

Association of metformin, elevated homocysteine and methylmalonic acid levels, and clinically worsened diabetic peripheral neuropathy

Running Title: Metformin and diabetic peripheral neuropathy

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Submitted 28 March 2009 and accepted 10 October 2009.

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Objective: The severity of peripheral neuropathy in diabetic patients varies for unclear reasons. Long-term use of metformin is associated with malabsorption of vitamin B₁₂ (cobalamin; Cbl) and elevated homocysteine (Hcy) and methylmalonic acid (MMA) levels, which may have deleterious effects on peripheral nerves. This study intends to clarify the relationship between metformin exposure, levels of Cbl, Hcy and MMA, and severity of peripheral neuropathy in diabetic patients. We hypothesized that metformin exposure would be associated with lower Cbl levels, elevated Hcy and MMA levels, and more severe peripheral neuropathy.

Research design and methods: A prospective case-control study of patients with type 2 diabetes and concurrent symptomatic peripheral neuropathy comparing those who had received more than 6 months of metformin therapy (N=59) to those without metformin exposure (N=63). Comparisons were made using clinical (Toronto Clinical Scoring System, Neuropathy Impairment Score), laboratory (serum Cbl, fasting Hcy, fasting MMA), and electrophysiological measures (nerve conduction studies).

Results: Metformin-treated patients had depressed Cbl levels and elevated fasting MMA and Hcy levels. Clinical and electrophysiological measures identified more severe peripheral neuropathy in these patients; cumulative metformin dose correlated strongly with these clinical and paraclinical group differences.

Conclusions: Metformin exposure may be an iatrogenic cause for exacerbation of peripheral neuropathy in patients with type 2 diabetes. Interval screening for Cbl deficiency and systemic Cbl therapy should be considered upon initiation of, as well as during, metformin therapy to detect potential secondary causes of worsening peripheral neuropathy.

Diabetes is an increasingly prevalent disorder with a range of systemic complications including diabetic peripheral neuropathy (DPN), which occurs in up to 50% of diabetic patients and causes sensory, motor and/or autonomic dysfunction (1). Several pathogenic mechanisms contribute to DPN severity, including microangiopathy, oxidative stress, polyol flux, mitochondrial dysfunction, insulin deficiency, and advanced glycation end products and ligand activation of their receptor (2-5). The course and severity of DPN is further affected by a wide range of comorbid conditions.

Vitamin B₁₂ (cobalamin; Cbl) deficiency may co-occur with diabetes. Although it is most classically associated with subacute combined degeneration, an exclusive peripheral neuropathy (PN) presentation can occur, typically manifesting as axonal neuropathy based upon electrophysiology and pathology (6-8). Accumulating evidence suggests that Cbl-associated metabolites methylmalonic acid (MMA) and homocysteine (Hcy) are more sensitive (MMA, Hcy) and specific (MMA) indicators of early symptomatic Cbl deficiency than serum Cbl itself (9-10).

Metformin, a biguanide, is perennially reported as a pharmacologic cause of Cbl deficiency (11-13). The responsible mechanism has been controversial; proposed contributors have included competitive inhibition or inactivation of Cbl absorption, alterations in intrinsic factor levels, bacterial flora, gastrointestinal motility, or ileal morphological structure, and interaction with the cubulin endocytic receptor (11, 14-15). Biguanides have recently been shown to impair calcium-dependent

membrane activity in the ileum, including uptake of the Cbl-intrinsic factor complex (16).

Metformin is recommended by the American Diabetes Association and the European Association for the Study of Diabetes as initial medical therapy for type 2 diabetes at diagnosis (17). Despite its wide use and its known effects upon Cbl, metformin has not been systematically studied as a potential iatrogenic cause of or contributor to DPN. The potentially reversible effect of cobalamin deficiency may increase the clinical burden for a population of patients with DPN whose sensory function, gait and balance is already frequently compromised.

We designed a prospective case-control study to assess the effects of prolonged metformin intake in patients with type 2 diabetes matched for disease duration and disease control. We specifically examined the relationship between metformin use, levels of Cbl and its metabolites, and clinical and electrophysiological markers of PN severity. We hypothesized firstly that metformin use would be associated with biochemical evidence of Cbl deficiency (lower serum Cbl levels, elevated MMA and Hcy), and secondly that metformin use would be associated with more severe PN. Decreases in Cbl have been shown to depend on the dose and duration of metformin therapy in a case-control study (18); this finding led us to further hypothesize that biochemical abnormalities and severity of neuropathy would correlate with cumulative lifetime metformin dose.

RESEARCH DESIGN AND METHODS

Ethical approval for this study was received from the University of Calgary

Centre for Advancement of Health. From December 2002 until May 2007, patients with pre-existing type 2 diabetes and a primary diagnosis of PN were assessed within the Neuromuscular Clinic at the University of Calgary. These patients then underwent further clinical, laboratory and electrophysiological evaluation of their neuropathy. Presence of diabetes was verified by two separate positive results: two prior fasting glucose results of ≥ 7.1 mmol/L (126 mg/dL) or two oral glucose tolerance tests leading to a 2 hour serum glucose of ≥ 11.1 mmol/L (200 mg/dL) (based on Canadian Diabetes Association guidelines). The age of diagnosis of diabetes and the duration of symptoms of DPN were recorded. History of other systemic illnesses, alcohol use, toxin and medication exposures, and family history of neuropathy was documented to assess for other potential causes of PN. The duration of metformin therapy and dosage history was determined for each patient by review of their medical record, and these dosages were confirmed verbally by the patients; this data was used to calculate a cumulative lifetime dose of metformin for each patient. Use of other anti-diabetic agents was recorded.

All patients underwent laboratory testing including a complete blood count, electrolytes, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), albumin, total bilirubin, international normalized ratio (INR), thyroid stimulating hormone, erythrocyte sedimentation rate, antinuclear antibody, extracted nuclear antibody, serum protein electrophoresis, rheumatoid factor, lactate, and serum folate. Hemoglobin A1C was measured in all patients. The sensitivity for detection

of gammopathy in our centre is 2 g/L by serum protein electrophoresis, with immunofixation performed when peaks are found in the range of 2-4 g/L. Serum Cbl levels were measured by Cobas e immunoassay analysis (Roche Diagnostics), Hcy levels using high performance liquid chromatography, and MMA levels using mass spectrometry for all patients. The lower limit of normal for Cbl in our centre is 210 pmol/L (285 pg/mL); the upper limit for Hcy is 13.7 μ mol/L (1.85 mg/L) in adult males, 9.9 μ mol/L (1.34 mg/L) in adult females under 49, and 12.8 μ mol/L (1.73 mg/L) in adult females over 49; here we adopt a conservative upper limit of 13.7 μ mol/L (1.85 mg/L) for all participants. The upper limit of normal for MMA in our centre is 0.15 μ mol/L. All blood testing was performed by Calgary Laboratory Services.

Patients were excluded if potential causes for PN other than diabetes and Cbl deficiency were identified, if they had previously been treated with metformin and had discontinued therapy, if they had less than six months metformin treatment at the time of assessment, if they had impaired glucose tolerance only, or if they had a juvenile onset of diabetes or frank requirement for insulin at diagnosis (i.e. possible type I diabetes). Lastly, patients were excluded if they refused concurrent electrophysiological or laboratory testing. We did not exclude patients with renal failure concurrently using metformin, though this is often considered a contraindication to metformin use due to potential lactic acidosis; however, many patients do not discontinue metformin use after diagnosis of renal impairment (19) and lactate levels were unremarkable in all patients.

Complete standardized neurological examinations were carried out in all DPN

patients, including tone, power, deep tendon reflexes, sensory function, Romberg testing, gait and tandem gait. Tandem gait was recorded as the number of heel to toe steps along a straight line the patient could perform to a normal threshold value of six. Each patient was also scored using the Toronto Clinical Scoring System (TCSS) (20) by an unblinded investigator prior to knowledge of laboratory results. The TCSS was developed as a clinical screening tool for the presence and severity of DPN that emphasizes sensory deficits; though it introduces some subjectivity in scoring, it has been validated by sural nerve fiber density. We also determined the Neuropathy Impairment Score (NIS), a scale scoring weakness of groups of muscles of the head and neck, upper limbs, and lower limbs, tactile, vibratory, joint position, and pin prick of index fingers and great toes, and reflexes, for each patient (21).

Electrophysiological assessment was performed after clinical assessment and prior to knowledge of the laboratory results using a Dantec Datapoint (Dantec Dynamics Ltd., Bristol, UK). Sensory and motor nerves of the non-dominant upper and lower extremity were tested within 3 months of clinical assessment. Motor nerve conduction studies (NCS) were performed using stimulation of the median nerve (wrist, elbow), ulnar nerve (wrist, below elbow, above elbow), peroneal (ankle, below fibular head and above fibular head locations) and tibial (ankle, popliteal fossa locations) nerves. For each motor nerve, distal motor latencies, compound motor action potentials, and conduction velocities were obtained or calculated. F wave latencies were obtained from median, ulnar, peroneal and tibial nerves. Sensory NCS were performed using the median (digits

2 and 4), ulnar (digits 4 and 5), superficial radial, superficial peroneal and sural nerves, with sensory nerve action potentials (SNAP), onset latency, and conduction velocity obtained or calculated. Temperatures were maintained at $\geq 32^{\circ}\text{C}$ for the upper extremities, and $\geq 30^{\circ}\text{C}$ for the lower extremities during NCS testing. Though all included participants completed electrophysiological testing, some participants do not have complete data for all individual nerves.

After all clinical, electrophysiological and laboratory testing, those patients with abnormal Cbl, MMA, or Hcy levels were prescribed monthly intramuscular Cbl. Sural nerve biopsies were done only in clinical situations where vasculitis or another serious cause of PN was suspected.

Group equivalence for patient age, duration of type 2 diabetes, duration of PN symptoms, A1C, and alcohol exposure were compared by independent samples t-test; gender and proportion using other anti-diabetic agents were compared by chi-square test. Elements of the past medical history (e.g. iron deficiency anemia, hereditary spherocytosis) were broadly classified (e.g. hematologic disease) and are summarized in Table 1; these were not statistically compared due to their heterogeneity. These elements of the history are detailed in the Online-Only Appendix which can be found at <http://diabetes.diabetesjournals.org>. The primary outcome measures were Hcy, MMA and Cbl blood levels, clinical neuropathy severity (TCSS, NIS) and electrophysiological markers of neuropathy; of the latter we chose to test sensory NCS in the lower extremity (conduction velocity and SNAP for

superficial peroneal and sural nerves) as we felt these would be most in keeping with exacerbation due to Cbl deficiency. These data did not follow a normal distribution (by Shapiro-Wilk test) and comparisons were made using Mann-Whitney U test. Proportions of patients with deficiency of Cbl and upregulation of Hcy and MMA were compared using Chi square test. Bivariate correlations of clinical and laboratory variables with cumulative metformin dose were calculated using Spearman rho test. Lastly, a linear regression analysis was performed using NIS total score as the dependent variable and age, duration of diabetes, A1C and presence of metformin exposure as explaining variables.

RESULTS

Of 226 patients with type 2 diabetes and PN assessed for eligibility, 104 patients were excluded; 55 had brief (<6 months) metformin exposure, 46 patients discontinued metformin prior to assessment, and three were unable to perform laboratory or electrophysiological testing. 122 patients were eligible for analysis, 59 (48%) of whom received metformin therapy for more than 6 months (mean cumulative exposure 3389.5 g, σ = 2560.6 g); the remaining patients had no prior metformin exposure. There were no significant differences in demographic variables (age, gender) or disease severity (duration of type 2 diabetes, A1C, duration of PN symptoms) between the two groups (Table 1). A significantly higher number of patients in the metformin treated group were concurrently treated with glyburide and significantly fewer with insulin (Table 1). There were no other notable differences in prescription medication use, presence of other systemic conditions, or alcohol

exposure between groups (Table 1; details available in online-only appendix).

Analysis of laboratory testing is summarized in Table 2. Median serum Cbl was significantly lower in the metformin-treated group (231 vs. 486 pmol/L; U=299.0; $p<0.001$) with frank deficiency in 18 patients (31%) compared with 2 (3%) in the non metformin-treated group ($p<0.001$). Cumulative metformin dose was inversely correlated with serum Cbl (Spearman rho = -0.41; two-tailed $p=0.001$). Median fasting serum Hcy was significantly higher in the metformin-treated group (11.6 vs. 8.4 $\mu\text{mol/L}$; U=454.0; $p<0.001$) with upregulation of Hcy in 15 vs. 1 patients ($p<0.001$). Median MMA was significantly higher in the metformin-treated group (0.18 vs. 0.11 $\mu\text{mol/L}$; U=306.5; $p<0.001$) and upregulated in 43 of these patients compared with 7 patients with no metformin exposure ($p<0.001$). Cumulative metformin dose was positively correlated with fasting serum Hcy (Spearman rho = 0.50; two-tailed $p<0.001$) and fasting serum MMA (Spearman rho = 0.37; two-tailed $p=0.005$) (Figure 1).

Clinical and paraclinical markers of neuropathy severity are summarized in Table 2. The median TCSS total score was higher in the metformin-treated group (10 vs. 5; U=527.0; $p<0.001$), with a strong positive correlation to increasing cumulative metformin dose (Spearman rho = 0.80; two-tailed $p<0.001$). Median NIS total score was significantly higher in the metformin treated group (10 vs. 4; U=408.0; $p<0.001$) and also had a strong positive correlation with increasing cumulative metformin dose (Spearman rho = 0.79; two-tailed $p<0.001$, Figure 1). Left sural (metformin treated N=41; non metformin treated N=51) and superficial peroneal nerves (metformin treated N=39;

non metformin treated N=49) had lower median SNAP and slower median conduction velocity in the metformin-treated group; however, these measures were not significantly different between groups after corrections for multiple comparisons (Table 2).

In the linear regression analysis, using the enter method, a significant model emerged ($F_{4,117} = 47.7$, $p < 0.005$) with adjusted $R^2 = .61$. Metformin exposure ($\beta = .55$, $p < 0.001$) and duration of diabetes ($\beta = .41$, $p < 0.001$) were the only significant explaining variables in this model. Collinearity diagnostics did not suggest a problem with multicollinearity in this model ($VIF < 1.5$ for all included variables).

CONCLUSIONS

We found evidence that patients with DM2, PN, and more than 6 months' exposure to metformin had lower serum Cbl, higher serum Hcy and MMA, and higher scores on the NIS and TCSS indicating clinically more severe PN when compared to similar patients with no metformin exposure. These abnormalities were correlated strongly with cumulative metformin exposure. A linear regression analysis including age, duration of diabetes, A1C and metformin exposure to predict clinical status (NIS total score) found that metformin exposure and duration of diabetes were significant explaining variables. We were unable to demonstrate significant group differences in sural or superficial peroneal SNAP or conduction velocity.

Metformin-associated Cbl deficiency may contribute to the clinical burden of DPN; this contribution is both detectable and ameliorable. This deficiency and concomitant increase in serum Hcy has been demonstrated in a randomized controlled trial (22), and this relationship

depends on dose and duration of metformin therapy (18); however, the potential for clinical sequelae has been discussed only rarely (11, 16) and has not previously been formally studied. The present findings therefore add clinical relevance to the existing literature on metformin-associated Cbl deficiency. Given the prevalence of DM2 and of metformin use, these effects have the potential to be widespread.

The present findings should not be seen to discourage against treating diabetic patients with neurologic impairment with metformin; in addition to its effects on metabolic control, metformin has been shown to have beneficial effects on advanced glycation end product formation in peripheral nerves (23) and may prevent apoptosis involved in diabetes-associated neurodegenerative processes (24). Instead, we recommend screening for features of Cbl deficiency in diabetic patients on long-term metformin therapy. The American Academy of Neurology recommends serum Cbl and metabolites (MMA with or without Hcy) as investigations with high diagnostic yield in distal symmetric polyneuropathy (10); this yield may be further increased in the present population given their predilection for comorbid Cbl deficiency. The optimal screening frequency remains to be determined, but baseline tests at initiation of metformin therapy and at intervals of no more than 1-2 years seem prudent, since metformin may begin to depress serum Cbl levels after as few as three months (15).

It is unclear whether Cbl supplementation will prevent clinical worsening in this group, but supplementation carries a low risk of toxicity. Current therapy consists of intramuscular Cbl replacement therapy and possible long-term Cbl and folate

therapy; oral Cbl supplementation may be as effective as intramuscular therapy, although long term outcomes have not yet been examined in DPN patients (25). Oral calcium supplementation has also been effective in reversing bioavailable Cbl deficiency in metformin-treated patients (16).

Further studies should better define differences in the electrophysiological profile of these groups. Potential central nervous system complications of Cbl deficiency, including myelopathy and cognitive impairment, should be considered as contributors to clinical status. The relationship between Cbl deficiency, elevated Hcy and MMA levels and PN is controversial and remains to be proven, but both Cbl deficiency and elevation of its serum metabolites are associated with presence of a sensorimotor PN (7).

Our findings are presented with some limitations. Although we identified patients prospectively, they were not randomly selected from a population with type 2 diabetes with or without DPN. Our sample size was not based upon a pre-determined power analysis. We did not identify a separate group of patients with asymptomatic DPN. We excluded patients with type 1 diabetes due to their expected limited metformin exposure and the potential for distinct pathophysiological mechanisms. Though A1C was not significantly different between groups at the time of evaluation, we did not examine measures of metabolic control over time. Investigators were blinded to the laboratory results until clinical and electrophysiological studies were completed, but were not blinded to use of metformin therapy.

Metformin-treated patients were more commonly treated with glyburide, and less commonly with insulin therapy;

insulin may be beneficial in diabetic patients with PN due to mechanisms other than glycemic correction (3). Undetected group differences may also exist; additional factors such as inadequate dietary intake might in part explain lower Cbl levels in our metformin-exposed patients, and it is possible that patients using metformin may have more severe diabetes in spite of the similar duration of type 2 diabetes and similar A1C levels between groups. All participants had normal folate status, but pyridoxine levels were not measured. Laboratory testing for pernicious anemia (by Schilling test) was not available during this investigation, and therefore the prevalence of this alternate cause of Cbl deficiency in our population is not known. Our multiple regression analysis should be interpreted with caution as not all variables potentially relevant to NIS total score were measured in our population, and our participants were grouped in a nonrandom manner.

The current findings suggest an association between metformin, elevated Cbl metabolites and exacerbation of DPN, but further work is needed to prove a direct causal relationship and its mechanism. Metformin may exacerbate PN due to other unknown mechanisms; a clear understanding of its role necessarily awaits further research on the pathogenesis of DPN. Despite these limitations, we believe that metformin exposure is a potential iatrogenic contributor to severity of PN in the population described. Recognition of this readily identifiable and potentially treatable component of disease might improve quality of life for this large population of diabetic patients.

ACKNOWLEDGMENTS

Dr. Cory Toth receives funding from the Alberta Heritage Medical Research Foundation (research into diabetic complications of the nervous system). This agency had no role in design, conduct of the study, collection,

management, analysis or interpretation of the data, review, or manuscript approval.

DISCLOSURE

The authors have no competing interests to disclose.

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Table 1 - Patient Characteristics

| Characteristic | Metformin-treated (N = 59) | Non-metformin treated (N = 63) | P value |
|--|----------------------------|--------------------------------|---------|
| Demographic variables | | | |
| Age (y) | 66.6 +/- 11.9 | 64.8 +/- 12.0 | NS |
| Female (%) | 24 (41%) | 29 (46%) | NS |
| Disease severity | | | |
| Duration of diabetes (y) | 5.5 +/- 3.3 | 4.7 +/- 2.9 | NS |
| Duration of peripheral neuropathy symptoms (y) | 3.8 +/- 2.1 | 3.8 +/- 2.4 | NS |
| A1C (%) | 6.7 +/- 1.0 | 6.8 +/- 1.1 | NS |
| Other diabetes pharmacotherapy | | | |
| Glyburide use (n(%)) | 38 (64%) | 22 (35%) | <0.001* |
| Glicazide use (n(%)) | 18 (31%) | 15 (24%) | NS |
| Insulin therapy (n(%)) | 7 (12%) | 29 (46%) | <0.001* |
| Clinical history | | | |
| Alcohol exposure (drinks /week) | 3.0 +/- 4.5 | 2.2 +/- 4.0 | NS |
| History of first degree relatives with peripheral neuropathy | 4 (5%) | 0 | |
| Rheumatologic disease (osteoarthritis or rheumatoid arthritis) (%) | 7 (12%) | 4 (6%) | |
| Elevated creatinine with renal failure (%) | 6 (10%) | 7 (11%) | |
| Thyroid disease (%) | 11 (19%) | 16 (25%) | |
| Hematologic disease (%) | 5 (8%) | 0 | |
| Cancer (%) | 7 (12%) | 7 (11%) | |
| Other illness (%) | 42 (71%) | 29 (46%) | |

A1C: hemoglobin A1C; NS: non-significant at the $\alpha = 0.05$ level

Values presented represent mean +/- standard deviation

*Significant at $\alpha = 0.05$ level for chi-square test

Table 2 – Markers of Cbl deficiency and neuropathy severity

| Marker | Metformin treated (N = 59) | Non-metformin treated (N = 63) | P value | Correlation with Cumulative Metformin Dose | P value |
|---|----------------------------|--------------------------------|---------|--|----------|
| Biochemical markers of cobalamin deficiency | | | | | |
| Serum Cbl (pmol/L) | 231 [343] | 486 [863] | <0.001* | -0.41 | 0.001** |
| Cbl deficiency (< 210 pmol/L) | 18 (31%) | 2 (3%) | <0.001* | | |
| Fasting serum Hcy (µmol/L) | 11.6 [17.7] | 8.4 [24.9] | <0.001* | 0.50 | <0.001** |
| Hcy upregulation (> 13.7 µmol/L) | 15 (25%) | 1 (2%) | <0.001* | | |
| Fasting serum MMA (µmol/L) | 0.18 [0.47] | 0.11 [0.14] | <0.001* | 0.37 | 0.005** |
| MMA upregulation (> 0.15 µmol/L) | 43 (73%) | 7 (11%) | <0.001* | | |
| Clinical markers of neuropathy severity | | | | | |
| TCSS total score | 10 [17] | 5 [12] | <0.001* | 0.80 | <0.001** |
| NIS total score | 10 [32] | 4 [12] | <0.001* | 0.79 | <0.001** |
| Electrophysiological markers of neuropathy severity | | | | | |
| Left sural nerve SNAP amplitude (µV) | 3.0 [11.4] | 4.4 [12.9] | 0.038 | | |
| Left sural nerve sensory conduction velocity (m/s) | 33.3 [15.0] | 33.0 [15.7] | 0.69 | | |
| Left superficial peroneal nerve SNAP amplitude (µV) | 2.5 [7.1] | 3.6 [7.8] | 0.12 | | |
| Left superficial peroneal nerve sensory conduction velocity (m/s) | 34.2 [18.4] | 36.8 [18.8] | 0.071 | | |

Cbl: cobalamin; Hcy: homocysteine; MMA: methylmalonic acid; TCSS: Toronto Clinical Scoring System; NIS: Neuropathy Impairment Score; SNAP: Sensory nerve action potential
Values presented represent median [range]

*Significant at Bonferroni-corrected $\alpha = 0.05$ level (markers of cobalamin deficiency: $\alpha = 0.0083$; markers of neuropathy severity: $\alpha = 0.0083$) using Mann-Whitney U testing

□ Significant at Bonferroni-corrected $\alpha = 0.05$ level (markers of cobalamin deficiency: $\alpha = 0.0083$) using Fisher exact test

**Two-tailed correlation coefficient significant at Bonferroni-corrected $\alpha = 0.05$ level ($\alpha = 0.01$) using Spearman rho testing

Figure 1 - Correlational Analysis

Correlation of lifetime cumulative metformin dose with serum cobalamin (A), fasting homocysteine (B), methylmalonic acid (C), TCSS total score (D) and NIS total score (E). Each point represents an individual. Correlation coefficients (Spearman rho) were: cobalamin, -0.41; P=0.001; homocysteine, 0.50; P<0.001; methylmalonic acid, 0.37; P=0.005; TCSS total score, 0.80; P<0.001; NIS total score, 0.79; P<0.001. Dashed lines indicate lower limit of normal (cobalamin) and upper limits of normal (homocysteine, methylmalonic acid) for our centre.

