were used for plasma folate and plasma vitamin B_6. The "additional covariates" included educational status, history of stroke, smoking status, alcohol intake, diabetes mellitus, body-mass index, and systolic blood pressure (as recorded at the base-line examination in which plasma total homocysteine was measured). CI denotes confidence interval, and *APOE* apolipoprotein E.

magnitude of the increases in the risks of death from cardiovascular causes and stroke associated with a similar increment in the plasma homocysteine level, which have been previously described in the Framingham cohort.[6,10]

The observed association appeared to be independent of age, sex, *APOE* genotype, plasma vitamin levels, and other putative risk factors for dementia and Alzheimer's disease. The prospective nature of this study and the strong association between newly diagnosed dementia and Alzheimer's disease and plasma homocysteine levels measured eight years before base line suggest that the elevation in the homocysteine level preceded the onset of dementia. Finally, subjects with a sustained elevation of plasma homocysteine had the greatest risk of dementia.

Two case–control studies have specifically addressed the relation between homocysteine levels and the risk of Alzheimer's disease.[17,18] Both studies found a significant elevation of the serum homocysteine level in patients with Alzheimer's disease as compared with age-matched controls. A report from the Rotterdam Study did not show an association between the base-line homocysteine level and a decline in the score on the Mini–Mental State Examination, perhaps because the follow-up period was only 2.7 years.[19] In our study population, an elevated homocysteine level at base line was related to a decline in the scores on the Mini–Mental State Examination, but only after a follow-up period of at least four years (data not shown).

Elevated plasma homocysteine levels are associated with carotid atherosclerosis and an increased risk of stroke.[8,10] Atherosclerosis and stroke, in turn, increase the risk of clinical Alzheimer's disease.[2,4] Hyperhomocysteinemia has been related to cerebral microangiopathy,[44] endothelial dysfunction,[45] impaired nitric oxide activity,[46] and increased oxidative stress[47] — all factors associated with the aging of the brain.[48,49] Increased concentrations of homocysteic acid, an *N*-methyl-D-aspartate receptor agonist and a metabolite of homocysteine, may result in excitotoxic damage to neurons.[50] Homocysteine promotes copper-mediated and β-amyloid-peptide–mediated toxic effects in neuronal cell cultures[51] and induces apoptosis in hippocampal neurons in rats.[52]

The strengths of our investigation include its prospective design, the large community-based sample, the long follow-up period, and the availability of pre-

TABLE 4. MULTIVARIABLE COX PROPORTIONAL-HAZARDS REGRESSION MODELS FOR THE RISK OF DEMENTIA AND ALZHEIMER'S DISEASE ACCORDING TO AGE-SPECIFIC QUARTILE OF PLASMA TOTAL HOMOCYSTEINE LEVEL.*

QUARTILE OF PLASMA HOMOCYSTEINE LEVEL	ANY DEMENTIA			ALZHEIMER'S DISEASE		
	NO. OF CASES/NO. OF SUBJECTS†	RR (95% CI)	P VALUE	NO. OF CASES/NO. OF SUBJECTS	RR (95% CI)	P VALUE
4 at base line (reference group, 1, 2, and 3 at base line)	105/1012	1.9 (1.3–2.9)	0.003	79/1012	1.9 (1.2–3.1)	0.008
With additional adjustment for plasma levels of folate and vitamins B_{12} and B_6‡	77/789	2.5 (1.5–4.4)	<0.001	54/789	2.8 (1.4–5.4)	0.003
4 at 8 yr before base line (reference group, 1, 2, and 3 at 8 yr before base line)	82/864	1.7 (1.0–2.8)	0.04	63/864	1.7 (1.0–3.1)	0.06
1, 2, or 3 at 8 yr before and at base line	48/555	1.0		38/555	1.0	
4 at 8 yr before base line and 1, 2, or 3 at base line	7/88	1.4 (0.6–3.1)	0.44	5/88	1.5 (0.6–3.8)	0.44
1, 2, or 3 at 8 yr before base line and 4 at base line	12/116	1.7 (0.9–3.3)	0.09	9/116	1.7 (0.8–3.6)	0.15
4 at 8 yr before and at base line	15/105	2.2 (1.2–4.1)	0.009	11/105	2.2 (1.1–4.4)	0.03

*All analyses were adjusted for age, sex, and apolipoprotein E genotype. The relative risks (RRs) indicate the risk as compared with that in the reference group during the follow-up period between the 20th biennial examination and December 31, 2000. The base-line plasma homocysteine level was estimated on the basis of plasma samples collected from nonfasting subjects at the 20th biennial examination; the level eight years before base line was estimated on the basis of plasma samples collected from nonfasting subjects at the 16th biennial examination. The 75th percentile of the plasma homocysteine level (the cutoff point for quartile 4) was 13.2 μmol per liter for subjects 65 to 69 years old, 13.8 μmol per liter for subjects 70 to 74 years old, 14.5 μmol per liter for subjects 75 to 79 years old, 16.5 μmol per liter for subjects 80 to 84 years old, 19.3 μmol per liter for subjects 85 to 89 years old, and 26.6 μmol per liter for subjects 90 to 95 years old. CI denotes confidence interval.

†The denominator (number of subjects at risk) is lower than the total number of subjects because 80 of the 1092 subjects evaluated at base line and 71 of the 935 subjects evaluated eight years before base line did not have *APOE* genotype data available and were excluded from the analyses shown.

‡Log-transformed values were used for plasma folate and plasma vitamin B_6.

study plasma homocysteine levels and base-line values for plasma B vitamins and other covariates. A limitation of this study is the lack of racial diversity in the overwhelmingly white Framingham cohort. It is possible that our use of samples obtained from nonfasting subjects resulted in estimates of plasma homocysteine levels that were up to 20 percent higher than they would have been in fasting subjects,[53] but any increase in the variability in plasma homocysteine values caused by this approach is likely to be random and is unlikely to have altered the results.

Vitamin therapy with folic acid, alone or in combination with vitamins B_6 and B_{12}, and dietary supplementation with enriched cereal-grain products and breakfast cereals containing folate can reduce plasma homocysteine levels.[54-56] The U.S. government now mandates folic acid fortification of the food supply.[55] Current plasma homocysteine levels in the Framingham Study population are significantly lower than those that were estimated at the 16th and 20th biennial examinations.[56] However, only 20 cases of dementia were diagnosed between 1997 and the time the levels were remeasured, and therefore it is not possible to assess the effect of recent increases in folic acid fortification on the risk of dementia in this cohort. Furthermore, since there have been no prospective trials of the effect of vitamin supplementation on the incidence of dementia, our findings cannot be used as a basis for setting health policy or treatment recommendations.

The relation between elevated plasma homocysteine levels and dementia must be evaluated in other cohort studies. If such studies confirm our findings, proof of a causal association between plasma homocys-

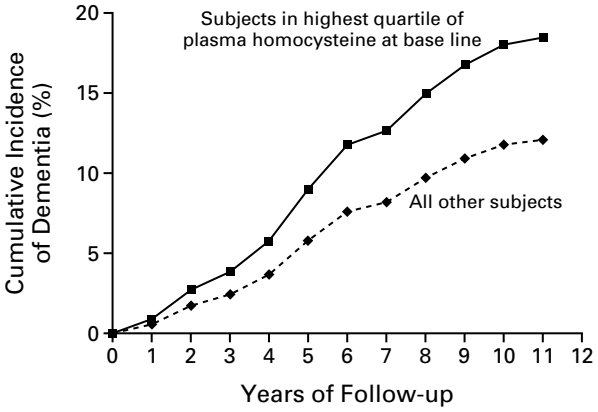

Figure 1. Crude Cumulative Incidence of Dementia among Subjects with Base-Line Plasma Homocysteine Levels in the Highest Age-Specific Quartile and among All Other Subjects.

The 75th percentile of the plasma homocysteine level (the cutoff point for quartile 4) was 13.2 μmol per liter for subjects 65 to 69 years old, 13.8 μmol per liter for subjects 70 to 74 years old, 14.5 μmol per liter for subjects 75 to 79 years old, 16.5 μmol per liter for subjects 80 to 84 years old, 19.3 μmol per liter for subjects 85 to 89 years old, and 26.6 μmol per liter for subjects 90 to 95 years old.

teine and the development of dementia and Alzheimer's disease will require further elucidation of the pathophysiologic mechanisms and direct evidence from controlled clinical trials in humans that interventions that reduce plasma homocysteine levels can reduce the risk of clinical dementia and Alzheimer's disease.

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Genome-Wide Meta-Analysis of Homocysteine and Methionine Metabolism Identifies Five One Carbon Metabolism Loci and a Novel Association of *ALDH1L1* with Ischemic Stroke

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Abstract

Circulating homocysteine levels (tHcy), a product of the folate one carbon metabolism pathway (FOCM) through the demethylation of methionine, are heritable and are associated with an increased risk of common diseases such as stroke, cardiovascular disease (CVD), cancer and dementia. The FOCM is the sole source of *de novo* methyl group synthesis, impacting many biological and epigenetic pathways. However, the genetic determinants of elevated tHcy (hyperhomocysteinemia), dysregulation of methionine metabolism and the underlying biological processes remain unclear. We conducted independent genome-wide association studies and a meta-analysis of methionine metabolism, characterized by post-methionine load test tHcy, in 2,710 participants from the Framingham Heart Study (FHS) and 2,100 participants from the Vitamin Intervention for Stroke Prevention (VISP) clinical trial, and then examined the association of the identified loci with incident stroke in FHS. Five genes in the FOCM pathway (*GNMT* [$p = 1.60\times10^{-63}$], *CBS* [$p = 3.15\times10^{-26}$], *CPS1* [$p = 9.10\times10^{-13}$], *ALDH1L1* [$p = 7.3\times10^{-13}$] *and PSPH* [$p = 1.17\times10^{-16}$]) were strongly associated with the difference between pre- and post-methionine load test tHcy levels (ΔPOST). Of these, one variant in the *ALDH1L1* locus, rs2364368, was associated with incident ischemic stroke. Promoter analyses reveal genetic and epigenetic differences that may explain a direct effect on *GNMT* transcription and a downstream affect on methionine metabolism. Additionally, a genetic-score consisting of the five significant loci explains 13% of the variance of ΔPOST in FHS and 6% of the variance in VISP. Association between variants in FOCM genes with ΔPOST suggest novel mechanisms that lead to differences in methionine metabolism, and possibly the epigenome, impacting disease risk. These data emphasize the importance of a concerted effort to understand regulators of one carbon metabolism as potential therapeutic targets.

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Introduction

As the fourth leading cause of death and the leading cause of disability in American adults, stroke constitutes a major public health burden. Epidemiological data consistently demonstrate an association between elevated plasma homocysteine (tHcy) and increased risk for stroke [1], cardiovascular disease [2], and dementia [3], but clinical trials of interventions to lower homocysteine have failed to demonstrate global benefit, with B12 supplementation helping to reduce risk only in subsets of the populations studied [4–6]. Collectively, these data support a more complicated relationship than simple biomarker and disease risk and indicate the need for new targets for risk reducing therapies. This begs the question, "Have we already missed the target of greatest clinical benefit by the time we lower homocysteine levels?" The folate one-carbon metabolism pathway (FOCM) is not only involved in the regulation of homocysteine, methionine and B-vitamin levels but also the methylation of proteins, histones, DNA and RNA. To this end, the demethylation of S-adenosyl-methionine, which gives rise to S-adenosyl-homocysteine, is the sole source of *de novo* methyl groups for the cell. Dysregulation of this step in the FOCM could have broad implications on many cellular processes, including risk for stroke and cardiovascular disease.

The post-methionine load test is a more sensitive tool for diagnosing hyperhomocysteinemia than circulating plasma homocysteine levels [7–10]. Additionally, "ΔPOST", or the difference in tHcy levels before and after methionine loading in the clinic, gives a measurement of one's ability to convert methionine to homocysteine in real time and likely reflects methyl group availability in the cell. We utilized this test to analyze genetic determinants of methionine metabolism and how these differences between individuals may be functionally regulated.

Here we present a genomic, genetic and epigenetic investigation into the regulation of methionine metabolism in the Vitamin Intervention for Stroke Prevention Trial (VISP) and the Framingham Heart Study (FHS). We first present genome-wide association (GWAS) data linking five loci to differences in ability to convert methionine to homocysteine. Strikingly, all of the most significant genes identified within these loci are members of the same pathway (FOCM), a feature rarely observed in GWAS studies.

We observed haplotype differences at the *GNMT* [MIM 606628] locus, our most significant GWAS finding, and devised a scheme to test methionine loading *in vitro* based on *GNMT* genotype. Additionally, we have shown epigenetic regulation of the *GNMT* promoter, based on a CpG-SNP rs11752813, which likely contributes to *GNMT* transcription and methionine metabolism. These data may one day contribute to identification of new targets for stroke and cardiovascular disease prevention as well as other complex diseases where epigenetics play a role.

Results

Framingham Heart Study (FHS) and Vitamin Intervention for Stroke Prevention Trial (VISP) cohorts

The FHS cohort is a community based longitudinal study to determine the risk for cardiovascular disease and is comprised of randomly recruited participants and their family members in the town of Framingham, Massachusetts (Table 1). VISP was a multicenter, double-blind, randomized, controlled clinical trial designed to determine if vitamin supplementation reduced recurrent cerebral infarction, nonfatal myocardial infarction or mortality and is made up of unrelated individuals. The VISP cohort has a higher proportion of men when compared to the FHS, which is not surprising when considering the VISP participants have all had a stroke (Table 1). Likewise, VISP also has a greater percentage of diabetics and hypertensive individuals (Table 1). The VISP cohort consists of individuals in the top quartile of circulating tHCY levels, which was part of the recruitment requirements; whereas FHS is made up of a normal distribution of tHCY levels (Table 1). FHS participants have higher vitamin B6, B12, and folate levels on average than VISP participants. BMI and smoking status are approximately the same between VISP and FHS.

The VISP study consisted of 1725 (82.1%) individuals of European descent, 258 (12.2%) individuals from African descent and 117 (5.6%) individuals of other ancestral populations. All VISP participants are unrelated. FHS samples are primarily Caucasian. In FHS the 3110 individuals contributing to GWAS belong to 1055 families with extended family size ranging from 1 to 140. In FHS, 1772 individuals have at least one blood relative in the family, 279 individuals have at least one first degree relative, 278 have at least one second degree relative, and 586 have at least one third degree relative.

Association of Folate One-Carbon Metabolism genes with methionine metabolism

In a GWAS of 2,710 persons from the FHS study, five loci (*GNMT, CBS* [MIM 613381], *CPS1* [MIM 608307], *ALDH1L1* [MIM 600249] and *PSPH* [MIM 172480]) reached our predetermined genome-wide significance threshold of 5×10^{-8} for the ΔPOST phenotype. These findings were confirmed in the VISP study sample of 2,100 persons for whom two of these loci (*GNMT* and *CBS*) independently reached genome wide significance (Figure S1, S2 and Table S1). The results of a sample size-weighted meta-analysis consisting of 4,810 subjects from both FHS and VISP confirm and strengthen the independent GWAS findings (Figure 1 and Table 2). Strikingly, all five loci identified are involved in the FOCM pathway (Figure S3). The most significant association was with *GNMT* (rs9296404, $p = 1.60\times10^{-63}$) located on 6p21.1, a region associated with large artery atherosclerotic stroke [11,12].

Haplotype analysis of *GNMT* locus

Using ten genotyped single nucleotide polymorphisms (SNPs) on chromosome 6 in the *GNMT* region, which were significantly-associated with ΔPOST in the VISP population, (Figure S1A), we conducted a haplotype analyses (Haploview software) [13]. Two major haplotypes emerged, encompassing ~81% of the individuals in the VISP population (n = 2,100) (Figure 2A,B) and corresponding to a high methionine metabolizing haplotype (ΔPOST = 19.4 μmol/L) and a low methionine metabolizing haplotype (ΔPOST = 14.5 μmol/L) (Figure 2C). One SNP,

Author Summary

Elevated homocysteine (tHcy) is strongly associated with risk for common disorders such as stroke, cardiovascular disease and Alzheimer disease. Lowering tHcy levels has proven to have variable success in reducing clinical risk, so the question remains, "Are we correctly targeting these disorders by lowering tHcy?" Understanding folate one-carbon metabolism pathway (FOCM) genetic variation will aid us in developing new targets for therapy. The FOCM is essential in regulation of the epigenome, which controls genes in ways beyond nucleotide sequence. We present data generated from stroke-only and general populations where we identify strong association of genetic risk factors for variation in one-carbon metabolism function, characterized by the post-methionine load test. We show that *GNMT* harbors genetic and epigenetic differences that influence gene function, which may have downstream effects on the epigenome of the cell, affecting disease risk. We developed a genetic risk score that predicts post-methionine load homocysteine levels that may be useful in clinic. Finally, we identified a novel association between ischemic stroke and *ALDH1L1*, which emphasizes the clinical importance of this work. Our results highlight the importance of a concerted effort to target the FOCM (beyond tHcy) and parallel pathways in future pharmacogenetic work using the genetic variation we describe here.

rs10948059, which is a genotyped and located in the *GNMT* promoter, captures 100% of alleles with a mean max r^2 of 0.722 (range 0.512–0.850). These data suggest functional differences in the *GNMT* gene impact an individual's ability to metabolize dietary methionine. The lack of a disruptive coding mutation identified by GWAS, or in sequencing of *GNMT* in 24 high and 24 low methionine metabolizers (data not shown), and the expectation that a higher rate of transcription of the *GNMT* gene should lead to higher tHcy levels, suggest a regulatory mechanism for the differences in ΔPOST rather than protein dysfunction. This metabolic difference mediated by genetic variation is of functional significance in both the general population (FHS) and a population with tHcy above the 25th percentile as required by the inclusion criteria for the clinical trial (VISP).

Promoter analysis of *GNMT*

The known *GNMT* promoter [14] from the high methionine metabolizing haplotype and the low methionine metabolizing haplotype were cloned giving rise to GNMT$^{\Delta HighLuc}$ and GNMT$^{\Delta LowLuc}$ constructs (Sequence alignments in Figure S4). *GNMT* is most highly expressed in the liver; therefore HepG2 cells were used to test promoter activity in the two haplotype groups. There was a ~30% difference in gene promoter activity between GNMT$^{\Delta HighLuc}$ and GNMT$^{\Delta LowLuc}$ constructs (Figure 3A), when cultured with L-methionine, which correlates with the differences seen between the average ΔPOST levels in our haplotype analysis (Figure 2C).

The quantitative trait, ΔPOST, is dependent on methionine dosing, therefore we starved HepG2 cells of L-methionine for 24 hours and then treated them with L-methionine for 24 hours. As seen in Figure 3C, the GNMT$^{\Delta HighLuc}$ construct responded to methionine starvation and treatment with ~2× greater activity than the GNMT$^{\Delta LowLuc}$ construct. Above standard L-methionine culturing conditions (0.2 mM) a feedback mechanism appears to be induced, which reduces *GNMT* expression (Figure 3B). These data suggest a mechanism by which elevated levels of tHcy may arise.

Epigenetic analysis of C/G SNP rs11752813

Given the differences in promoter activity seen in Figure 3, we sought to identify functional variants that may play a role transcriptional activity. SNP rs11752813 was significantly associated in our meta-analysis (Figure 1A, $p = 7.99\times10^{-32}$), and either creates or eliminates a CpG site that can be methylated depending on genotype (rs11752813 and flanking sequence: C(**C/G**)A). The high methionine metabolizing haplotype (Figure 2) harbors the C/C genotype (known as GNMT$^{\Delta HighLuc}$ in Figure 3) at rs11752813, and the low methionine metabolizing haplotype (Figure 2) harbors the G/G genotype (known as GNMT$^{\Delta LowLuc}$ in Figure 3) at rs11752813. The presence of a "G" at the rs11752813 locus creates a CpG site while the presence of a "C" eliminates this CpG site. Analysis of ΔPOST values in the VISP (n = 2100) study shows that the individuals that harbor the "G" genotype at rs11752813 have significantly lower ΔPOST on average, indicating a less active *GNMT* gene (Figure 4A). Bisulfite pyrosequencing of the rs11752813 locus show that the G/G genotype can be methylated whereas the C/C genotype is not methylated (Figure 4B). These results are consistent with the central dogma of DNA methylation that only CpG sites can be methylated. Finally, 23 individuals harboring the G/G genotype at rs11752813 were bisulfite pyrosequenced and percent methylation status was plotted against individual ΔPOST values (Figure 4C). As seen in Figure 4C, even between individuals with the G/G genotype there is a strong correlation between percent methylation and ΔPOST values. These data indicate that the G/G genotype at rs11752813 creates a closed chromatin state that inhibits *GNMT* transcription and methionine metabolism.

Genetic risk score of most significant ΔPOST variants

We next investigated cumulative effects of ΔPOST risk variants by generating a combined genetic risk score using the most significant SNPs from Table 2. Risk scores are normally distributed in both VISP and FHS (Figure 5A,C). As the risk variant load increases, the average ΔPOST levels in the VISP and FHS samples increases (Figure 5B,D). This score explains 13% of the variability of ΔPOST in FHS and 6.3% of the variability in VISP (Table 3). Because the SNPs used in this analysis are all imputed, for both the VISP and FHS studies, we repeated the analysis utilizing the most significant genotyped SNPs from each locus. This analysis yielded similar results indicating that using imputed SNPs for this risk score does not distort the analysis (Figure S5).

ALDH1L1 is associated with ischemic stroke in the Framingham Heart Study

Importantly, when interrogating the most significant five SNPs associated with ΔPOST, we also identified an association between the aldehyde dehydrogenase 1 family member L1 gene (*ALDH1L1*) and incident ischemic stroke in the FHS cohort (rs10934753, hazard ratio = 1.26, p = 0.015, n = 168 cases 4008 controls, analyses adjusted for age, sex and family relationships). The protein encoded by *ALDH1L1* converts 10-formyltetrahydrofolate to tetrahydrofolate and is an essential component of the FOCM pathway. These results provide a new and significant link between the FOCM pathway and risk of initial ischemic stroke. It is important to note that the VISP population consists of exclusively ischemic stroke patients examined for recurrent stroke over a 2 year period; rs10934753 was not associated with recurrent stroke. Additionally, we did not observe an association of genetic variation in *GNMT* or the other 3 loci with incident ischemic stroke in FHS but our sample has limited power to detect moderate effect size

Table 1. Summary statistics for Framingham Heart Study (FHS) and Vitamin Intervention for Stroke Prevention (VISP) subjects.

	FHS			VISP		
	N	**Male(%)**	**Female(%)**	**N**	**Male(%)**	**Female(%)**
Sex	3110	1461(46.98%)	1649(53.02%)	2100	1315(63.57%)	785(37.38%)
Trait	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**
Age	3110	58.52	9.68	2100	67.19	10.75
tHcy (μmol/L)	3108	9.75	4.00	2090	13.32	4.83
POST tHcy (μmol/L)	2711	24.47	8.19	1947	29.71	10.11
ΔPOST tHcy (μmol/L)	2710	14.80	6.55	1944	16.50	8.81
Vitamin B_6 (pm/mL)	3110	84.89	83.33	1897	42.45	37.38
Vitamin B_{12} (pg/mL)	3110	417.97	167.84	1954	358.79	181.91
Folate Assay with L.Casei (ng/mL)	3102	10.89	7.84	1954	25.86	15.91
BMI (kg/m^2)	3105	27.89	5.10	1992	28.85	5.29
	N	**Events(%)**		**N**	**Events(%)**	
Current Smoker	3107	477(15.35%)		2100	328(15.62)	
Prevalent Diabetes	3110	354(11%)		2074	562(27.10)	
Prevalent Hypertension (JNC-7 Stage 1 or greater)	3103	1267(40.83%)		2069	1491(71.00)	
Stroke at baseline	3110	82(2.6%)			(100%) All Ischemic Stk.	
ABI	3110	104 (3.3%)				
CE	3110	64 (2.1%)				
ICH	3110	28 (0.9%)				
SAH	3110	3 (0.0009%)				
OTH	3110	1 (0.0003%)				

tHCY-Circulating total plasma homocysteine. POST tHcy-Post-methionine load plasma homocysteine. ΔPOST tHcy-Difference between pre-methionine load homocysteine and post-methionine load homocysteine. BMI-Body Mass Index. Stroke subtypes: ABI-Acquired Brain Injury, CE- Cardioembolism, ICH- Intracerebral Hemorrhage, SAH-Subarachnoid Hemorrhage, OTH- stroke of other determined etiology.
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(e.g. power ranges from 20–40% to detect a hazard ratio of 1.20 for a variant with minor allele frequency ranging from 0.1–0.5).

Discussion

Elevated tHcy has long been associated with increased risk for stroke and cardiovascular disease but to date functional evidence for the driving genetic forces behind elevated tHcy levels have only been attributable, in part, to dysfunction in the methylenetetrahydrofolate reductase gene (*MTHFR* [MIM 607093]) and *CBS* genes [15–18]. *MTHFR*, which also participates in the FOCM pathway, is tightly related to all the genes products identified in our study and has been implicated in susceptibility to vascular disease, neural tube defects, colon cancer and acute leukemia [19–24]. It is interesting to note that a prior GWAS in the individual FHS and VISP cohorts or in a meta-analysis yielded no significant results for baseline tHcy alone. This highlights the usefulness of the post-methionine load test in the diagnosis of hyperhomocysteinemia as well as the fact that we can specifically detect genetic variations that lead to differential methionine metabolism.

In the current study we followed up with a functional evaluation of our GWAS findings. These independent GWAS analyses and a meta-analysis of FHS and VISP find the *GNMT* locus as the top result. However, there were differences in the GWAS results from FHS and VISP are likely attributable to two factors: one being power (FHS consists of 610 more individuals), and two, VISP is a more homogenous population than FHS lacking a normal distribution of tHcy. Our functional studies start with the *GNMT* gene as it contributes to the majority of the variance in both VISP and FHS. The *GNMT* association makes biological sense given that GNMT catalyses the conversion of S-adenosyl-methionine (SAM) to S-adenosyl-homocysteine (SAH), using SAM as the methyl donor [25], and affects global cellular epigenetic status as the sole source of methyl groups for the cell including those used in DNA, histone, protein and RNA modifications. Additionally, it is known that global hypomethylation is seen in atherosclerosis [26], and we suspect that variation in *GNMT* could affect risk status. Further, hyperhomocysteinemia is a risk factor for stroke and cardiovascular disease, and these data indicate *GNMT* may represent a new pharmacogenetic target for reducing stroke risk. It is our assertion that targeting the FOCM pathway before methionine is converted to homocysteine may allow us to modulate parallel pathways, such as DNA and histone methylation, which directly impact stroke risk. A recent review by Krishna et al. [27] describes in detail a "tHcy memory effect" that may alter the epigenetic state of the cell and promote deleterious chances after tHcy is lowered. This further strengthens the argument to identify genetic risk, through use of our risk score, and examine parallel targets for therapy.

We did not observe any of the deleterious *GNMT* mutations associated with Glycine N-Methyltransferase Deficiency [MIM 606664] (characterized by elevated levels of plasma S-adenosyl-methionine and normal plasma sarcosine) in sequencing of *GNMT*

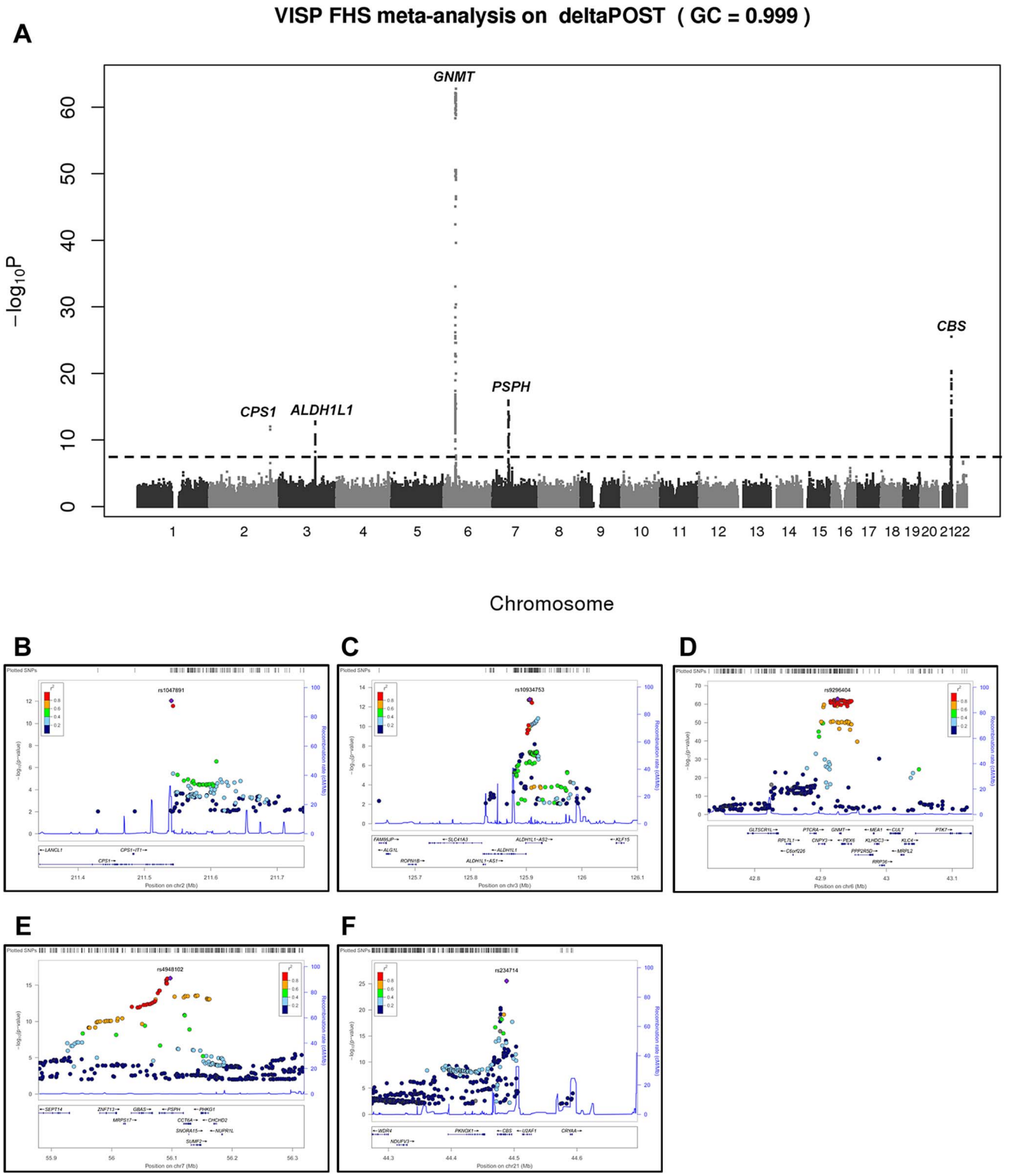

Figure 1. Meta-analysis and chromosome 2, 3, 6, 7 and 21 regional association of single nucleotide polymorphisms (SNPs) for ΔPOST in both VISP and FHS cohorts. A sample size-weighted meta-analysis was used. (A) Manhattan plot of meta-analysis association results for the combined VISP and FHS samples. Association p-values are noted on the Y axis (-$\log_{10}$ P value), with points above the dashed line indicating SNPs reaching or exceeding genome-wide significance ($P \leq 5 \times 10^{-8}$). (B–F) Locus Zoom plots showing the regional association of chromosomes 2, 3, 6, 7 and 21. Y-axis shows $-\log_{10}$ p-value≤0.01. X-axis shows Mb position on each chromosome. Each circle represents an independent SNP and color shading represents r^2 values.
doi:10.1371/journal.pgen.1004214.g001

Table 2. Most significant meta-analysis SNPs.

RS#	Chr	BP	Associated Gene	Gene Region	Alleles (Minor/Major)	MAF FHS	P-Value FHS	MAF VISP	P-Value VISP	N Meta	MAF Meta	Z-score	Direction	IQ FHS	IQ VISP	P-Value
rs9296404	6	42925803	*GNMT*	5′	C/T	0.48	1.29E-42	0.46	3.40E-23	4810	0.47	16.83	++	0.96	0.98	1.60×10^{-63}
rs234714	21	44488033	*CBS*	Intron 4/5′ UTR	T/C	0.20	2.28E-18	0.22	1.03E-09	4810	0.21	10.6	++	0.49	0.98	3.15×10^{-26}
rs4948102	7	56097265	*PSPH*	Intron 3	C/G	0.25	1.40E-15	0.28	5.21E-04	4810	0.26	8.29	++	0.77	0.99	1.17×10^{-16}
rs10934753	3	125906179	*ALDH1L1*	5′	A/G	0.42	4.90E-13	0.40	0.0033	4810	0.42	7.37	++	0.99	Genotyped	7.3×10^{-13}
rs1047891	2	211540507	*CPS1*	Ser (ACC)/Phe (AAC)	A/C	0.30	1.35E-08	0.37	1.30E-05	4810	0.32	7.14	++	0.73	Genotyped	9.10×10^{-13}

RS#-SNP annotation, dbSNP build 137. Chr-Chromosome. Position from dbSNP build 137. MAF-minor allele frequency from 4810 VISP and FHS subjects used in meta-analysis. All SNPs seen here are imputed with 1000Genomes data. IQ = Imputation Quality.

doi:10.1371/journal.pgen.1004214.t002

Figure 2. Haplotype analysis of Chr6 SNPs significantly-associated with ΔPOST in the VISP cohort. Haplotype analyses of the 10 most-associated SNPs, which are all genome wide significant, from chromosome 6, were performed using Haploview version 4.2 [13]. (A) Shows results for the chromosome 6 genomic haplotype structure in VISP, encompassing the *GNMT* gene. Genetic coordinates, Entrez gene structure, haplotype blocks and linkage disequilibrium (LD) pattern between SNPs are shown. Within LD pattern, r^2 values are shown and are represented by shading. The darker the shading the closer the r^2 value is to 1.0. All SNPs assessed were genotyped. (B) Shows haplotypes generated by Haploview for haplotype blocks 1 and 2. Haplotype block

2 is characterized by two major haplotypes, which account for 80% of haplotypes observed. (C) Shows the mean ΔPOST values in the VISP sample for each of the 2 major haplotype block 2 haplotypes. (*) p≤0.001 by student's T-test; error bars represent standard deviation from the mean (SD).
doi:10.1371/journal.pgen.1004214.g002

in 24 high and 24 low methionine metabolizers. Additionally, a lookup of large GWAS studies of cardiovascular and cerebrovascular disease, identified many of our most significant chromosome 6 findings in a meta-analysis for blood lipids [28], with rs2274517 being the most significant result ($p = 1.37 \times 10^{-4}$), suggesting that *GNMT* may play a broader role in risk traits for CVD beyond tHcy measures.

Our functional studies support the role of *GNMT* in variation of methionine metabolism. The consistent and biologically plausible results from the individual GWAS and meta-analysis emphasize that the other significant associations observed in the FOCM pathway cannot be ignored. The cystathionine-beta-synthase (*CBS*) gene has been associated with stroke [20] and methionine metabolism [29]. Additionally, *CBS* mutations are associated with homocystinuria, iridodonesis and agitated motion of the iris [MIM 236200] [30–33]. Within the carbamoyl phosphate synthetase one (*CPS1*) gene, ΔPOST rs1047891 was found to be associated with a

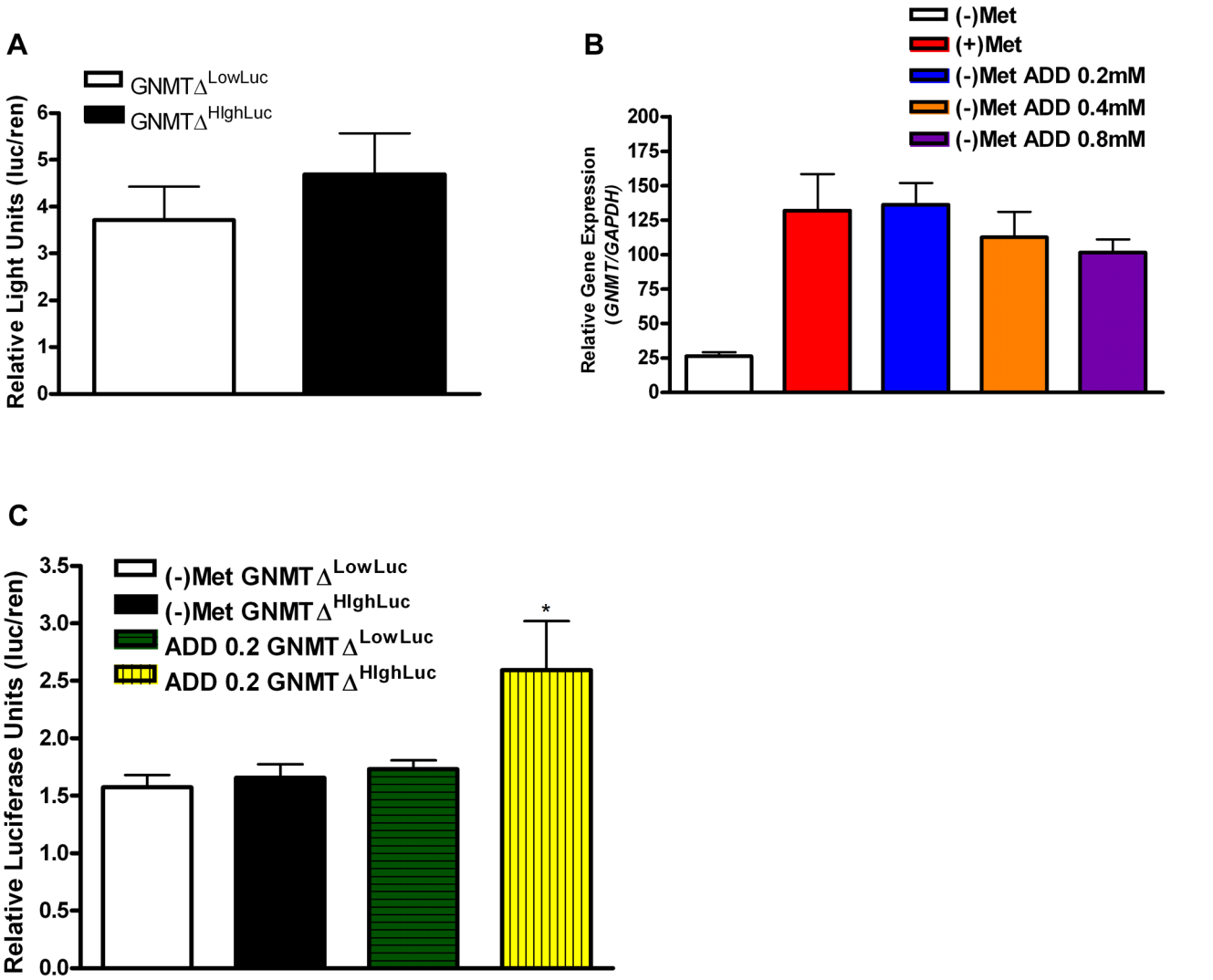

Figure 3. *GNMT* promoter analysis of major haplotype groups. (A) Histogram shows mean luciferase activity of the high-methionine-metabolizing haplotype (TCCGGAT) and the low-methionine-metabolizing haplotype (CGTCTGC) represented by constructs $GNMT^{\Delta HighLuc}$ and $GNMT^{\Delta LowLuc}$ cultured in standard DMEM with L-methionine (0.2 mM). (B) Shows *GNMT* qPCR analysis in HepG2 cells. Culturing conditions are as follows: (+)Met = standard complete DMEM cultured for 48 hours, (−)Met = complete DMEM without L-Methionine culture for 48 hours, (−)Met ADD 0.2 = DMEM without L-Methionine culture for 24 hours, addition of L-Methionine at 0.2 mM for 24 hours, (−)Met ADD 0.4 = DMEM without L-Methionine culture for 24 hours, addition of L-Methionine at 0.4 mM for 24 hours, (−)Met ADD 0.8 = DMEM without L-Methionine culture for 24 hours, addition of L-Methionine at 0.8 mM for 24 hours. (C) (−)Met $GNMT^{\Delta HighLuc}$ and (−)Met $GNMT^{\Delta LowLuc}$ represent HepG2 cells transfected with $GNMT^{\Delta HighLuc}$ and $GNMT^{\Delta LowLuc}$ constructs and cultured without L-methionine for 48 hours. ADD 0.2 $GNMT^{\Delta LowLuc}$ and ADD 0.2 $GNMT^{\Delta HighLuc}$ represent HepG2 cells transfected with $GNMT^{\Delta HighLuc}$ and $GNMT^{\Delta LowLuc}$ constructs cultured without L-methionine for 24 hours and with 0.2 mM L-methionine for 24 hours. * $P<0.05$ by students t-test. N = 3 biological replicates for all analyses. Error bars represent standard deviation from the mean (SD).
doi:10.1371/journal.pgen.1004214.g003

missense Ser(ACC)/Phe(AAC) mutation ($p = 9.10 \times 10^{-13}$). These findings are related to a sex-specific association of *CPS1* with tHcy and women, performed in the Woman's Health Genome Study [34] and a Filipino population [35]. We meta-analyzed ΔPOST, rather than tHcy, and included both men (54%) and women (46%). Phosphoserine phosphatase (*PSPH*) mutations have been associated with phosphoserine phosphatase deficiency [MIM 614023], which results in pre- and postnatal growth retardation, moderate psychomotor retardation, and facial features suggestive of Williams syndrome [36,37].

Taken together, these data present a new link to the genetics of the FOCM pathway with methionine metabolism both in stroke and non-stroke populations. Because of the impact that the FOCM pathway has on the biology of the cell, including overall epigenetic state and DNA methylation, gluconeogenesis, and DNA repair, understanding how individual genetic composition impacts this pathway is essential. The FOCM has also been implicated in many aspects of human health and the work presented may be relevant to several key biological mechanisms, affecting tumorogenesis [38], B-vitamin utilization [39], as well as cardiovascular and cerebrovascular disease risk. Additionally, it is necessary to repeat these analyses in studies of different ethnicities as both FHS and VISP are comprised of mainly individuals of European descent.

While a direct link between tHcy levels and stroke and cardiovascular disease remains debated, we have shown that understanding sequence variation in the FOCM pathway may provide a link to functional differences in the population, that in turn tie one carbon metabolism to a broad range of disease risk factors. Additionally, because tHcy-lowering therapies have had variable success in reducing stroke risk in subtype populations [6,40,41] and have in some cases been harmful [42], we believe that understanding how these genetic variants impact the overall FOCM and related pathways is essential to understanding the pathogenesis of stroke. Methionine metabolism provides a first clue to the impact of this pathway on cell biology.

Materials and Methods

Ethics statement

All human research was approved by the relevant institutional review boards, and conducted according to the Declaration of Helsinki. The Framingham Heart Study protocol was approved by the institutional review board (IRB) of the Boston University

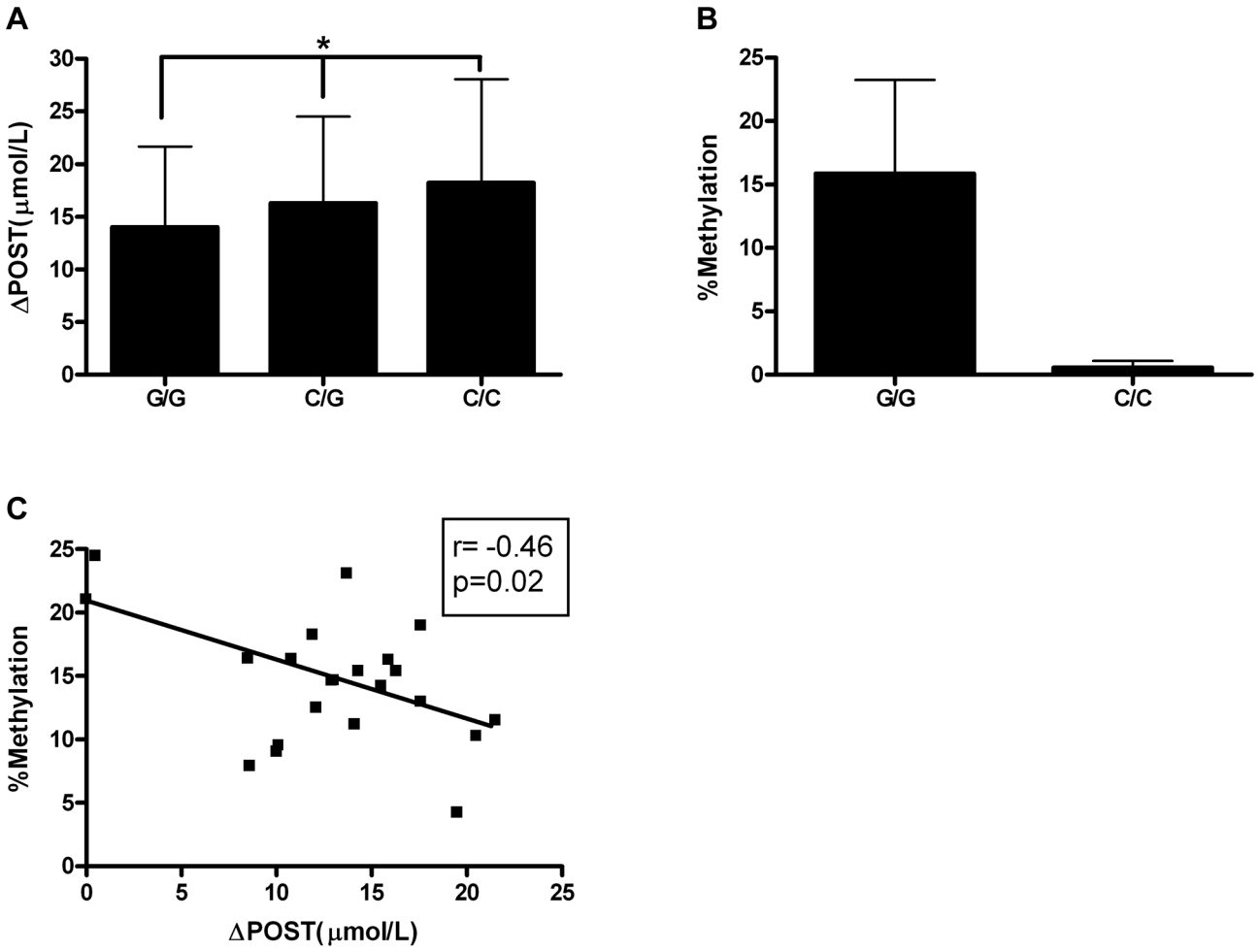

Figure 4. Epigenetic Evaluation of rs11752813. (A) Average ΔPOST (μmol/L) in VISP trial based on rs11752813 genotype. (*) $p < 0.001$ by one-way ANOVA and all genotypes are significantly different by students t-test. (B) Percent methylation analyzed by bisulfite pyrosequencing of n = 23 individuals (G/G) and n = 6 individuals (C/C). (C) Linear regression analysis of percent methylation of rs11752813 based on genotype and ΔPOST (μmol/L).
doi:10.1371/journal.pgen.1004214.g004

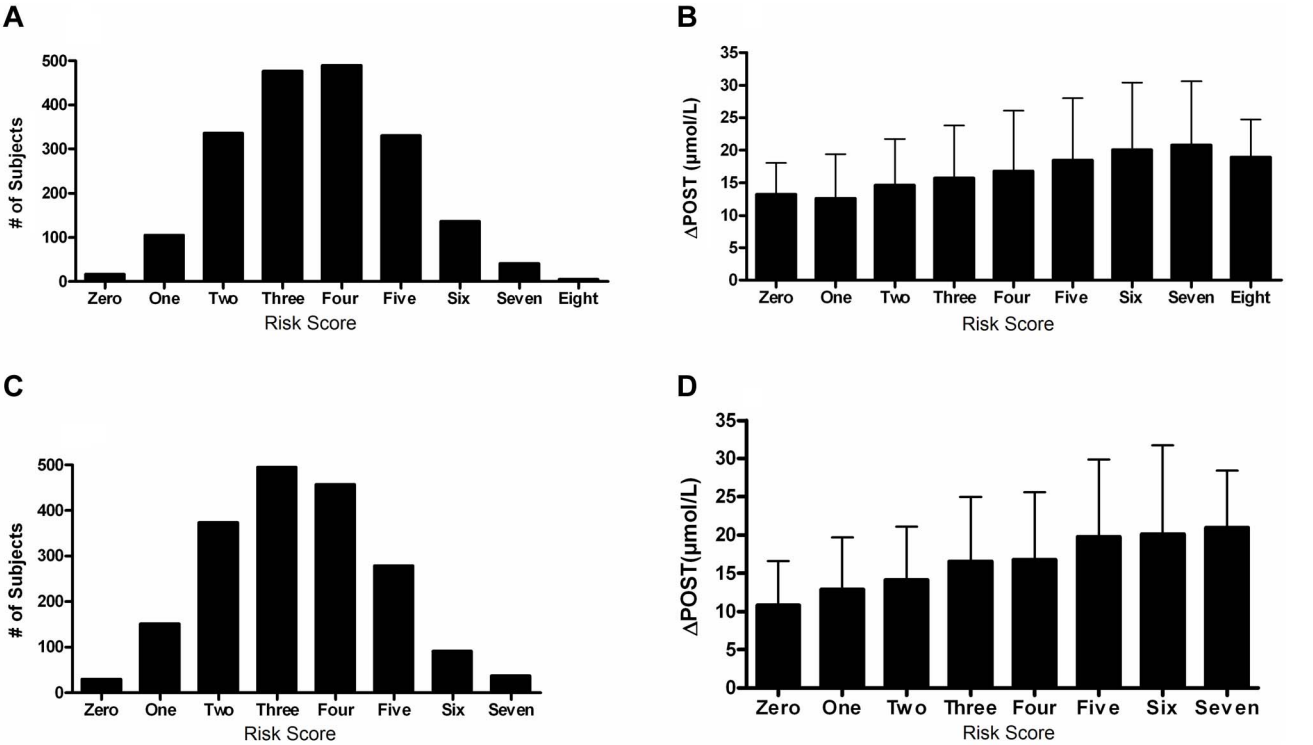

Figure 5. Risk score in FHS and VISP studies. (A) Distribution of risk scores among the FHS sample shows a normal distribution. Y-axis represents the number of individuals who have the given risk score seen on the x-axis. (B) X-axis represents the number of risk variants per subject in FHS. Y-axis represents the average ΔPOST value for each group containing a specific risk score. (C) Distribution of risk scores among the VISP sample shows a normal distribution. Y-axis represents the number of individuals who have the given risk score seen on the x-axis. (D) X-axis represents the number of risk variants per subject in VISP; Y-axis represents the average ΔPOST value for each group containing a specific risk score. Risk variants considered were SNPs at each of the 5 loci most significantly associated with ΔPOST in the meta-analysis. For each SNP a score of 0 was applied for homozygous non-risk variant, 1 for heterozygous at risk variant and 2 for homozygous at risk variant, derived from dosage values of 1000genomes imputation.
doi:10.1371/journal.pgen.1004214.g005

School of Medicine and all participants provided written, informed consent. The VISP study protocol was approved by the IRBs of the Wake Forest University School of Medicine (coordinating center) and University of North Carolina Chapel Hill School of Medicine (statistical center). The local IRB for each of the individual recruiting sites approved the VISP protocol and

Table 3. Variance and effect size explained by risk score.

Variance Explained							
RS#	**Chr**	**Position (bp)**	**Associated Gene**	**Gene Region**	**FHS V.E.**	**VISP V.E.**	**P-Value**
rs9296404	6	42925803	*GNMT*	5′	0.059651	0.042	1.60×10^{-63}
rs234714	21	44488033	*CBS*	Intron 4/5′ UTR	0.025319	0.017	3.15×10^{-26}
rs1047891	2	211540507	*CPS1*	Ser (ACC)/Phe (AAC)	0.010857	0.0062	9.10×10^{-13}
rs10934753	3	125906179	*ALDH1L1*	5′	0.01746	0.0061	7.3×10^{-13}
rs4948102	7	56097265	*PSPH*	Intron 3	0.021223	0.0023	1.17×10^{-16}
					Total = 0.13	Total = 0.06	
Effect Size							
		Estimate*	**SE**	**T**	**P-Value**		
FHS		1.7(μmol/L)/risk var.	0.087	19.52	1.68×10^{-79}		
VISP		1.6(μmol/L)/risk var.	0.130	11.85	2.4×10^{-31}		

RS#-SNP annotation, dbSNP build 137. Chr-Chromosome. Position from dbSNP build 137. V.E.-Variance explained.
* = Average ΔPOST increase per risk variant added.
doi:10.1371/journal.pgen.1004214.t003

all participants provided written, informed consent. The Genomics and Randomized Trial Network (GARNET) analysis of the VISP data was approved by University of Virginia School of Medicine IRB.

Genome wide analysis of Framingham Heart Study

FHS started in 1948 for evaluation of cardiovascular diseases and risk factors [43]. In 1971, 5124 children of the original cohort, and spouses of these children, referred to as Offspring cohort, were enrolled and have been examined approximately every four years [44]. Genotyping was performed on the Affymetrix 500K mapping array and the Affymetrix 50K supplemental array. Circulating homocysteine levels were measured on 3465 Offspring participants (N = 3464 with tHcy and N = 2999 with POST) during examination cycle 6 (1995–1998). The study sample for GWAS consists of a subset of 3110 individuals with at least one phenotype and GWAS data (N = 3108 with tHCY, N = 2711 with POST, N = 2710 with Δ POST). The sample used to examine the association between the SNPs identified in GWAS of homocysteine phenotypes and incident stroke includes 4176 original cohort and Offspring individuals (N stroke = 200 stroke; N ischemic stroke = 168).

Homocysteine measurement and stroke classification

Plasma tHcy levels were measured using high-performance liquid chromatography with fluorescence detection [45].

Clinical stroke was defined as rapidly developing signs of focal neurologic disturbance of presumed vascular etiology lasting more than 24 hours. Additional details of stroke classification and diagnosis can be found in prior publications [45–48].

Imputation and statistical analyses

Imputation of about 11 million 1000 Genomes SNPs (1000G Phase I Integrated Release Version 3 Haplotypes: 2010–11 data freeze, 2012-03-14 haplotypes) was performed using MACH version 1.0.16 (http://www.sph.umich.edu/csg/abecasis/MACH/) based on 412,053 good quality SNPs (excluded SNPs were characterized by call rate <97%, pHWE<1E-6, Mishap p<1e-9, >100 Mendel errors, MAF<1%).

Prior to association analysis, homocysteine phenotypes were normalized by replacing its observed value with the corresponding quantile under normal distribution. For GWAS, linear mixed effects models were fitted with the transformed phenotypes as dependent variables, individual SNP genotype as a fixed effect, and person specific random effects with correlation coefficient between two individuals being twice their kinship coefficient to account for correlation within extended families [49]. FHS GWAS has adjusted for age, sex and first 10 eigenstrat principal components in the linear mixed effects mode (Table S2). Cox proportional hazard model with a robust variance to account for familial relationship was fitted to relate SNPs identified in GWAS with stroke outcomes [50].

VISP Imputation

Imputation was performed using all SNPs and samples passing basic quality filters. In brief, SNPs were selected using the recommended composite quality filter that emerged from the genotype data cleaning process. Samples were selected to have an overall missing call rate <2%, while certain sample-chromosome combinations were also excluded where a gross chromosomal anomaly was detected or when the chromosome-specific missing call rate was >5%. These study data were imputed to a phase 1 interim release from the 1000 Genomes (1000G) Project [51]. Imputation target variants were defined as those with MAF≥0.005 across all 629 1000G samples.

Imputation was carried out using BEAGLE imputation software [52] (v3.3.1) for chromosomes 1–22 and the X chromosome. The imputed dataset contained total 7,500,450 variants; 766,577 of which (10.2%) were observed from the array genotyping. In addition to the primary imputation analysis, additional imputations were run on chromosome 22 and the X chromosome, masking a random 10% of observed SNPs to empirically assess imputation quality. The squared correlation between observed and imputed allelic dosages (dosage r^2) was used to summarize the imputation quality. The median dosage r^2 was 0.933 for chromosome 22 masked SNPs and 0.930 for X chromosome masked SNPs. The imputed dataset, along with a detailed report on imputation methodology, is available through the authorized access portion of the VISP dbGaP posting.

Genotyping was performed on the Illumina HumanOmni1-Quad-v1 array (Illumina, Inc.) at the Center for Inherited Disease Research, Johns Hopkins University. The genome-wide association analysis was conducted using PLINK v1.0.7. Multivariate linear regression model was used to test correlation of quantitative traits and SNP markers. Using the KING software, the top 10 principle components were derived from genotype data [53] and subsequently used to adjust for population heterogeneity, in addition, age and gender were also included as covariates in the model. The VISP population consists of genetically confirmed unrelated individuals and no adjustments were made to the analysis for relatedness. To normalize phenotypic traits, inverse normal transformation was applied to values of POST and ΔPOST. The same regression model was employed to perform association tests between the phenotype and expected allele counts.

Meta-analysis of Δ post methionine load test homocysteine levels

Meta-analysis of the 2100 VISP and 3110 FHS cohorts was conducted using the METAL software [54]. The sample size of each study was used as weight, and the sign of the beta value of each SNP coded allele was used as the direction for association (Table S3).

Regional association plots

Figure 1 regional association plots were created using the locus zoom "plot your own data" function (https://statgen.sph.umich.edu/locuszoom/genform.php?type = yourdata). Plots were created utilizing the genome build/LD population hg19/1000 Genomes Mar 2012 EUR.

Cell culture

Unless otherwise noted, HepG2 cell lines were cultured in complete media containing high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) with 10% (v/v) FBS, 2 mM L-glutamine supplemented with 1× nonessential amino acids, 1 mM sodium pyruvate (Invitrogen) and 1X antibiotic-antimycotic (Invitrogen). Cells were maintained at 37°C in a 5% CO_2 incubator.

In vitro L-methionine treatment

HepG2 cells were culture in complete media as described above for 24 hrs in 25 cm^2 tissue culture flasks or 6 well culture plates. For methionine starvation, after 24 hrs media was removed and replaced with either complete media or complete media lacking

L-methionine. After 24 hrs, cells starved of methionine were supplemented with L-methionine (Ameresco) at concentrations of (0.2 mM, 0.4 mM, 0.8 mM).

Real time qPCR

RNA isolation was conducted using the Qiagen RNeasy kit according to standard manufacture's protocols. cDNA synthesis was conducted using 1 ug of total RNA and the Verso cDNA Synthesis Kit (Fisher Scientific). A 3:1 mix (v/v) of random hexamers and anchored oligo-dT was used following standard theromocycling conditions. For *GNMT* quantitative real-time PCR, Taqman MGB probes and primers were used (Hs002219089) (Applied Biosystems). All samples were run in triplicate in 10 µl reaction volumes. PCR conditions were the default settings of the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) using the standard curve setting to achieve raw data, which was analyzed in Microsoft Excel. The cycle threshold (Ct) was determined during the geometric phase of the PCR amplification plots as automatically set by the 7900 software. Relative differences in transcript levels were quantified using the ΔΔCt method with *GAPDH* [MIM 138400] (probe 4333764F) mRNA as an endogenous control.

Creation of luciferase plasmids

GNMT promoters were PCR amplified using primers (Forward: 5′-CGGGGTACCACAGAGCGAGACTGTGTC-3′, Reverse: 5′-GCGAGATCTCCTGCGCCGCGCCTGGCT-3′) as previously described [14]. One VISP case was chosen for TA cloning from the high ΔPOST and low haplotype ΔPOST haplotypes. Promoters were cloned into the StrataClone PCR Cloning vector according to the standard protocols (Agilent Technologies). Promoters were next restriction digested and ligated into the pGL3 luciferase plasmid (Promega) using KpnI and SacI enzymes (New England Biolabs) giving rise to $GNMT^{\Delta HighLuc}$ and $GNMT^{\Delta LowLuc}$.

Haplotype analysis

Haploview version 4.2 [13] was used to analyze haplotype blocks on chromosome 6 from the VISP population. 10 SNPs with $p \leq 5\times10^{-8}$ were assessed using version 3, release 27, analysis panel CEU+TSI.

Average ΔPOST values were taken from the VISP population, and any individuals with missing data from 1 or more SNPs were excluded from the analysis. The top two haplotypes, encompassing 80% of the total VISP population were assessed.

GNMT promoter analysis

2×10^4 HepG2 cells were transfected in quadruplicate with $GNMT^{\Delta HighLuc}$ or $GNMT^{\Delta LowLuc}$ and pGL4.74[hRluc/TK] (Promega) at a 10:1 ratio using TransIt-LT1 transfection reagent (Mirus biosciences) in 96 well plates. Luciferase assays were conducted following the Dual-glo kit standard protocol (Promega). Luciferase readings were taken using the Beckman Coulter DTX880 luminometer at a 1 second integration time. Firefly luciferase measures from $GNMT^{\Delta HighLuc}$ or $GNMT^{\Delta LowLuc}$ were taken for each well, followed by treatment for renilla luciferase activity and renilla measurement. Relative luciferase activity of each promoter was calculated by dividing the average firefly luciferase counts from $GNMT^{\Delta HighLuc}$ or $GNMT^{\Delta LowLuc}$ by pGL4.74[hRluc/TK] for each independent condition. L-Methionine treatment used 0.2 mM reagent. Total relative luciferase activity for each plasmid encompasses the average of 3 biological replicates.

Pyrosequencing

Pyrosequencing was performed as previously described [55]. Primers are as follows, Forward: AGTAGAGAAGTGTTAGTTAGGTTTTAT, Reverse (Biotin labeled): ACCCATACAAAAAAAACAAAAAAAATCTC, Sequencing primer: TTTGGATTAGGTGGATAG.

Risk score analysis

Scores were determined by using imputation dosage measures from VISP and FHS. Alleles were assessed for the average ΔPOST values in FHS and VISP. If a dosage for a homozygous SNP was associated with high homocysteine on average (i.e. close to the value 2) the number was not changed. However, if the homozygous allele was not associated with the risk variant (i.e. close to 0) but was represented as a number above 1.5, 2 was subtracted from that number and made positive. After correction, homozygous risk variants would have a dosage value near 2, heterozygous variants would have a value near 1, and non risk variants would be assigned a number near 0. All imputation dosage values were summed. The sum of each risk value was then taken for each individual to give a score from 0 to a possible 10, and that score was rounded to the nearest integer.

For calculation of the variance explained by the risk score, linear regression was used for VISP, and a linear mixed effects model was used for FHS, as these methods were used in the initial GWAS for each study.

Statistics

All statistics for Figures 2–4 were performed using GraphPad Prism 4. Tests used are indicated in Figure legends. Significance threshold was set at $p = 0.05$.

Supporting Information

Figure S1 Association of single nucleotide polymorphisms (SNPs) with ΔPOST. In Panels A and B, each SNP is represented by a point. The higher the point, the lower the negative -log10 p-value seen on the y-axis and the more significant the association with ΔPOST. Points above the dashed line indicate SNPs with a p-value of less than 5×10^{-8}. (A) GWAS of the VISP cohort for ΔPOST imputed with 1000 Genomes. (B) GWAS of the FHS cohort for ΔPOST using imputation with 1000 Genomes.
(PDF)

Figure S2 QQ plots of meta-analyzed data of ΔPOST. Minus logarithm to base 10 of the p-values are plotted against the minus logarithm to base 10 of the quantiles of uniform (0,1) distribution to compare the p-value distribution with expected uniform distribution with all SNPs with 1 Mb of the top SNP of each associated loci removed. A diagonal line was drawn to show any departure of the p-value distribution from expected uniform distribution. The plotted genomic control parameter (lambda) is the ratio of median chi-squared test statistics to the median of an expected 1 degree-of-freedom chi-squared distribution. (A) All data from meta-analysis. (B) All data excluding meta-analysis significant SNPs and all SNPs within 1MB to rule out LD.
(PDF)

Figure S3 Folate one-carbon metabolism pathway. Diagram shows all genome wide significant genes and their role in FOCM.
(PDF)

Figure S4 Alignment of GNMT promoters from HighDeltaPOST and LowDeltaPOST constructs. Sequences were aligned

using Bioedit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and the pairwise alignment function.
(PDF)

Figure S5 Genetic risk score in VISP using top genotyped SNPs. (A) Distribution of genetic risk scores in VISP. (B) Risk score vs. ΔPOST in VISP. Error bars represent standard error.
(PDF)

Table S1 Independent GWAS results for ΔPOST phenotype in VISP and FHS.
(PDF)

Table S2 Correlation coefficients of principal components and ΔPOST. As an exploratory analysis, we calculated the Pearson correlation coefficients of ΔPost with all the covariates (age, first 10 principal components from EIGENSTRAT analysis (FHS) or KING (VISP)) and corresponding p-values in FHS and VISP.
(PDF)

Table S3 Test of heterogeneity for VISP-FHS meta-analysis of ΔPOST. We use Cochrane's Q statistic to test heterogeneity between FHS and VISP results. Cochrane's Q statistic is the sum of squared deviations of each study's effect estimate from the overall meta-analyzed effect estimate, weighted by inverse variance of the corresponding estimates.
(XLSX)

Acknowledgments

Members of Framingham Heart Study

Aleksandra Pikula, MD, Jose Rafael Romero, MD, Carlos S. Kase, MD, Ralph D'Agostino, Sr., PhD, Margaret Kelly-Hayes, EdD, RN, Daniel Levy, MD, Vasan Ramachandran, MD, Sander Robins, MD.

Members of the Genomics and Randomized Trials Network

Alex Reiner, MD, MSc, Charles Kooperberg, PhD, Richard Weinshilboum, MD, Peter Fasching, MD, Bruce Weir, PhD, Teri Manolio, MD, PhD, Patrick Heagerty, PhD.

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Author Contributions

Conceived and designed the experiments: SRW QY FC XL KLK PJ WMC GW FCH AB LW EB KFD PAW MZ JS SN SMG SS MMS BBW. Performed the experiments: SRW FC QY XL KFD SN WMC. Analyzed the data: SRW FC QY XL FCH WMC KLK SS BBW MMS. Wrote the paper: SRW QY SS MMS BBW. Edited the manuscript: SRW QY FC XL KLK PJ WMC GW FCH AB LW EB KFD PAW MZ JS SN SMG SS MMS BBW.

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Association of Plasma Homocysteine Levels with Subclinical Brain Injury: Cerebral Volumes, White Matter Hyperintensity and Silent Brain Infarcts on Volumetric MRI in the Framingham Offspring Study

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Abstract

Objective—To evaluate the relation between plasma homocysteine (tHcy) and brain MRI in a community-based sample.

Background—Elevated tHcy levels have been associated with an increased risk of dementia and stroke, but it is uncertain if the mediating mechanisms are predominantly cellular, vascular or both.

Design—Our sample comprised 1965 Framingham Offspring participants (1050 women; age 62±9 yrs) who were free of clinical stroke, dementia, or other neurological disease affecting brain MRI and who had at least one measurement of plasma tHcy (1991-2001) and a brain MRI (1999-2002). We used multivariable regressions to relate initial (1991-95) and concurrent (1998-2001) plasma tHcy concentrations to total cerebral brain volume (TCBV) and lobar volumes as measures of neuronal loss and atrophy; and to the presence or absence of silent brain infarcts (SBI) and extensive white matter hyperintensity (log-WMH ≥1 SD above the age-adjusted mean) as separate measures of vascular injury.

Results—Mean TCBV was 78%. 218 participants had SBI; 250 had extensive WMH. Participants with a plasma tHcy level in the highest age-, sex-specific quartile had a smaller TCBV (-0.37% and -0.48%; p=0.01 and <0.001 respectively), compared to participants with lower levels. Initial tHcy levels were associated with an increased prevalence of SBI (RR: 1.5; 95% CI: 1.1-2.1; p=0.02) and concurrent tHcy levels with smaller frontal and temporal lobar volumes (-0.14% and -0.10%; p=0.001 and 0.04 respectively). Prevalence of extensive WMH did not differ according to initial or concurrent plasma tHcy levels (RR: both 1.0, 95% CIs: 0.7-1.4 and 0.8-1.4, respectively).

Conclusion—Higher plasma tHcy levels are associated with smaller brain volumes and presence of silent infarcts on MRI, even in healthy, middle-aged adults. Thus, both cellular and vascular

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mechanisms may underlie the association of plasma tHcy with brain aging, as reflected by the effects on subclinical as well as overt disease.

Keywords

magnetic resonance imaging; homocysteine; brain volume; silent brain infarcts; white matter hyperintensity; epidemiology

Elevated plasma total homocysteine (tHcy) levels have been associated with an increased risk of clinical stroke,[1] dementia and Alzheimer's disease (AD).[2] The mechanisms underlying the association with clinical dementia are uncertain and may involve both vascular and neuronal pathways. MRI brain imaging provides subclinical markers that may reflect vascular or nonvascular brain injury. Thus, the presence or absence of silent brain infarcts (SBI) and extensive white matter hyperintensity (WMH) are considered indicators of subclinical macro- and microvascular injury respectively, whereas the total cerebral brain volume (TCBV), hippocampal volume (HV) and lobar volumes are accepted as measures of neuronal loss and other generalized brain changes, although such loss could be secondary to vascular changes. These MRI measures have been associated with the risk of clinical stroke (SBI and WMH)[3] and with impaired cognitive function and the risk of AD (TCBV, HV).[4] Prior studies have suggested that an elevated plasma tHcy level may be associated with MRI changes in each of these measures[5-15] but the techniques used to assess WMH and brain volume were, with two exceptions,[7,12] qualitative rather than quantitative. Further several of these studies were hospital-based series of patients with stroke or psychiatric illness.[10,13] In the present investigation, we related both prior and concurrent plasma tHcy levels to these various MRI measures in a large community-based, stroke- and dementia-free cohort of middle-aged adults (younger than those described in previous studies).

METHODS

Study participants

The Framingham Offspring cohort comprises 5124 participants who were enrolled in 1971 and have been evaluated 7 times; the 8th examination is currently underway.[16] Plasma tHcy was estimated at the 5th (1991-95), 6th (1995-99) and 7th (1998-2001) examinations. At the 7th examination, all participants (n=3,539) were invited to undergo brain MRI. As of 2002, n=2,014 participants had completed the MRI, while 1525 had a contraindication to MRI or had declined or deferred the test. Participants with an MRI were excluded if they were known to have a neurological illness that could affect MRI measurements such as a clinical stroke (n=29), dementia (n=2), or other relevant neurological condition (multiple sclerosis, brain tumor or head injury; n= 18). Our study sample consists of the remaining n=1,965 participants.

Plasma tHcy levels were measured, using high performance liquid chromatography with fluorescence detection.[17] Levels were measured at the 5th (initial or prior tHcy level, n=1663) and/or the 7th Offspring examinations (concurrent tHcy level, n=1923) in all 1965 eligible participants (1050 women).[18] We did not relate tHcy levels measured at the 6th examination to brain MRI measures because folate fortification has been mandated since half-way through this examination. Hence, persons who underwent the 6th Offspring examination before and after the initiation of folate fortification differed in their mean tHcy levels.[19]

Participant age at the 5th examination, the time of initial plasma tHcy measurement was mean (± SD) 54 ± 10 years (age range: 26-81 years). The interval between this examination and MRI was 7.5 years (SD 1.0, range 4.5-10.8 years), while the interval between the concurrent plasma tHcy measurement and MRI was 0.6 years (SD 0.5, range -2.3 – 3.0). The study protocol was

approved by the Institutional Review Board of Boston University and informed consent was obtained from all participants.

Brain Imaging

MRI acquisition and measurement techniques and inter-rater reliability have been described previously.[4,20-22] The images were analyzed by operators blinded to the participant's identity, age, sex, plasma tHcy levels and exposure to stroke risk factors. Brain volume was determined by manual outlining of the intracranial vault to determine the total cranial volume (TCV) and subsequent mathematical modeling to determine total brain parenchymal volume (TCB).[20] We computed the Total Cerebral Brain Volume (TCBV) as the ratio of TCB to TCV; thus this is a measure of brain parenchymal volume correcting for differences in head size.

Lobar volumes were computed by rotating the images into anatomical standard space followed by operator-defined outlining of the frontal, temporal, parietal and occipital lobes using standard anatomical landmarks. The average of the left and right lobar volume was expressed as a ratio to TCV. Hippocampal volume was estimated using operator defined, manually traced boundaries to define the region of interest. Intra and inter-rater reliability using this method was very good with coefficient of variation of 0.96. Hippocampal data were available in a subset of the population (n=661).

The volume of abnormal white matter hyperintensity (WMH) was determined according to previously published methods[22] and participants were categorized as having extensive WMH if the log-WMH volume was more than 1 SD above the age-adjusted mean in this cohort. The presence or absence of silent brain infarcts (SBI) was determined manually by the operator, based on the size (≥3 mms), location and imaging characteristics of the lesion.[23] We chose TCBV, SBI and WMH as our primary MRI measures and the lobar volumes as secondary measures.

Definitions of covariates

Education was dichotomized at high school graduation and alcohol use as 0 or >0. Persons were categorized according to the presence or absence of ≥1 apolipoprotein E ε4 allele. Serum creatinine was estimated using the modified Jaffe method and fasting plasma cholesterol using standard enzymatic methods. Plasma folate was estimated by a microbial (*Lactobacillus casei*) assay; cyanocobalamin (vitamin B_{12}) levels were estimated using a radioassay kit (Magic, Ciba—Corning, Medfield, MA); and pyridoxal-5′-phosphate (vitamin B6) was measured by the tyrosine decarboxylase apoenzyme method. We used log-normalized values of folate and pyridoxal-5′-phosphate in our analyses.

The Framingham Stroke Risk Profile (FSRP) has been previously described and validated for predicting stroke risk.[24,25] The components include systolic blood pressure (SBP) recorded as the average of two physician-recorded measurements, use of antihypertensive therapy, diabetes mellitus (defined by a fasting blood glucose >126 mg/dl or 7 mmol/L, a previous diagnosis of diabetes mellitus, or the use of a hypoglycemic agent or insulin), current smoking status, presence or absence of prior cardiovascualr disease (a diagnosis of coronary heart disease, congestive heart failure or peripheral vascular disease), atrial fibrillation and ECG-LVH (based on a standard 12-lead EKG obtained at, or prior to, the initial examination).

Statistical analysis

We used multivariable linear (for continuous outcomes) and logistic (for binary outcomes) regression models to examine the association between plasma tHcy levels (the predictor variable) and various primary and secondary brain MRI measures (outcome variables). Plasma tHcy was categorized using sex- and age-specific quartiles, defined within 10-year age groups

at each examination. Since we had previously shown that a plasma tHcy in the highest quartile (Q4) was associated with an increased risk of stroke, dementia and AD, we decided *a priori* that our primary analysis would utilize threshold models to compare the various MRI parameters in participants with plasma tHcy in the top quartile (Q4) with the rest of the sample (Q1-3). However, we additionally modeled plasma tHcy as a continuous variable (after log-transformation to normalize the distribution) and also examined the trend across quartiles. All analyses were adjusted for age (at MRI examination), sex, the time elapsed in each subject between the baseline examination and the date of brain MRI; the TCBV and lobar volume analyses were additionally adjusted for age squared. Since our sample is overwhelmingly Caucasian, the analyses were not adjusted for race. This constituted our basic Model A. We found no effect modification by sex and therefore all analyses were sex-pooled (but sex-adjusted). We conducted age-stratified analyses categorizing participants as age <55 years or ≥55 years at the time of MRI, based on our prior observations that risk factor relations to brain MRI measures may be stronger in the older age group.[4]

Vascular risk factors have been independently associated in the Framingham study with the examined MRI variables, and may lie along the causal pathway, hence in secondary analyses, we re-examined the relations between plasma tHcy and MRI parameters after accounting for the FSRP score at the time of plasma tHcy estimation. We also adjusted for covariates that influence plasma tHcy levels (serum folate, vitamins B_6 and B_{12}, body mass index [BMI], and serum creatinine) or have been postulated to influence brain MRI measures (education, APOE ε4 genotype, serum cholesterol and alcohol consumption).

We chose plasma tHcy levels at the 5th Offspring examination as our primary predictor variable, since we believed that a prolonged exposure to vascular risk factors was more likely to be reflected in MRI changes. Further we have shown that following mandated folic acid fortification of all enriched grain products which began in 1997, mean plasma tHcy levels have declined in the Framingham cohort; hence plasma tHcy levels at the 7th Offspring examination might not accurately reflect long-term plasma tHcy levels in individuals.[19] We then examined the relations between plasma tHcy levels at the 7th examination and MRI parameters in the same manner to see if the patterns observed with the original analysis were also seen when relating concurrent plasma tHcy levels to MRI parameters. The adjustment for vitamin and serum creatinine levels was not made in these analyses as they were not available at the 7th examination. All analyses were performed using Statistical Analyses System (SAS® Institute, Cary, North Carolina)©

RESULTS

Mean plasma tHcy levels for the entire group were 9.8 μmol/L (range 3.9 to 97) at the 5th Offspring examination and 8.3 μmol/L (range 3.3 to 93) at the 7th examination. Mean plasma tHcy levels and the range of values within each age-, sex-specific quartile are shown in Table 1. Mean plasma tHcy was 14.3±5.9 μmol/L and 11.8±5.3 for participants in the top age-, sex-adjusted quartile (Q4) and was 8.4±1.8 μmol/L and 7.2±1.5 for participants with plasma tHcy in the lower 3 quartiles at the 5th and 7th Offspring examinations respectively.

The distributions of demographic and vascular risk factors across the quartiles of plasma tHcy are summarized in Table 2. Participants in the highest quartile of plasma tHcy were more likely to have a higher mean SBP, to be on antihypertensive medication, to be diabetic, currently smoking and to have a history of prior CVD. The mean FSRP score and mean BMI were higher and mean plasma levels of folate, vitamins B12 and B6 were lower in this group.

Table 3 presents the results of analyses relating various measures of plasma tHcy, as a continuous variable and examining the trend across quartiles, to the primary MRI variables

(TCBV, WMH and SBI). The results of our primary analyses relating the plasma tHcy concentrations (Q4 versus Q1-3) with our primary MRI variables using sex-pooled models adjusted for age, sex and time-interval between plasma tHcy measurement and brain MRI are shown. Table 4 presents results of subgroup analyses relating plasma tHcy to TCBV and SBI among persons age ≥55 years and the results of secondary analyses adjusting for vascular and other covariates. In Table 5 we examine the effect of initial, concurrent and sustained hyperhomocysteinemia on these brain MRI measures defining hyperhomocysteinemia as a level in the highest age- and sex-specific quartile.

Mean TCBV in the 1965 participants was 78%. Elevated plasma tHcy at either the 5th or the 7th Offspring examination was associated with a smaller TCBV and quartile specific analyses showed a threshold effect; participants with a plasma tHcy in the highest quartile (Q4) at each examination had a lower TCBV (Table 3). We observed a stronger association in persons aged ≥55 years (Table 4). This association between elevated plasma tHcy and TCBV persisted after adjusting for multiple covariates, FSRP score *and the presence or absence of SBI*. The decrease in TCBV in participants with an initial plasma tHcy in the highest quartile was equivalent to that associated with an increase of 2 years in age for persons in the overall study sample and was equivalent to 3 years of .aging among persons aged >55 years at the time of MRI. The impact of concurrent plasma tHcy was greater than that of prior tHcy level ($p<0.05$) and the effect was greatest in subjects who had a plasma tHcy in the highest quartile at both the 5th and the 7th Offspring examinations (-0.43±0.20, p= 0.029 compared to all others, also data in Table 5).

Two hundred and eighteen participants had a SBI.. An elevated *initial* plasma tHcy was associated with an increased risk of SBI and in models evaluating multiple tHcy thresholds, participants with a plasma tHcy above the median had a greater prevalence of SBI (Table 3). The association was strongest in persons aged ≥55 years and in this subgroup *concurren*t plasma tHcy was also associated with an increased prevalence of SBI (Table 4). Initial hyperhomocysteinemia had a more powerful effect than concurrent hyperhomocysteinemia (Table 5). ; this is not surprising since we were looking at *prevalent* rather than incident SCI.

Extensive WMH was seen in 250 participants. However, the prevalence of extensive WMH did not differ across quartiles of initial or concurrent plasma tHcy (odds-ratio, OR for Q4 vs. Q1, 2, 3: 1.01, 95% CI 0.72-1.42, p= 0.96) (Table 3). We also failed to find any effect in the subgroup aged ≥55 years (data not presented) and in persons with sustained hyperhomocysteinemia (OR: 1.2, 95% CI:0.75-1.85).

Elevated plasma tHcy (Q4) at the 7th (but not the 5th) examination was associated with a lower mean frontal and temporal lobe volume but we failed to find an association between elevated plasma tHcy at either examination and parietal or occipital lobar volumes (Table 6).

DISCUSSION

We found a strong, independent cross-sectional association between higher plasma tHcy levels and lower MRI total brain volume using volumetric brain MRI in our community-based sample of middle-aged adults. This intriguing observation reaffirms data from some previous, smaller studies that used semi-quantitative MRI techniques[8,11,14] and extends their observations to younger participants. We also found an increased risk of subclinical (or covert) infarcts in these participants, again in accordance with prior studies showing an association between plasma tHcy levels and clinical stroke[1,26-29] as well as SBI. [6] We observed a greater impact in older adults, consistent with our prior observations that the relation between elevation in plasma tHcy and poorer scores on cognitive testing was stronger in older adults.[30]

We observed that concurrent (but not initial) elevated plasma tHcy concentrations were related to a smaller frontal and temporal brain volume. To our knowledge this has not been previously reported. While our results require replication, they are interesting since these areas are maximally affected by Alzheimer pathology. Prior reports have described a cross-sectional association of plasma tHcy levels with smaller hippocampal volumes,[14,31,32] and we observed a similar trend although the result failed to reach statistical significance, perhaps because of our lower mean age (61±9 years in our sample versus 73±8 years in a prior report) [14] and the healthy sample (persons with clinical stroke were not excluded in the prior report). [14] While we had hippocampal data available for only a subset of our sample, our sample size was larger than in any previous report. The association of lobar brain volumes with concurrent rather than initial plasma tHcy levels may reflect the identification of a subgroup of participants resistant to correction of plasma tHcy levels with increased dietary folate.

We did not find an association between plasma tHcy levels and volume of WMH and this is consistent with the results of some prior studies that described a relation between plasma tHcy levels, or the MTHFR TT genotype, and the risk of SBI or the combined risk of SBI and WMH, but not with WMH alone.[5,33] The different relations of plasma tHcy levels to SBI versus WMH may reflect differences in the pathophysiology of these two conditions. While SBI may be partly due to similar vascular mechanisms as clinical infarcts, the pathological correlates of WMH include ependymal and matrix changes, evidence of inflammation, fluid accumulation and demyelination in addition to ischemic changes.[34]

It is also possible that we failed to observe an association between plasma tHcy and extensive WMH in our relatively young and healthy population, but that such a relationship may exist in older subjects, in those with a greater cardiovascular risk factor burden, or in subjects with clinical stroke or dementia.[35] Prior epidemiological studies that observed a relationship between plasma tHcy and WMHI[7,12,15] have studied older populations with a greater prevalence of cardiovascular risk factors than was seen in our population;[12,15] further two of the studies did not exclude participants with clinical stroke.[7,15] However, we failed to find an association between plasma tHcy levels and extensive WMH, even in a subset of older participants (age >70 yrs at time of MRI) within our population. Other reasons for the differences between these and our results may include residual confounding by age in previous studies (none of them defined age-specific quartiles of plasma tHcy), differences in the techniques used to measure WMH[12,15] and ethnic differences between the study populations. [12] Our results support data from tissue and animal studies suggesting that both cellular and vascular pathways, or their combination, mediate the observed association of elevated plasma tHcy levels with brain aging.

The strengths of our study are the inclusion of younger participants than previously studied, the use of volumetric brain MRI techniques, and the availability of both concurrent and remote plasma tHcy levels. Limitations include the predominantly Caucasian population and the availability of only a single MRI so that we are unable to relate initial plasma tHcy levels to changes in brain volume or to incident (rather than prevalent) silent infarcts. The subset of participants who underwent brain MRI were healthier than the entire group of surviving Framingham Offspring.[4] This bias may be inevitable in epidemiological studies and in fact our enrollment of participants over 25 years prior to the MRI may have minimized the healthy volunteer bias as compared to other studies that undertook MRI at enrollment.

In prior studies we have also demonstrated an effect of elevated plasma tHcy levels on cognitive function in dementia- and stroke-free persons.[30] Overall our findings suggest that plasma tHcy levels may play a sustained role in the changes of brain aging and dementia, affecting not only the incidence of clinically overt stroke and dementia as we have previously demonstrated but also the prevalence of subclinical brain MRI changes in an apparently healthy population.

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Table 1

Age and Plasma Homocysteine Levels in Framingham Offspring Undergoing MRI.

Age at Exam (years)	Plasma tHcy at 5th Offspring Examination (mean and range in µmol/L)				Plasma tHcy at 7th Offspring Examination (mean and range in µmol/L)			
N at exams 5 and 7	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
<40 (n= 101 and 0)	5.9 4.0-7.6	7.6 5.8-8.8	9.5 8.1-11.0	13.6 10.9-26.4				
40-49 (n= 481 and 219)	6.3 4.0-8.3	8.2 6.6-10.0	9.8 8.3-11.4	13.4 9.1-45.0	5.3 3.6-6.7*	6.6 5.7-7.7*	7.7 6.6-9.0*	11.8 7.7-92.6*
50-59 (n= 543 and 708)	6.4 3.9-8.5	8.4 7.0-10.1	10.1 8.3-11.9	14.8 10.6-97.1	5.6 3.3-7.3	7.0 5.8-8.3	8.1 6.8-9.8	11.0 8.1-65.2
60-69 (n= 464 and 590)	6.8 4.3-8.3	8.7 7.4-10.2	10.6 8.9-12.4	14.8 11.1-35.0	5.8 3.3-7.2	7.4 6.2-8.8	8.9 7.6-10.7	11.9 9.2-28.9
70+ (n= 74 and 406)	7.4 5.4-8.9	9.6 8.6-11.2	11.4 10.2-12.5	13.7 11.9-16.8	6.2 4.0-7.7	7.8 6.7-9.2	9.3 8.0-10.9	12.9 10.-37.2
All ages (n= 1663 and 1923)	6.5 3.9-8.9	8.4 5.8-11.2	10.2 8.1-12.5	14.3 9.9-97.1	5.8 3.3-7.7	7.2 5.7-9.2	8.6 6.6-92.6	11.8 7.7-92.6

* age group <50 years at the time of MRI

† tHcy values in adjacent quartiles overlap since the range of values with in each quartile differed in men and women.

≠ Number of subjects at this age range at time of MRI who had plasma tHcy measurements available at 5th and 7th Offspring examinations respectively

Table 2

Description of Stroke Risk Factors (Prevalence and Levels) and Other Covariates in Subjects Grouped By Quartile Of Baseline Plasma Homocysteine Level.

	5th Offspring Examination			7th Offspring Examination		
	Q1-Q3	Q4	P value	Q1-Q3	Q4	P value
Framingham Stroke Risk Profile Variables	**N-1247**	**N=416**		**N=1442**	**N=481**	
Age (in years)*	54 ± 10	55 ± 10	0.598	61 ± 9	61 ± 9	0.705
Systolic Blood Pressure (mmHg)*	124 ± 18	125 ± 19	0.375	125 ± 18	127 ± 20	0.046
Treatment with antihypertensive medication (%)	14.3%	19.1%	0.020	28.0%	37.6%	<0.001
Diabetes (%)	5.1%	4.6%	0.647.	7.7%	11.7%	0.008
Current Smoker (%)	13.1%	25.8%	<0.001	10.8%	15.2%	0.010
History of cardiovascular disease (%)	5.5%	7.5%	0.136	8.0%	12.9%	0.001
Atrial fibrillation (%)	1.0%	1.2%	0.786	2.8%	3.7%	0.322
ECG-LVH (%)	1.7%	2.7%	0.209	1.8%	2.3%	0.435
FSRP Score*	4.0 ± 4.7	4.5 ± 5.5	0.069	6.3 ± 7.3	7.6 ± 8.9	0.005
Other Covariates						
Education: High school graduate (%)	96.5%	96.0%	0.654	96.9%	95.3%	0.106
Folate level (ng/ml)	9 ± 6	5 ± 4	<0.001			
Vitamin B12 level (pg/ml)*	470 ± 241	390 ± 234	<0.001			
Vitamin B12 < 150	4.5%	6.4%	0.151			
Pyridoxal 5′-phosphate level (nmol/ml)*	80 ± 65	61 ± 48	<0.001			
Serum creatinine (mg/dl)*	1.0 ± 0.2	1.1 ± 0.2	0.004			
Body Mass Index (kg/m^2)*	27 ± 5	28 ± 5	0.091	28 ± 5	29 ± 5	0.001
Plasma cholesterol (mg/dl)*	203 ± 36	207 ± 39	0.069	200 ± 36	202 ± 39	0.475
Apolipoprotein genotype: ε4 +ve (%)	23.6%	20.9%	0.256	22.6%	22.0%	0.796
Any Alcohol Use	72.3%	68.7%	0.158	67.0%	66.2%	0.744

*: Mean ± SD

Table 3

Results of Multivariable Regression Analyses of Plasma Homocysteine (tHcy) levels at the 5th and 7th Offspring Examination on Primary MRIV ariables: Total Cerebral Brain Volume (TCBV), Risk Of Extensive White Matter Hyperintensity On Brain MRI (WMH) And Risk Of Silent Cerebral Infarcts (SCI) — analyses using different measures of tHcy

Outcome variable*	Observed value in entire group	per log elevation of tHcy		Quartiles of homocysteine (age-specific)				p (trend across quartiles)	Q4 vs. Q1-3; change and p value
		Change	p	Q1	Q2	Q3	Q4		
Plasma homocysteine (tHcy) measure at 5th Offspring Examination (N=1663)									
TCBV	Mean ± SD: 77.8 ± 3.2	β= -0.40	0.05	77.9	77.9	77.9	77.5	0.08	β= -0.38; p= 0.01
				(adjusted means ± SE)					
Extensive WMHI (%)	12.2%	OR: 1.06	0.81	1.00	1.23	1.05	1.10	0.85	OR: 1.01; p= 0.96
SCI (%)	11.2%	OR: 1.72	0.03	1.00	0.98	1.25	1.60	0.02	OR: 1.49; p= 0.02
Plasma homocysteine (tHcy) measure at 7th Offspring Examination (N =1923)									
TCBV	Mean ± SD: 77.9 ± 3.2	β= -0.68	0.001	78.0	78.2	78.0	77.6	0.004	β= -0.489; p<0.001
				(adjusted means ± SE)					
Extensive WMHI (%)	12.4%	OR: 0.98	0.92	1.00	0.73	0.80	0.87	0.58	OR: 1.04; p=0.82
SCI (%)	11.2%	OR: 1.42	0.16	1.00	1.34	1.29	1.51	0.08	OR; 1.25 ; p=0.17

*All analyses are adjusted for age, gender, and interval between the baseline examination and date of brain MRI; TCBV is addit ionally adjusted for age-squared.

Table 4

Results Of Age-Stratified Multivariable Regression Analyses of Plasma Homocysteine (tHcy) levels on Primary MRI Parameters Related to tHcy in overall analyses : Total Cerebral Brain Volume (TCBV) And Risk Of Silent Cerebral Infarcts (SCI) - Results expressed as Q4 versus Q1-3

	Initial (5th) Offspring Examination		Final (7th) Offspring Examination	
Dependent Variable (and covariates in model)	**Entire Group**	**Age ≥55 years at time of MRI**	**Entire Group**	**Age ≥55 years at time of MRI**
	N=1663	**N=1229**	**N=1923**	**N=1408**
	TCBV expressed as β±SE and p value			
TCBV (Model A: age, age squared, sex, interval between exam and MRI)	-.37 ±.15, p= 0.011	-.56 ±.18, p= 0.001	-.48 ±.14, p<0.001	-.67 ±.16, p<0.001
TCBV (Model A and FSRP score)	-.34 ± .15, p=0.021	-.53 ± .18, p=0.003	-.44 ± .14, p=0.001	-.63 ± .17, p<0.001
TCBV (Model A, FSRP score and presence of SCI)	-.34 ± .15, p=0.023	-.53 ± .18, p=0.003	-.44 ± .14, p=0.001	-.63 ± .17, p<0.001
TCBV (Model A and vitamin levels, body mass index and creatinine)	-.38 ± .17, p= 0.027	-.59 ± .21, p= 0.005		
TCBV (Model A and alcohol intake, education, plasma cholesterol, presence of APOE ε4 allele)	-.36 ± .15, p=0.017	-.54 ± .18, p=0.003	-.46 ± .14, p=0.001	-.63 ± .17, p<0.001
	SCI expressed as OR, 95% CI			
SCI (Model A: age, sex, interval between exam and MRI)	1.49, 1.07-2.08	1.56, 1.08-2.24	1.25, 0.91-1.72	1.44, 1.02-2.03
SCI (Model A and FSRP score)	1.48, 1.06-2.07	1.54, 1.06-2.23	1.22, 0.88-1.69	1.39, 0.98-1.98
SCI (Model A and vitamin levels, body mass index and creatinine)	1.39, 0.93-2.08	1.42, 0.90-2.22		
SCI (Model A and alcohol, education, plasma cholesterol, presence of APOE ε4 allele)	1.61, 1.14-2.25	1.68, 1.16-2.44	1.26, 0.91-1.74	1.43, 1.00-2.04

β=Regression coefficient- unstandardized beta weights adjusted for specified predictor variables; each row represents a separate multivariable model. S.E. refers to the standard error of beta.

Table 5

Results of Multivariable Regression Analyses of Plasma Homocysteine (tHcy) levels at Both Initial and Concurrent Examinations with Total Cerebral Brain Volume Ratio (TCBV), And Risk Of Silent Cerebral Infarcts (SCI) - adjusted for age, gender, and interval between exam 5 and MRI (TCBV analyses additionally adjusted for age squared)

Dependent Variable (and covariates in model)	Entire Group N =1634	Age ≥55 years at time of MRI N =1210
TCBV (β±SE [*†])		
Plasma tHcy Q1-3 at both initial and concurrent examinations (Low-Low)	0	0
Plasma tHcy Q1-3 at initial and Q4 at concurrent examination (Low-High)	-0.57±.19, p=0.003	-0.74±.23, p=0.001
Plasma tHcy Q4 at initial and Q1-3 at concurrent examinations (High-Low)	-0.34±.20, p=0.08	-0.48±.23, p=0.039
Plasma tHcy Q4 at both initial and concurrent examinations (High-high)	-0.56±.20, p=0.005	-0.85±.24, p=<0.001
SCI (OR and 95% CI)		
Plasma tHcy Q1-3 at both initialand concurrent examinations (Low-Low)	1.00	1.00
Plasma tHcy Q1-3 at initial and Q4 at concurrent examination (Low-High)	1.35, 0.86-2.13	1.41, 0.86-2.33
Plasma tHcy Q4 at initial and Q1-3 at concurrent examinations (High-Low)	1.73, 1.13-2.66	1.65, 1.02-2.66
Plasma tHcy Q4 at both initial and concurrent examinations (High-high)	1.36, 0.85-2.18	1.61, 0.97-2.67

[*] Regression coefficient- unstandardized beta weights adjusted for specified predictor variables; each row represents a separate multivariable model.

[†] S.E. refers to the standard error of beta. P values are given for all significant results.

Table 6

Results Of Multivariable Regression Analyses Of Plasma homocysteine (tHcy) levels on Secondary MRI Measures

	5th Offspring Examination			7th Offspring Examination		
Predictor variable*	**Mean ± SD**	**Q4 vs. Q1-3 Change**	**P value**	**Mean ± SD**	**Q4 vs. Q1-3 Change**	**P value**
Frontal Brain Volume	35.6 ± 3.3	-0.07	0.160	35.6 ± 3.3	-0.14	0.001
Occipital Brain Volume	10.4 ± 2.8	-0.02	0.731	10.4 ± 2.7	0.01	0.901
Parietal Brain Volume	21.5 ± 3.5	-0.03	0.631	21.6 ± 3.5	0.02	0.722
Temporal Brain Volume	10.4 ± 0.9	-0.01	0.924	10.4 ± 0.9	-0.10	0.037
Hippocampal Volume (n=661)	0.31 ± 0.04	-0.11	0.192	0.31 ± 0.04	-0.15	0.065

*All analyses are adjusted for age, age squared, gender, and tim e between Offspring examination and MRI; Frontal, occipital, parietal, temporal and hippocampal brain volumes are all expressed in standard deviation units.

REVIEW

Homocysteine and Risk of Ischemic Heart Disease and Stroke

A Meta-analysis

The Homocysteine Studies Collaboration

Context It has been suggested that total blood homocysteine concentrations are associated with the risk of ischemic heart disease (IHD) and stroke.

Objective To assess the relationship of homocysteine concentrations with vascular disease risk.

Data Sources MEDLINE was searched for articles published from January 1966 to January 1999. Relevant studies were identified by systematic searches of the literature for all reported observational studies of associations between IHD or stroke risk and homocysteine concentrations. Additional studies were identified by a hand search of references of original articles or review articles and by personal communication with relevant investigators.

Study Selection Studies were included if they had data available by January 1999 on total blood homocysteine concentrations, sex, and age at event. Studies were excluded if they measured only blood concentrations of free homocysteine or of homocysteine after a methionine-loading test or if relevant clinical data were unavailable or incomplete.

Data Extraction Data from 30 prospective or retrospective studies involving a total of 5073 IHD events and 1113 stroke events were included in a meta-analysis of individual participant data, with allowance made for differences between studies, for confounding by known cardiovascular risk factors, and for regression dilution bias. Combined odds ratios (ORs) for the association of IHD and stroke with blood homocysteine concentrations were obtained by using conditional logistic regression.

Data Synthesis Stronger associations were observed in retrospective studies of homocysteine measured in blood collected after the onset of disease than in prospective studies among individuals who had no history of cardiovascular disease when blood was collected. After adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, a 25% lower usual (corrected for regression dilution bias) homocysteine level (about 3 μmol/L [0.41 mg/L]) was associated with an 11% (OR, 0.89; 95% confidence interval [CI], 0.83-0.96) lower IHD risk and 19% (OR, 0.81; 95% CI, 0.69-0.95) lower stroke risk.

Conclusions This meta-analysis of observational studies suggests that elevated homocysteine is at most a modest independent predictor of IHD and stroke risk in healthy populations. Studies of the impact on disease risk of genetic variants that affect blood homocysteine concentrations will help determine whether homocysteine is causally related to vascular disease, as may large randomized trials of the effects on IHD and stroke of vitamin supplementation to lower blood homocysteine concentrations.

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THE HYPOTHESIS THAT ELevated blood concentrations of the sulfur-containing amino acid homocysteine may be a risk factor for cardiovascular disease was suggested by the observation that children with homozygous homocystinuria, a rare inborn error of metabolism causing markedly elevated blood total homocysteine concentrations, had a high incidence of premature occlusive vascular disease.[1] The initial epidemiological evidence in support of this hypothesis came from retrospective case-control studies.[2-4] More recently, however, inconsistent results have been reported from prospective observational studies, including cohort and nested case-control studies, with some showing highly significant associations but others showing none.[5]

The aim of this collaborative meta-analysis was to combine individual participant data from all relevant observational studies to produce reliable estimates of the associations of total plasma homocysteine with ischemic heart disease (IHD) and stroke, with adjustment for confounding caused by known cardiovascular risk factors and correction for regression dilution caused by random variation in homocysteine measurements.[6,7] The chief emphasis in this report is on combined

See also pp 2023 and 2042.

Author Members and Additional Collaborators of the Homocysteine Studies Collaboration are listed at the end of this article.

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analyses of the prospective studies (which involve the measurement of homocysteine in blood collected before the onset of disease) rather than on the retrospective studies, since the former should be less prone to artifacts produced by any effects of preexisting vascular disease on homocysteine levels (referred to as *reverse causality*).

METHODS

Study Populations

Relevant studies were identified by systematic searches of the scientific literature for all reported observational studies of associations between IHD or stroke risk and homocysteine concentrations (using the terms *coronary heart disease, myocardial infarction, cerebrovascular disease, stroke, cardiovascular disease, homocysteine* or *homocyst(e)ine*, and *hyperhomocystinemia*). We searched the MEDLINE database for articles published from January 1966 to January 1999 and identified additional studies by hand-searching references of original articles or review articles on this topic and by personal contact with relevant investigators in that period. Studies were included if they had data available by January 1999 on total blood homocysteine concentrations, sex, age at entry (for prospective studies), and age at event (G. S. Omenn, unpublished data, September 2002).[8-37] Studies were excluded if they measured only blood concentrations of free homocysteine or of total homocysteine only after a methionine-loading test[3,38-40] or if relevant data were unavailable[41-48] or incomplete.[49-51] To avoid confounding by disease, prospective studies of participants selected on the basis of existing cardiovascular disease, diabetes mellitus, renal impairment, or some other disease were also excluded,[52-56] as were participants with preexisting cardiovascular disease in prospective studies of apparently healthy populations.

Data Collection

Investigators who agreed to collaborate were asked to provide data for each participant on date of birth, sex, blood homocysteine concentration (with the date of blood collection), any nonfatal or fatal myocardial infarction or occlusive coronary artery disease, and any nonfatal or fatal stroke or transient cerebral ischemic attack (with the dates of all such events). If available, data were also collected on history of heart disease event, prior cerebrovascular disease event, diabetes mellitus, smoking (current vs not), alcohol consumption, blood concentrations of total and high-density lipoprotein cholesterol and of creatinine, systolic and diastolic blood pressure, weight, and height. Studies were classified as prospective if the blood sample used to measure homocysteine was collected before the IHD or stroke event (whether homocysteine was measured in all individuals or in a nested manner among cases and matched controls) and as retrospective if the blood sample was collected after the event in cases. Retrospective studies were further classified as population-based if the controls were selected randomly from the same source population as the cases or as other if the controls were spouse controls or patients with some other illness.

Statistical Analyses

Cases and controls were restricted to people who were at least 40 years of age at baseline (ie, when the blood sample for homocysteine measurement was taken) and had measured homocysteine concentrations between 3 and 40 μmol/L (0.41 and 5.41 mg/L). Analyses were conducted for all ages together and for 3 age-at-event bands (40-54, 55-64, and ≥65 years), with prospective and retrospective studies considered separately to assess the possible impact of reverse causality and of any bias caused by control selection or participation.[57] Results from prospective studies in which all participants had had homocysteine measured in a baseline sample were combined with those from prospective studies in which only the cases and a matched random sample of controls had had homocysteine measured in a baseline sample (ie, nested case-control studies). For prospective studies that provided data as nested case-control studies, the controls originally used for each case were retained in this study. For the other prospective studies, up to 8 controls per case were selected in each age-at-event band (matched for sex and age at baseline in 5-year bands), with age at last follow-up having to be greater than the lower limit of the age-at-event band. Controls could be selected only once within an age-at-event band but could be selected again for a later age-at-event band. For anyone experiencing multiple vascular events, only the first event was considered.

All analyses were based on logarithmically transformed homocysteine values because homocysteine values are positively skewed, with $\log_2$ used so that a unit increase in $\log_2$ of homocysteine is equivalent to a doubling in homocysteine. Conditional logistic regression analyses stratified for study were used 2 ways to describe the dose-response relationships. First, to investigate the shape of the association, the odds ratios (ORs) for groups defined by quintiles of baseline values of homocysteine within each study were calculated, with 95% confidence intervals (CIs) estimated from floated variances reflecting the amount of information underlying each group (including the reference group).[58] Second, assuming a log-linear association, regression coefficients were calculated for the percentage of difference in risk associated with a 25% lower homocysteine concentration (which is equivalent to the average change in plasma total homocysteine concentration achieved by folic acid supplementation).[59] Heterogeneity between the results of prospective and retrospective study designs and between studies within each type of design was assessed by a χ^2 statistic. Heterogeneity between the results of prospective studies was investigated with respect to mean age at baseline, percentage of men, percentage of current smokers, mean homocysteine concentrations in controls, and mean time between blood collection and vascular events. Analyses of the prospective studies were

adjusted for the effects of smoking (tobacco use vs not), total cholesterol level, and systolic blood pressure by using multivariable logistic regression, with the stepwise change in the χ^2 statistic after making these adjustments providing a quantitative indication of the potential confounding effects of these factors. All analyses were performed with SAS version 8.1 (SAS Institute Inc, Cary, NC).

Correction for Regression Dilution Bias

The analyses of the prospective studies relate vascular disease risk to the estimated usual concentrations of homocysteine at approximately the time of the event by using remeasurements of homocysteine in samples collected at an appropriate interval after baseline to correct for regression dilution.[6,7] Random fluctuations in a measured value of homocysteine will underestimate the strength of the real association between the usual (ie, long-term average) level of homocysteine during a particular exposure period and disease. This so-called regression dilution effect may be caused by measurement error or transient fluctuations in homocysteine levels caused by treatment, disease, age, or changes in diet. Information from repeated homocysteine

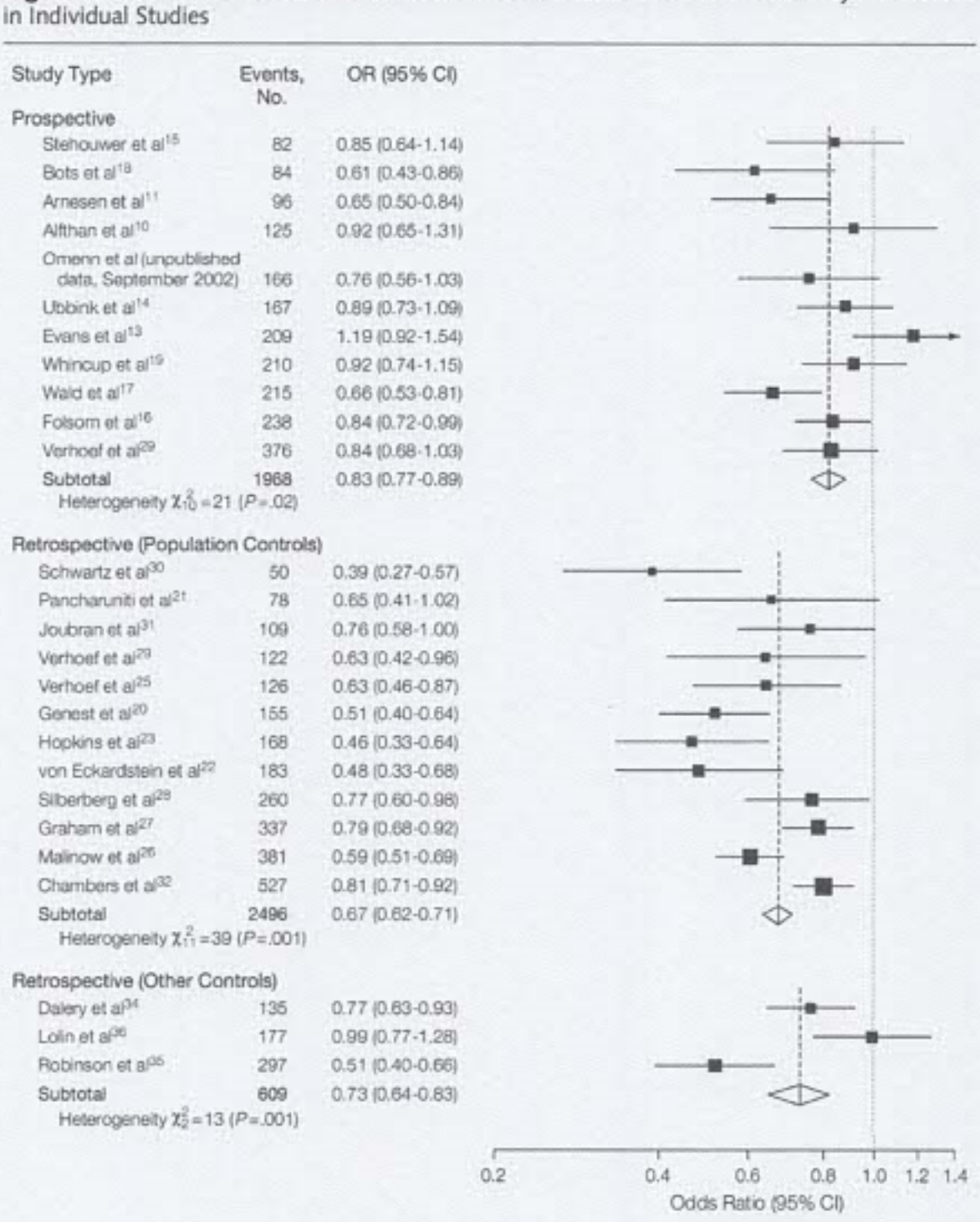
Table 1. Characteristics of Included Studies*

Source	Total Population†	Age at Screening, Mean (SD), y	Homocysteine Level in Controls, Mean (SD), µmol/L‡	Ischemic Heart Disease Events, No.	Stroke Events, No.	Study Type
Prospective (12 studies)						
Stampfer et al[8] and Verhoef et al[9]	985	60 (9)	10.7 (3.6)	376	121	I
Alfthan et al[10]	407	54 (7)	9.5 (3.0)	125	51	F
Arnesen et al[11]	527	53 (6)	11.4 (3.7)	96	. . .	I
Perry et al[12]	212	54 (5)	12.3 (3.8)	. . .	95	F
Evans et al[13]	646	48 (5)	12.9 (4.7)	209	5	I
Ubbink et al[14]	1778	57 (5)	12.1 (4.1)	167	12	P
Stehouwer et al[15]	643	71 (5)	14.5 (4.7)	82	67	P
Folsom et al[16]	758	56 (5)	9.6 (3.9)	238	. . .	F
Wald et al[17]	1268	54 (6)	11.8 (3.6)	215	. . .	I
Bots et al[18]	746	69 (8)	14.9 (3.8)	84	93	F
Whincup et al[19]	540	51 (6)	13.4 (4.3)	210	. . .	F
Omenn et al (unpublished data, September 2002)	515	62 (5)	11.5 (4.2)	166	19	I
Subtotal	**9025**	58 (9)	12.1 (4.2)	**1968**	**463**	
Retrospective: population controls (13 studies)						
Genest et al[20]	408	50 (6)	10.8 (4.3)	155	. . .	
Pancharunti et al[21]	157	46 (4)	12.3 (3.4)	78	. . .	
von Eckardstein et al[22]	320	51 (5)	8.1 (1.7)	183	. . .	
Hopkins et al[23]	317	53 (7)	10.3 (3.0)	168	. . .	
Lindgren et al[24]	211	74 (11)	14.3 (4.6)	. . .	164	
Verhoef et al[25]	241	58 (9)	9.5 (3.3)	126	. . .	
Malinow et al[26]	855	55 (7)	13.0 (4.6)	381	. . .	
Graham et al[27]	1169	50 (5)	10.9 (3.9)	337	144	
Silberberg et al[28]	455	55 (11)	13.1 (5.2)	260	. . .	
Verhoef et al[29]	219	53 (7)	12.0 (3.1)	122	. . .	
Schwartz et al[30]	265	42 (1)	10.9 (3.4)	50	36	
Joubran et al[31]	219	53 (9)	12.9 (5.3)	109	. . .	
Chambers et al[32]	1501	51 (7)	10.9 (3.6)	527	. . .	
Subtotal	**6337**	52 (9)	11.3 (4.1)	**2496**	**344**	
Retrospective: other controls (5 studies)						
Coull et al[33]	225	66 (8)	10.3 (2.7)	. . .	189	
Dalery et al[34]	350	48 (5)	9.7 (4.8)	135	. . .	
Robinson et al[35]	503	59 (11)	11.0 (3.5)	297	. . .	
Lolin et al[36]	200	61 (8)	13.3 (7.9)	177	. . .	
Evers et al[37]	146	57 (9)	11.3 (3.0)	. . .	177	
Subtotal	**1424**	57 (11)	10.5 (4.3)	**609**	**306**	
Total	**16 786**	56 (9)	11.8 (4.2)	**5073**	**1113**	

*Ellipses indicate data not computed; I, individually matched nested case-control study; F, frequency-matched nested case-control study; and P, prospective study.
†Total population includes participants who had homocysteine values between 3.0 and 40.0 µmol/L (0.41 and 5.41 mg/L), were 40 years or older when screened, and had no history of vascular disease.
‡To convert µmol/L to mg/L, divide by 7.397.

measurements in a representative sample of individuals can be used to correct for the effects of regression dilution. Most of the studies did not have such repeat measurements of homocysteine, but 3 studies involving a total of 2318 individuals had remeasured participants at 3 to 8 years after baseline,[60-62] and 1 other study had done so in 500 individuals at 3, 6, 9, and 12 years.[63] Baseline measurements of homocysteine were used to divide people into quintiles, and the ratio of the range between the mean values of the repeat measurements in the top vs the bottom quintile to the range of the baseline measurements was used to estimate regression dilution ratios for various intervals. The regression dilution ratio declined with increasing intervals between measurements,[6] with a value of 0.75 for the mean interval of 5 years from the initial homocysteine measurement to IHD events in prospective studies and of 0.77 for the mean interval of 4 years to stroke events. In the retrospective studies, homocysteine was measured at or shortly after the event, so a regression dilution ratio of 0.83 (which makes allowance for short-term variability only) was used for IHD and stroke analyses. The regression coefficient (and its SE) relating risk to usual concentrations of homocysteine was then estimated as the uncorrected regression coefficient (and its SE) that related risk to single measurements of baseline homocysteine concentrations (1 divided by the regression dilution ratio).

Figure 1. Odds Ratios of Ischemic Heart Disease for a 25% Lower Usual Homocysteine Level in Individual Studies



Data were adjusted for study, sex, and age at enrollment and were corrected for regression dilution. The size of the square is inversely proportional to the variance of the log odds ratio (OR). The horizontal lines represent the 95% confidence intervals (CIs). The combined ORs in the subtotals for each study design and their 95% CIs are indicated by the diamonds.

RESULTS

Study Populations

Individual participant data were obtained for 30 studies, which include 18 of 28 eligible retrospective studies and 12 of 13 eligible prospective studies (TABLE 1). Of 5073 IHD events, 1968 came from prospective studies, 2496 from retrospective studies with population controls, and 609 from retrospective studies with other controls. Of 1113 stroke events, 463 came from prospective studies, 344 from retrospective studies with population controls, and 306 from retrospective studies with other controls. The median (and interquartile range [IQR]) (SD) homocysteine level among controls in the different studies varied from 7.8 µmol/L (1.05 mg/L; IQR, 7.0-8.9) to 14.3 µmol/L (1.93 mg/L; IQR, 12.3-17.0), with an overall median of 11.0 µmol/L (1.49 mg/L; IQR, 9.0-13.6) (SD, 4.2 µmol/L [0.57 mg/L]). Eighty-five percent of the participants were men (88% in prospective studies, 83% in retrospective studies with population controls, and 73% in retrospective studies with other controls), and 36% were current smokers (41%, 31%, and 24%, respectively). In the prospective studies, the mean age at IHD was 62 years (SD, 8 years), and these events occurred at an average of 5 years (range, 2-9 years) after baseline. The mean age at stroke was 68 years (SD, 11 years) in prospective studies, and these events occurred at an average of 4 years (range, 2-11 years) after baseline. The mean age of IHD cases in retrospective studies with population controls was 53 years (SD,

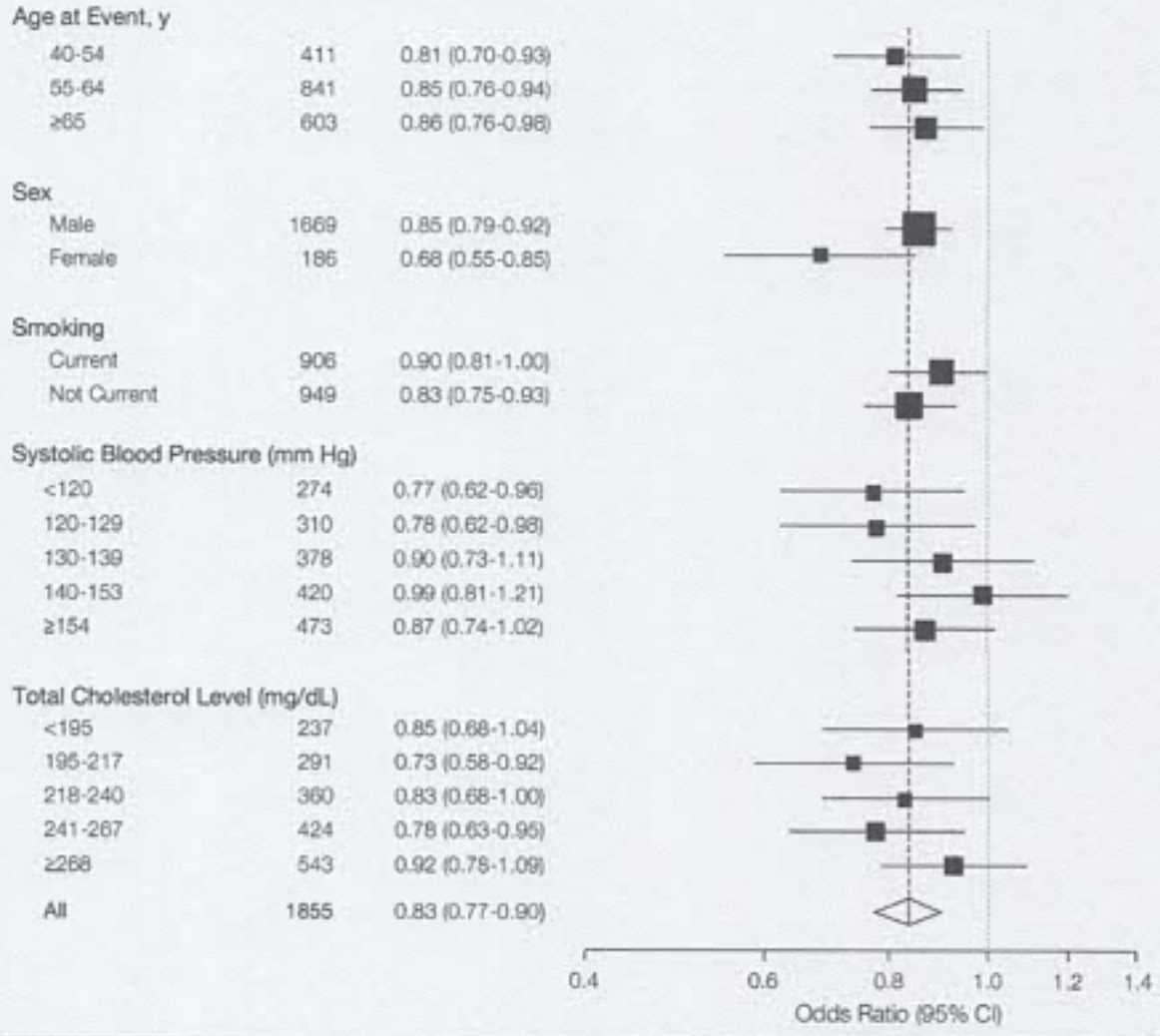
7 years); in retrospective studies with other controls, 59 years (SD, 10 years); and of stroke cases, 61 years (SD, 16 years) and 63 years (SD, 9 years), respectively.

Risks of IHD in Relation to Differences in Homocysteine Levels

Before correction for regression dilution, the ORs for IHD associated with a 25% lower baseline homocysteine level after adjustment for study, age, and sex were 0.87 (95% CI, 0.82-0.92) in prospective studies, 0.71 (95% CI, 0.68-0.75) in retrospective studies with population controls, and 0.77 (95% CI, 0.69-0.86) in retrospective studies with other controls. There was significant heterogeneity between the results from these study designs (χ^2_2=27; P<.001), which was attenuated only slightly by correction for regression dilution (χ^2_2=21; P<.001). After correction for regression dilution (separately within each study), the adjusted ORs for IHD associated with a 25% lower usual homocysteine level were 0.83 (95% CI, 0.77-0.89) in prospective studies, 0.67 (95% CI, 0.62-0.71) in retrospective studies with population controls, and 0.73 (95% CI, 0.64-0.83) in retrospective studies with other controls (FIGURE 1). Among the prospective studies, there was only marginally significant heterogeneity between the adjusted ORs for IHD (χ^2_{10}=21; P=.02) after correction for regression dilution, with a slight trend toward attenuation of the ORs with increasing mean time to event in the studies (test for trend, χ^2_1=4.1; P=.04). In contrast with previous suggestions,[27] however, there was no evidence that the association of homocysteine level with IHD was influenced by age, sex, smoking, levels of blood pressure or blood cholesterol (FIGURE 2), or mean homocysteine concentrations in controls (or size of study, which might have reflected a tendency not to report small studies with less extreme findings).

Homocysteine concentrations were correlated with current smoking, total cholesterol levels, and systolic blood pressure, and the study-, age-, and sex-adjusted ORs for IHD in prospective studies were attenuated after further adjustment for these risk factors in individuals with all relevant data (to 0.89 [95% CI, 0.83-0.96]) (TABLE 2). The substantial change in the χ^2 statistic with these adjustments (from 24 to 9) suggests that a large part of the asso-

Figure 2. Odds Ratios of Ischemic Heart Disease for a 25% Lower Usual Homocysteine Level Among People in Prospective Studies



Data were adjusted for study, sex, and age at enrollment. Studies are grouped by age at event, sex, smoking, and quintiles of systolic blood pressure and total cholesterol level. A global test for heterogeneity between all of these subgroups was not significant (χ^2_{16}=14; P=.60). Symbols and conventions are as for Figure 1. To convert cholesterol to mmol/L, multiply by 0.02586. OR indicates odds ratio; CI, confidence interval.

Table 2. Odds Ratios for Ischemic Heart Disease (IHD) and for Stroke Associated With 25% Lower Usual Homocysteine Levels in Prospective Studies

	Events, No.*	Adjusted Odds Ratio (95% Confidence Interval)			
		Age and Sex	Age, Sex, and Smoking	Age, Sex, Smoking, and Systolic Blood Pressure	Age, Sex, Smoking, Systolic Blood Pressure, and Total Cholesterol Level
IHD	1855	0.83 (0.77-0.90) χ^2_1 = 24	0.85 (0.78-0.91) χ^2_1 = 20	0.89 (0.82-0.96) χ^2_1 = 10	0.89 (0.83-0.96) χ^2_1 = 9
Stroke	435	0.77 (0.66-0.90) χ^2_1 = 11	0.78 (0.67-0.91) χ^2_1 = 10	0.81 (0.69-0.96) χ^2_1 = 6	0.81 (0.69-0.95) χ^2_1 = 6

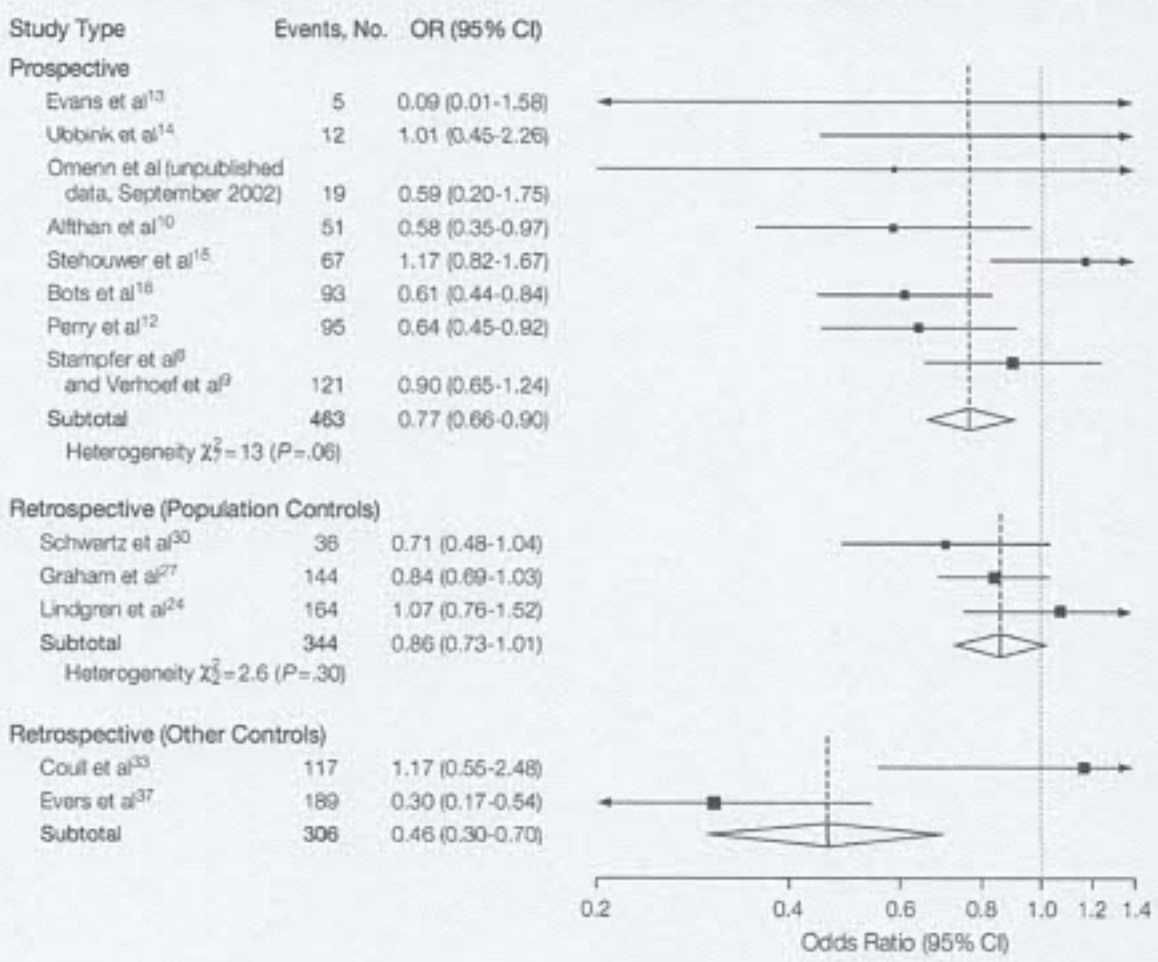
*Among people with all available data used for adjustment for known cardiovascular risk factors.

Table 3. Odds Ratio (95% Confidence Interval) of Ischemic Heart Disease for Quintiles of Baseline Homocysteine Levels in Prospective Studies*

	Quintiles				
	1	2	3	4	5
Adjusted for age, sex, and study	1.00 (0.87-1.15)	1.05 (0.92-1.19)	1.05 (0.92-1.20)	1.20 (1.06-1.36)	1.44 (1.28-1.62)
Adjusted for age, sex, study, smoking, systolic blood pressure, and cholesterol level	1.00 (0.86-1.16)	1.05 (0.92-1.21)	0.91 (0.79-1.04)	1.10 (0.97-1.26)	1.16 (1.02-1.32)

*Based on 1855 cases of ischemic heart disease for whom data on each of homocysteine level, systolic blood pressure, total cholesterol level, and current smoking were available.

Figure 3. Odds Ratios of Stroke for a 25% Lower Homocysteine Level in Individual Studies



Data were adjusted for study, sex, and age at enrollment and were corrected for regression dilution. Symbols and conventions are as for Figure 1. OR indicates odds ratio; CI, confidence interval.

ciation was due to confounding. Moreover, because these confounding factors will necessarily have been measured with some error, substantial residual confounding will remain.

TABLE 3 compares ORs of IHD in quintiles of homocysteine levels determined separately within each study before combined analyses. The study-, age-, and sex-adjusted ORs of IHD appeared to increase with increasing homocysteine concentrations in a graded relationship, but this pattern was attenuated after further adjustment for current smoking, systolic blood pressure, and total cholesterol levels.

Risk of Stroke in Relation to Differences in Homocysteine Concentrations

After adjustment for study, age, and sex and correction for regression dilution, the ORs for stroke were 0.77 (95% CI, 0.66-0.90) in prospective studies, 0.86 (95% CI, 0.73-1.01) in retrospective studies with population controls, and 0.46 (95% CI, 0.30-0.70) in retrospective studies with other controls (FIGURE 3). The heterogeneity between the results from these designs for stroke ($\chi^2_2 = 7.4$; $P = .02$) was less extreme than that observed for IHD. Among the prospective studies, there was no significant heterogeneity between the adjusted ORs for stroke ($\chi^2_7 = 13$; $P = .06$), and there was no good evidence that the association of homocysteine level with stroke was influenced by age or sex. As for IHD, there was a substantial reduction in the χ^2 statistic (from 11 to 6) for the association of homocysteine level with stroke risk after further adjustment for current smoking, systolic blood pressure, and cholesterol level (OR, 0.81; 95% CI, 0.69-0.95) (Table 2), suggesting that confounding may have inflated the estimated strength of this association.

COMMENT

This meta-analysis indicates that homocysteine level is less strongly related to IHD and stroke risk in healthy populations than has been suggested.[4] The chief strength of this study is that inclusion of data from individual participants has allowed adjustment for possible confounding caused by known cardiovascular risk factors and appropriate correction for regression dilution bias. Data from 1 prospective study of homocysteine and cardiovascular disease (involving 244 cases)[48] that fulfilled the inclusion criteria were unavailable, as were data from 5 relevant prospective studies that were completed after January 1999 (involving about 600 IHD cases and 50 stroke cases).[64-68] These prospective studies reported results similar to those of the prospective studies that were included in this study (based on 1968 IHD cases and 463 strokes), suggesting that our findings were probably not materially altered by their exclusion.

The risks of IHD and stroke associated with homocysteine level were significantly weaker in the prospective studies than the retrospective studies,

which may reflect bias in retrospective studies because of the difficulties of selecting appropriate controls or the effects of changes in treatment, renal function, or other factors after the onset of disease that produce increases in homocysteine concentrations among the cases. To minimize such bias, the chief emphasis in this study was on the results from prospective studies (in which blood for homocysteine measurements had been collected before the clinical onset of disease) among individuals with no recorded history of cardiovascular disease at enrollment. The risks of IHD associated with given differences in homocysteine level observed in the prospective studies of such individuals were remarkably consistent, with the exception of the Multiple Risk Factor Intervention Trial.[13] The reasons for the apparent discrepancy in that study are unclear, although the mean time from baseline homocysteine measurement to IHD events was about twice that of the other prospective studies.[13] Homocysteine concentrations were strongly correlated with current smoking and systolic blood pressure, and the strength of the association of homocysteine with vascular disease was reduced substantially after adjustment for these known cardiovascular risk factors. Moreover, because these confounding factors will necessarily have been measured with some error, substantial residual confounding may well remain. Indeed, because systolic blood pressure (on remeasurement a few years later) has a self-correlation of only about two-thirds,[7] adjustment of the relationship between homocysteine and vascular disease should reduce the χ^2 value by about two thirds, as full adjustment for usual blood pressure would have done. Hence, the results in Table 2 are also consistent with the suggestion that the relationship of homocysteine to disease is largely due to confounding by the usual blood pressure. Thus, among prospective studies of individuals with no history of cardiovascular disease, and after appropriate adjustment for known cardiovascular risk factors and correction for regression dilution bias, a 25% lower usual homocysteine level was associated with about an 11% lower IHD risk and about a 19% lower stroke risk.

If the modest associations observed in this study are causal, then the implications for public health of decreasing the population mean levels of homocysteine could still be substantial. Studies of genetic variants affecting blood homocysteine concentrations and risk of IHD may well help to assess the nature of this association.[69,70] Individuals who have a C-to-T substitution at base 677 (amino acid change alanine 222 valine) of the gene that encodes the methylenetetrahydrofolate reductase enzyme have reduced enzyme activity and, as a consequence, have homocysteine levels that are about 25% higher than those with the *CC* genotype.[70,71] An accompanying article in THE JOURNAL (2002;288:2023-2031) describes a meta-analysis of 40 studies of this genetic polymorphism in which individuals with the *TT* polymorphism have a 16% (95% CI, 5%-28%) higher risk of IHD than those with the *CC* polymorphism.[72] The concordance of the IHD risks associated with genetically determined differences in homocysteine and of those observed in the population studies of such homocysteine differences provides support for these associations being causal.[69] Results from large randomized trials of the effects on vascular disease of lowering homocysteine with folic acid–based vitamin supplementation should provide further information about the relevance of homocysteine levels to the risks of IHD and stroke.[73]

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Intrajejunal vs oral levodopa-carbidopa therapy in Parkinson disease

A retrospective cohort study

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Abstract

Levodopa-carbidopa intestinal gel (LCIG) is a method of continuous administration of levodopa – the standard treatment in Parkinson disease (PD, a neurodegenerative disorder characterized by resting tremor, rigidity, gait impairment, and bradykinesia), thought to reduce the short-life and pulsatile problems of oral administration. We aimed to study the effects of Levodopa-Carbidopa therapy in 2 separate groups: one with intrajejunal administration of Levodopa-Carbidopa gel and the second with oral therapy.

We performed an observational retrospective Romanian cohort study on 61 patients diagnosed with PD patients, with Hoehn and Jahr 3 and 4 stages, recruited from a single regional tertiary center in Cluj-Napoca, Romania, between 2009 and 2019.

The mean adjusted UPDRS III (and similarly for UPDRS II) improved in the LCIG compared to the oral therapy group with 15.6 (95% CI 12.0–19.2, $P < .001$), and with 18.4 (95% CI 13.8–22.9, $P < .001$), stratified for the Hoehn and Jahr stages 3 and 4. There was a 41.7% (10) reduction in dyskinesia, and 29.2% reduction in wearing off/on-off at 1 year in the LCIG group compared to 0% (0) dyskinesia reduction, and 2.7% reduction in wearing off/on-off in the oral therapy group.

Continuous intrajejunal infusion of LCIG ensures a significant and clinical reduction in motor fluctuations compared to oral therapy in advanced PD, even after adjustment for important confounders.

Abbreviations: CI = confidence interval, COMT = catechol-O-methyltransferase inhibitor, IQR = interquartile range, LCIG = levodopa-carbidopa intestinal gel, MAO-B = monoamine oxidase-B, OMT = oral medical therapy, PD = Parkinson disease, PEG-J = percutaneous endoscopic transgastric jejunostomy, SD = standard deviation, STN-DBS = subthalamic nucleus deep brain stimulation, UPDRS = the unified Parkinson disease rating scale.

Keywords: LCIG, levodopa-carbidopa intestional gel, Parkinson disease, PD

1. Introduction

Parkinson disease (PD) is a neurodegenerative disorder characterized by resting tremor, rigidity, gait impairment, bradykinesia, sleep dysfunction, mood disorders, cognitive impairment, and dementia.[1] The underlying pathogenesis PD is not yet fully understood. It is thought to consist of the interaction between many genetic and environmental factors. This lack of knowledge explains the inability to make a precise diagnosis in the early stages and the limitations of treatment success in the later stages.

Levodopa is the amino-acid precursor of dopamine and has the function of recharging the depleted dopamine. For more than 4 decades, levodopa was described as the most efficient treatment in PD. Because of its short plasma half-life, oral levodopa may cause pulsatile striatal receptor stimulation, which leads to dyskinesias and a wide range of complications.[2–4] To diminish these types of complications, researchers developed levodopa-carbidopa intestinal gel (LCIG). It is delivered by using a percutaneous pump, set in place through an endoscopic intervention. This way, it leads to a constant plasma level of levodopa, therefore delivering a continuous dopaminergic stimulation.[4]

The intrajejunal administration of LCIG is one of the most efficient and frequently recommended pharmacological combination in PD. Nevertheless, studies found a wide range of motor and non-motor complications with the treatment.[4]

Because relevant studies engaged in the comparison between new therapeutical methods are still limited, we aimed to study the effects of levodopa-carbidopa in 2 separate groups: one with oral therapy and the second with intrajejunal administration of levodopa-carbidopa gel.

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2. Methods

2.1. Study design and setting

We performed an observational retrospective Romanian cohort study on 61 patients diagnosed with PD recruited from a single regional tertiary center in Cluj-Napoca, Romania, between 2009 and 2019.

2.2. Patients

We included PD patients with Hoehn and Jahr 3 and 4 stages, receiving oral administration of levodopa-carbidopa, or levodopa-carbidopa intrajejunal treatment. We excluded patients with an unclear diagnosis of PD, other Parkinsonian syndromes, neurodegenerative diseases, concomitant narrow-angle glaucoma, having contraindications for the placement of a nasogastric sonde or jejunal tube and oncological diseases.

2.3. Variables

We gathered the data from medical files and the hospital database. We set our outcome of interest the unified Parkinson disease rating scale (UPDRS) II and III reductions, and secondary the improvement in dyskinesia and wearing off/on-off in 1 year follow-up. Our exposure variable was the intrajejunal treatment compared to oral therapy. Besides these variables, we collected predictors and potential confounders, as well as variables to describe the sample better: demographic data (age, gender, place of residence), PD symptoms and evolution (disease duration, treatment duration, Hoehn and Yahr at baseline, UPDRS II and III, dyskinesia, Wearing off/on-off at baseline and 1 year follow-up), hallucinations, drug-induced psychosis, PD connex problems (mixed anxiety-depressive disorder, mild cognitive impairment, Parkinson dementia), oral treatment, additional treatments (deep brain stimulation), death, comorbidities (hypertension, atrial fibrillation, ischaemic stroke/cerebral lacunarism, type 2 diabetes, dyslipidemia, polyneuropathy), anemia related data (iron deficiency anemia, folate-deficiency anemia, B12 vitamin deficiency). UPDRS is one of the most frequently used questionnaires that follows the longitudinal course of Parkinson disease, but also the most commonly used scale in the clinical study of Parkinson disease and provides insight into the patients disease in a more objective manner.[5,6]

All subjects in both groups were assessed with the same scales that are commonly used in the hospital practice.

To minimize selection bias, we included subjects from the same hospital and excluding similar medical entities to PD. To minimize confounding, we performed adjustments in multiple regression analyses for important potential confounder variables, and stratified analyses.

2.4. Statistical analysis

Categorical data were presented as counts and percentages. Continuous data were presented as means and standard deviations (for normally distributed data) or medians and quartiles (1 and 3, for non-normally distributed data). Comparisons between the 2 groups for categorical data were made with the Chi-Squared test or with Fisher exact test, while for continuous data were made with *t* test for independent samples (for normally distributed data), or with Wilcoxon rank-sum test (for non-normally distributed data). To further assess the relationship between intrajejunal treatment compared to oral one, we used multivariate linear regression models, adjusted for age, Parkinson disease duration, treatment duration, Hoehn, and Yahr stage at the beginning. Since the Hoehn and Yahr stage appears to be a confounder, we also performed the same multivariate analysis, stratified by its 2 stages, 3 and 4. For all models, we checked the assumptions of residuals normality, heteroskedasticity (using the Breusch Pagan test of heteroskedasticity), linearity (using component residual plots), outliers and leverage points (Cooks D distance, studentized residuals). For multivariate models, we checked the assumptions of multicollinearity (using variance inflation factors), confounding (checking for a marked change in models coefficients when adding new variables to the model).

We removed 2 outliers/leverage points to correct for homoskedasticity – although the models were similar. Missing data was not imputed. For all statistical tests, the significance level was 0.05, and the two-tailed *P* value was calculated. All statistical analyses were performed with the R environment for statistical computing and graphics (R Foundation for Statistical Computing, Vienna, Austria), version 3.6.1.[7]

2.5. Ethics statement

The study was performed in agreement with the Declaration of Helsinki and was approved by the "Iuliu Hatieganu" University of Medicine and Pharmacy Ethics Committee.

3. Results

A total of 61 subjects with a mean age of 70.4 years (8.5 - standard deviation, ranging from 55 to 85 years), were enrolled in the study.

The characteristics of the intrajejunal therapy group and oral therapy group are compared in Table 1. Demographically the subjects were similar except for a higher frequency of female subjects in the intrajejunal group. The intrajejunal therapy group had a statistically significant longer PD history (6 years median difference), while the duration from the initiation of treatment was not significantly different.

The baseline clinical manifestations (Hoehn and Yahr stage; UPDRS II, and III; dyskinesia; Wearing off/On-Off) of PD disease were significantly worse in the intrajejunal group compared to the oral therapy group. The observed cognitive impairment and anxiety-depressive disorder were more frequent in the intrajejunal group than the oral therapy group, but only the latter reached a statistically significant level.

Regarding comorbidities, the cardiovascular, metabolic, were not significantly different between groups, although observed values were higher in the oral therapy group, except hypertension that was significantly different. Anemia-wise, the observed deficits were higher in the intrajejunal group, but not significantly different (except for iron deficiency anemia).

3.1. Intrajejunal therapy group-specific characteristics

Hallucinations and drug-induced psychosis were exceptional before the therapy, but a quarter of the subjects developed them after the therapy (see Table 2).

Table 1

Comparative analysis of Parkinson disease subjects receiving intrajejunal therapy and oral therapy group.

	Intrajejunal therapy (n = 24)	Oral therapy (n = 37)	*P* value
Age (years), mean (SD)	70.12 (7.66)	70.59 (9.11)	.835
Gender (female), n (%)	11 (45.83)	8 (21.62)	.046
Place of residence (rural), n (%)	3 (12.5)	2 (5.41)	.373
Duration of PD (years), median (IQR)	15 (13.75–20.25)	9 (6–13)	<.001
Duration from initiation of therapy (years), median (IQR)	5 (3.75–7)	4 (3–6)	.461
Hoehn şi Yahr stages la baseline			
3	2 (9.09)	29 (78.38)	<.001
4	20 (90.91)	8 (21.62)	
UPDRS II at baseline, median (IQR)	37 (33–39)	17 (13–24)	< .001
UPDRS II at 1 year, median (IQR)	27 (21–31.5)	24 (19–31)	.27
UDPRS II Diference at 1 year -baseline, median (IQR)	10 (6–12.5)	-7 (-8–-5)	<.001
UPDRS III at baseline, median (IQR)	41.5 (38–45)	24 (18–27)	<.001
UPDRS III at 1 year, median (IQR)	30 (28.5–35.5)	30 (26–34)	.307
UDPRS III Diference at 1 year-baseline, median (IQR)	11 (8.5–13)	−7 (−10–5)	<.001
Dyskinesia at baseline, n (%)	17 (70.83)	5 (13.51)	<.001
Dyskinesia at 1 year, n (%)	8 (33.33)	6 (16.22)	.12
Dyskinesia evolution in 1 year, n (%)			
dissapearing:	10 (41.67)	0 (0)	<.001
absent:	6 (25)	31 (83.78)	
persistent:	7 (29.17)	5 (13.51)	
newly occured:	1 (4.17)	1 (2.7)	
Dyskinesia evolution at 12 months (improvement vs. same or worsening), n (%)	10 (41.67)	0 (0)	<.001
Wearing off/On-Off at baseline, n (%)	24 (100)	10 (27.03)	<.001
Wearing off/On-Off at 1 year, n (%)	17 (70.83)	14 (37.84)	.012
Wearing off/On-Off evolution in 1 year, n (%)			
dissapearing:	7 (29.17)	1 (2.7)	<.001
absent:	0 (0)	22 (59.46)	
persistent:	17 (70.83)	9 (24.32)	
newly occured:	0 (0)	5 (13.51)	
Wearing off/On-Off evolution at 12 months (improvement vs. same or worsening), n (%)	7 (29.17)	1 (2.7)	.005
Deep brain stimulation, n (%)	1 (4.17)	0 (0)	.393
Decease, n (%)	6 (25)	3 (8.11)	.136
Mixed anxiety–depressive disorder, n (%)	13 (54.17)	8 (21.62)	.009
Mild cognitive impairment, n (%)	10 (41.67)	14 (37.84)	.765
Parkinson Dementia, n (%)	4 (16.67)	4 (10.81)	.7
Drug-induced psychosis, n (%)	5 (20.83)	0 (0)	.007
Hypertension, n (%)	6 (25)	21 (56.76)	.015
Permanent atrial fibrilation, n (%)	1 (4.17)	4 (10.81)	.64
Ischaemic Stroke / cerebral lacunarism, n (%)	5 (20.83)	16 (44.44)	.06
Diabetes type II, n (%)	1 (4.17)	8 (21.62)	.076
Polyneuropathy, n (%)	16 (66.67)	22 (61.11)	.662
Dyslipidemia, n (%)	3 (12.5)	7 (18.92)	.726
Iron deficiency anemia, n (%)	5 (20.83)	1 (2.78)	.033
Folate-deficiency anemia, n (%)	5 (20.83)	6 (16.67)	.741
Vitamin B12 deficiency, n (%)	2 (8.33)	4 (11.11)	1

SD = standard deviation, IQR = interquartile range, PD = Parkinson disease, UPDRS = the unified Parkinson disease rating scale.

3.2. Description of the oral therapy group specifics

The oral therapy group received in majority levodopa with carbidopa, about half of them received monoamine oxidase-B inhibitors and dopaminergic agonist, followed by amantadine, and the least frequent anticholinergic agents or catechol-O-methyltransferase inhibitors (see Table 3).

3.3. Comparative disease evolution under treatment

The evolution of PD clinical manifestations was statistically significant and clinically clearly better in the intrajejunal therapy group compared to the oral therapy regarding UPDRS II and III improvement, dyskinesia, and wearing off/On-Off at 1 year (see Table 1). Moreover, the oral therapy group had a diminishing of all the previously stated clinical manifestations at a year follow-up compared to the baseline evaluation. The difference in dyskinesia improvement in favor of intrajejunal therapy was of 47.47%, and statistically significant. While the difference in wearing off/On-Off improvement in favor intrajejunal therapy was of 26.47%, and statistically significant.

In order to check if the UPDRS II and III improvement, in 1 year, in the intrajejunal group, compared to oral therapy, was not due to other variables, we performed an adjustment in a multiple linear regression adjusting for age, Parkinson disease duration, treatment duration, Hoehn and Yahr stage at the beginning (see Table 4 and Table 5). In all the models the intrajejunal treatment had the most important effect in improving UPDRS II and III compared to all the other variables. Its effect was both important

Table 2

Characteristics of the intrajejunal therapy group.

Characteristic	Number (%) (n = 24)
Administration of Levodopa-Carbidopa on the nasogastric tube(test phase) but without administration of intrajejunal Levodopa-Carbidopa	4/24 (16.67)
Hallucinations	
Hallucinations before PEG-J	1/24 (4.17)
Number of years of hallucinations before PEG-J	0: 23/24 (95.83); 3: 1/24 (4.17)
Hallucinations after PEG-J	6/24 (25)
Hallucinations after PEG-J number of months, median (IQR)	0 (0–0.75)
Drug-induced psychosis	
Drug-induced psychosis before PEG-J	0/24 (0)
Drug-induced psychosis after PEG-J	6/24 (25)

IQR = interquartile range, PEG-J = percutaneous endoscopic transgastric jejunostomy.

Table 3

Drugs used for the patients under oral therapy.

Characteristic	Number (%) (n = 37)
Levodopa-Carbidopa	36 (100.00)
MAO-B Inhibitors	20 (55.56)
Dopaminergic Agonists	15 (41.67)
Anticholinergic Agents	2 (5.56)
Amantadine	7 (19.44)
COMT inhibitor (Entcapone)	4/35 (11.43)

MAO-B = monoamine oxidase-B, COMT = catechol-O-methyltransferase inhibitor.

and statistically significant. The determination coefficient for the univariate and for the multivariate models containing the intrajejunal treatment was important (above 0.74). All multivariate models were statistically significant. The relation between intrajejunal treatment and UPDRS II and III remained similar even after adjustment, and even on stratified analyses regarding the Hoehn and Yahr stage.

4. Discussions

Using the data collected during a decade in a tertiary clinical center, this analysis compared the clinical outcomes, side effects and complications between 2 separate groups of PD patients treated with LCIG and oral therapy. We found that UPDRS II and III scores statistically and clinically improved in the LCIG group compared to the oral therapy group, and the results stayed stable even after adjusting for age, disease duration, treatment duration, and stratified for Hoehn and Yahr stage at the beginning of the therapy. Dyskinesia, and wearing Off/On-Off diminished statistically and clinically in the LCIG group compared to the oral therapy group.

A study performed by Nyholm et al on 24 patients with advanced PD, compared daytime intraduodenal levodopa-carbidopa gel infusion as monotherapy with oral conventional combination therapies.[8] The median total UPDRS score at the end of each treatment arm was 53 with Conventional and 35 with Infusion ($P < .05$) and infusion provided lower median scores in all parts of the UPDRS, a result similar to ours.

A study on 11 patients with advanced PD analyzed the efficacy and safety of LCIG delivered continuously through an intrajejunal percutaneous tube (PEG-J).[9] LCIG contained a water-based suspension with micronized levodopa (20 mg/ml) and carbidopa (5 mg/ml) in methylcellulose (Duodopa) and was administered by continuous jejunal infusion for 12 hour/day using a portable pump (CADD-Legacy) by PEG-J.[9] The efficacy and safety outcomes were assessed by using the UPDRS parts II, III, and IV and were performed at baseline (T0) before LCIG initiation, and after 3 (T3) and 6 (T6) months of therapy.[9] The result was that patients showed statistically significant ($P < .05$) higher performances in activities of daily living, statistically significant ($P < .001$) lower incidence and severity of motor fluctuations, as rating by UPDRS part IV, compared to their best oral therapy and the success rate for PEG-J placement was 100%.[9] Previous research found that continuous intrajejunal infusion of LCIG provide a significant clinical improvement and improves UPDRS,[10–13] a result similar to ours. However, device and procedural complications, while generally of mild severity,

Table 4

The unified Parkinson disease rating scale II improvement (UPDRS II at therapy initiation minus UPDRS II at 12 months follow-up) assessment by univariate analyses, then in relation with therapy in multivariate regression, adjusted for age, Parkinson disease duration, treatment duration, Hoehn and Yahr stage at the beginning, and in stratified multivariate analysis by Hoehn and Yahr stage.

	Unstratified analyses							Stratified by Hoehn & Yahr = 3			Stratified by Hoehn & Yahr = 4		
	B	(95% CI)	P value	R2	B adjusted*	(95% CI)	P value	B adjusted**	(95% CI)	P value	B adjusted**	(95% CI)	P value
Age (years)	−0.17	(−0.42–0.08)	.183	0.03	−0.12	(−0.24–0)	.042	0.003	(−0.08–0.09)	.941	−0.33	(−0.58–-0.08)	.011
Parkinson's disease duration (years)	0.52	(0.2–0.84)	.002	0.16	−0.02	(−0.22–0.18)	.845	−0.08	(−0.25–0.09)	.342	0.06	(−0.30–0.41)	.741
Treatment duration (years)	0.24	(−0.71–1.19)	.614	0.005	−0.21	(−0.72–0.3)	.422	−0.09	(−0.58–0.41)	.724	−0.21	(−1.04–0.62)	.605
Hoehn and Yahr stage at the beginning	9.29	(5.7–12.88)	<.001	0.32	0.33	(−2.46–3.11)	.815		–	–	–	–	–
Therapy (intrajejunal vs. oral)	15.17	(13.17–17.18)	<.001	0.80	15.19	(12.37–18.01)	<.001	15.4	(12.46–18.34)	<.001	14.72	(10.25–19.19)	<.001
Adjusted R2					0.81			0.8			0.74		

* model containing all the variables in the table.

** model containing all the variables in the model, excepting the stratifying variable (Hoehn and Yahr stage); R^2 – coefficient of determination.

CI = confidence interval.

Table 5

The unified Parkinson disease rating scale III improvement (UPDRS III at therapy initiation minus UPDRS III at 12 months follow-up) assessment by univariate analyses, then in relation with therapy in multivariate regression, adjusted for age, Parkinson disease duration, treatment duration, Hoehn and Yahr stage at the beginning, and in stratified multivariate analysis by Hoehn and Yahr stage.

	Unstratified analyses							Stratified by Hoehn & Yahr = 3			Stratified by Hoehn & Yahr = 4		
	B	(95% CI)	P value	R2	B adjusted*	(95% CI)	P value	B adjusted**	(95% CI)	P value	B adjusted**	(95% CI)	P value
Age (years)	−0.22	(−0.51–0.07)	.138	0.04	-0.2	(−0.31–-0.08)	.001	−0.14	(−0.24–0.03)	.010	−0.33	(−0.58–0.07)	.014
Parkinson's disease duration (years)	0.68	(0.35–1.02)	<.001	0.23	0.12	(−0.07–0.3)	.209	0.07	(−0.14–0.27)	.500	0.14	(−0.19–0.46)	.385
Treatment duration (years)	0.12	(−0.98–1.23)	.824	0.001	-0.26	(−0.74–0.22)	.285	−0.06	(−0.66–0.54)	.827	−0.28	(−1.06–0.49)	.461
Hoehn and Yahr stage at the beginning	10.79	(6.57–15.02)	<.001	0.32	−0.54	(−3.33–2.25)	−.54	–	–	–	–	–	–
Therapy (intrajejunal vs oral)	18.14	(15.99–20.29)	<.001	0.84	17.76	(14.92–20.6)	17.76	15.6	(12.01–19.18)	<.001	18.35	(13.84–22.87)	<.001
Adjusted R^2					0.86			0.78			0.81		

* model containing all the variables in the table.
** model containing all the variables in the model, excepting the stratifying variable (Hoehn and Yahr stage); R^2 – coefficient of determination.
CI = confidence interval.

were present and were explained by the severity and progression of the disease.[10–13]

A long-term retrospective study analyzing advanced therapies in PD including oral medical therapy (OMT), LCIG and subthalamic nucleus deep brain stimulation (STN-DBS), found that OFF time improved to the same extent in STN-DBS and LCIG (−62% vs −54.5%; $P = .830$) and worsened with OMT (+78.6%; $P < .001$). Our study similarly found improvement in Wearing off/On-Off in LCIG compared to OMT. STN-DBS and LCIG yielded greater improvement on dyskinesia compared to OMT (dyskinesia duration: −66.1% vs −9.0% vs +24.2% [$P = .001$][14] similar to our study were dyskinesia at 1 year improved in the LCIG group compared to OMT group. The vast majority of studies have reported positive outcomes in motor complications with reduced duration of OFF time, increased ON time and plasma drug levels were maintained relatively stable in patients with LCIG therapy.[15–22]

4.1. Study limitations

The study has several limitations. Even though the sample size was not that large, the results are highly statistically significant, and the adjusted coefficients and confidence intervals for the main results are distant from the value of 0, thus suggesting a strong force of association. As with any observational study designs, residual confounding cannot be excluded even if we adjusted for several variables in the multivariate analysis. More extensive studies with more confounder adjusted models are warranted. Nevertheless, the large determination coefficient and the large adjusted coefficients suggest that this association is more likely to withstand adjustment for other confounders. Unknown confounders may diminish the association between intrajejunal treatment and disease progression.

The fact that the clinical status of PD patients was poorer in the intrajejunal group compared to the control group is normal since intrajejunal therapy is initiated in more advanced stages. Moreover, we tried to have subjects in both groups as homogenous as possible, thus limiting them to having only stage 3 and 4 for the Hoehn and Yahr stage. However, even with this initial difference, the improvement in outcomes in the intrajejunal group is important.

Since the cohort of PD patients has characteristics similar to patients from regional tertiary centers, the results are generalizable to this type of population. The reduced set of exclusion criteria helps to this generalizability.

Having taken into account the statistically significant and clinically important relation between intrajejunal treatment and clinical manifestations of PD, after adjustment for important confounders, for a cohort of similar subjects with advanced stage of PD, and also the similar findings of other studies, we have good arguments sustaining this relationship.

5. Conclusions

Continuous intrajejunal infusion of LCIG ensures a statistically significant and clinical important reduction of UPDRS II and III, compared to oral therapy in advanced PD patients, and the results stayed stable even after adjusting for age, disease duration, treatment duration, and stratified for Hoehn and Yahr stage at the beginning of the therapy. The same differences were found also for dyskinesia and wearing Off/On-Off that were diminished in the LCIG group.

Author contributions

Luminita Celia Popa carried out the study, analyzed the data, and wrote the paper; Daniel Corneliu Leucuta made substantial contributions to the analysis of the data, interpretation, and revised the drafts; Nicoleta Tohanean analyzed the data; Stefan-Lucian Popa made contributions to the conception of the manuscript; L. Perju-Dumbrava analyzed the data, supervised the work and critically revised the manuscript. All authors read and approved the final manuscript.

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