Immunologic Effects of Metformin and Pioglitazone Treatment on Metabolic Syndrome and Multiple Sclerosis

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**IMPORTANCE** Metabolic syndrome (MetS) is thought to influence several autoimmune diseases, including multiple sclerosis (MS). Anti-inflammatory effects of treatments used for MetS, such as metformin hydrochloride and pioglitazone hydrochloride, have been demonstrated, although clinical evidence supporting use of these treatments in MS is lacking.

**OBJECTIVES** To determine whether metformin and/or pioglitazone are associated with a reduction in disease activity as measured by brain magnetic resonance imaging in patients with MS and MetS and to evaluate the potential mechanisms underlying this anti-inflammatory effect.

**DESIGN, SETTING, AND PARTICIPANTS** A prospective cohort study was conducted from March 1, 2012, to December 30, 2014, at a private MS referral center among 50 obese patients with MS who also developed MetS. Twenty patients received metformin hydrochloride, 850 to 1500 mg/d, and 10 patients received pioglitazone hydrochloride, 15 to 30 mg/d; 20 untreated patients served as controls. Groups were comparable in terms of sex, age, body mass index, Expanded Disability Status Scale score, disease duration, annual relapse rate, and treatment status. Patients were followed up for a mean (SD) of 26.7 (2.7) months (range, 24-33 months).

**MAIN OUTCOMES AND MEASURES** Magnetic resonance imaging of the brain was performed at 6-month intervals, and the presence of new or enlarging T2 lesions or gadolinium-enhancing lesions was registered. Serum leptin and adiponectin levels were measured. The production of cytokines by peripheral blood mononuclear cells was assayed, as were regulatory T-cell numbers and function.

**RESULTS** Of 50 patients, after 6 months of treatment, 20 patients with MS who were treated with metformin and 10 who received pioglitazone showed a significant decrease in the number of new or enlarging T2 lesions (metformin, 2.5 at study entry to 0.5 at month 24; pioglitazone, 2.3 at study entry to 0.6 at month 24), as well as of gadolinium-enhancing lesions (metformin, 1.8 at study entry to 0.1 at month 24; pioglitazone, 2.2 at study entry to 0.3 at month 24). Compared with controls, both treatments led to a decrease in mean (SD) leptin levels (metformin, 5.5 [2.4] vs 10.5 [3.4] ng/mL, \( P < .001 \); pioglitazone, 4.1 [0.8] vs 11.0 [2.6] ng/mL, \( P < .001 \)) and increase in mean (SD) adiponectin serum levels (metformin, 15.4 [5.5] vs 4.5 [2.4] \( \mu \)g/mL, \( P < .001 \); pioglitazone, 12.6 [3.6] vs 4.8 [0.6] \( \mu \)g/mL, \( P < .001 \)). Mean (SD) number of myelin basic protein peptide–specific cells secreting interferon \( \gamma \) and interleukin (IL)-17 were significantly reduced in patients receiving metformin compared with controls (interferon \( \gamma \), 30.3 [11.5] vs 82.8 [18.8], \( P < .001 \); IL-17, 212.4 [85.5] vs 553.8 [125.9], \( P < .001 \)). Patients treated with pioglitazone showed significant decreases in the mean (SD) number of myelin basic protein peptide–specific cells secreting IL-6 and tumor necrosis factor compared with controls (IL-6, 361.6 [80.5] vs 1130.7 [149.2], \( P < .001 \); tumor necrosis factor, 189.9 [53.4] vs 341.0 [106.0], \( P < .001 \)). Both metformin and pioglitazone led to a significant increase in the number and regulatory functions of CD4+CD25+FoxP3+ regulatory T cells compared with controls (metformin, 6.7 [1.5] vs 2.1 [1.0], \( P = .001 \); pioglitazone, 6.9 [0.8] vs 3.0 [0.8], \( P = .001 \)).

**CONCLUSIONS AND RELEVANCE** Treatment with metformin and pioglitazone has beneficial anti-inflammatory effects in patients with MS and MetS and should be further explored.
Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease that affects the central nervous system. Although the pathogenesis of MS is not yet fully understood, there is considerable evidence to suggest it is an autoimmune disease mediated mainly, but not exclusively, by Th1 and Th17 lymphocytes. It is generally accepted that autoimmune diseases such as MS arise from complex interactions between genetic susceptibility and environmental factors. In recent decades, the incidence of autoimmune diseases, including MS, has increased dramatically in Western countries. Given the short time in which these changes in disease distribution have occurred worldwide, genetic factors seem an unlikely cause. Several epidemiologic studies have linked the risk of MS with environmental and lifestyle factors. During the same period, obesity levels have followed the same pattern of distribution as a result of more sedentary habits and changing dietary trends.

Metabolic syndrome (Mets) is a common feature of obesity in which a cluster of conditions, including increased blood pressure, atherogenic dyslipidemia, and insulin resistance, occur together, thereby ultimately increasing the risk of heart disease, stroke, and diabetes. The rising prevalence of both autoimmune diseases and obesity has led to a search for underlying mechanisms that have something in common. Indeed, evidence suggests that obesity during childhood and adolescence may influence later development of MS. A variety of adipokines, which are involved in the regulation of immunity and inflammation, are produced by adipose tissue. These mediators are secreted by adipocytes and by different immune cells. Several adipokines, such as leptin and adiponectin, participate in a wide range of biological functions to strengthen the link between immune function, metabolism, and nutrition. Leptin was initially identified as a major regulator of food intake and energy expenditure via the neuroendocrine system. In addition, leptin has been shown to influence immune function by favoring the secretion of interleukin (IL)-1 and tumor necrosis factor (TNF), as well as differentiation of Th1 cells. Plasma levels of leptin are elevated in obese individuals and associated with proinflammatory conditions and autoimmunity in humans. In contrast, plasma levels of adiponectin, consistently shown to be an anti-inflammatory adipokine, are decreased in obese individuals. Although the immune system can be influenced by adipocytes, the reverse also holds true; namely, proinflammatory cytokines, such as TNF and IL-6 released from lymphocytes or by activated macrophages residing within the adipose tissue, can cause development of insulin resistance by impairing adipocyte metabolism and insulin signal transduction. These observations suggest a link between metabolism and autoimmunity. This study evaluates the effects of metformin hydrochloride and pioglitazone hydrochloride, 2 compounds used in the treatment of Mets, on immune regulation in patients with MS.

**Methods**

**Patient Selection and Study Design**

Fifty obese (body mass index ≥30; calculated as weight in kilograms divided by height in meters squared) patients with a diagnosis of clinically definite relapsing-remitting MS according to the 2010 McDonald criteria who also developed Mets were included in this study, conducted from March 1, 2012, to December 30, 2014. Metabolic syndrome was defined according to modified World Health Organization criteria as the presence of hyperinsulinemia or hyperglycemia (fasting glucose level ≥110 mg/dL [to convert to millimoles per liter, multiply by 0.0555]) in addition to at least 2 of the following: waist circumference of 94 cm or more, dyslipidemia (triglyceride level ≥150 mg/dL [to convert to millimoles per liter, multiply by 0.0113] or high-density lipoprotein cholesterol level <40 mg/dL [to convert to millimoles per liter, multiply by 0.0259]), or blood pressure of 140/90 mm Hg or higher or requiring antihypertensive medication. Twenty patients received metformin hydrochloride, 850 to 1500 mg/d, and 10 patients received pioglitazone hydrochloride, 15 to 30 mg/d. Twenty patients who opted not to receive treatment for Mets were included as a control group. No patients had been prescribed corticosteroids for at least 6 months prior to study entry.

Patients were subjected to a comprehensive neurologic examination every 3 months, including physical assessment of disease activity and estimation of Expanded Disability Status Scale score. Brain magnetic resonance imaging was performed at 6-month intervals on a 1.5-T Sigma unit (General Electric). Axial slices, 5 mm thick, were obtained with T2-weighted, proton density, fast spin-echo, fluid-attenuated inversion recovery, and T1-weighted sequences before and after administration of gadolinium diethylenetriamine pentaacetic acid, 0.1 mmol/kg. Postcontrast images were completed within 15 minutes of injection with gadolinium.

Immunologic evaluations were performed during the last 18 to 24 months of follow-up. Serum samples for adipokine assay were collected at baseline (time 0) and after 18 months of treatment. The study protocol was approved by the Institute for Neurological Research Dr Raúl Carrea Institutional Ethics Committee, and written informed consent was obtained from all participants.

**Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis**

For quantitative assessment of relative messenger RNA levels, total RNA was prepared using TRIzol LS reagent (Invitrogen) fol-
lowing manufacturer instructions. The RNA was reverse transcribed using an M-MLV RT reverse transcription kit with random hexamer primers (Invitrogen). Relative levels of 5′-adenosine monophosphate–activated protein kinase (AMPK) and peroxisome proliferator–activated receptor γ (PPARγ) messenger RNA were determined by real-time polymerase chain reaction using an ABI 7000 sequence detection system (Applied Biosystems). Values obtained were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase. Primer sequences used were as follows: glyceraldehyde 3-phosphate dehydrogenase, forward 5′-CGGAAGAGTGTCTGGAGCAA-3′, reverse 5′-GAAGATGGT-GATGGGATTTC-3′; PPARγ forward 5′-AAAGAAGCGACAC-TAAACC-3′, reverse 5′-CTTCATTACGGAGA GATCC-3′; AMPK forward 5′-CGGAAGAGTGTCTGGAGCAA-3′, reverse 5′-GGATGAAGCGAGTCTGGA-3′.

Serum Adipokine Assay
Serum samples for leptin and adiponectin level assays were drawn between 8 and 10 AM and measured using commercially available enzyme-linked immunosorbent assay kits following manufacturer instructions (R&D Systems). Assay sensitivity levels were 8 pg/mL for leptin and 0.5 ng/mL for adiponectin. Intra-assay and interassay coefficients of variation levels were less than 7% and 6.5%, respectively.

Quantification of Secreted Cytokines
The number of peripheral blood mononuclear cells (PBMCs) secreting IL-4, IL-6, IL-10, IL-12, IL-17, interferon (IFN) γ, and TNF was measured using commercially available kits for single-cell resolution enzyme-linked immunospot (ELISPOT) assay as described elsewhere. The ELISPOT detection kits for IL-4, IL-6, IL-10, IL-17, and IFN-γ were purchased from R&D Systems, and the kits for TNF and IL-12 from Abcam.

Evaluation of CD4+CD25+FoxP3+ Regulatory T Cells
The number of CD4+CD25+FoxP3+ regulatory T (Treg) cells was evaluated by flow cytometry using commercially available regulatory Treg cell staining kits and following manufacturer instructions (eBioscience).

Small Interfering RNA Technique
In some experiments, IL-10 gene (OMIM 124092) expression was silenced using small, interfering RNA (siRNA). An IL-10 siRNA targeting the specific sequence ATAAAGCTCCAAGAGA-AAGGC was selected. The double-stranded RNA consisted of the sense strand 5′-UAAGCUCCAAGAGAAAGGcdTdT-3′ and the antisense strand 3′-dTdTAUUCCAGGUGUCCUUUUCG-5′. For the nonsilencing control, an irrelevant siRNA with random antisense strand 3′-dTdTAUUCGAGGUUCUUUCCG-5′. For the sense strand 5′-UAAGCUCCAAGAGAAAGGCdTdT-3′ and the AAGGC was selected. The double-stranded RNA consisted of siRNA targeting the specific sequence ATAAGCTCCAAGAGA-5′.

Statistical Analysis
Data analysis occurred from January 10 to March 31, 2015. Clinical and demographic data as well as immunologic variables between patients receiving treatment and the control group were compared using the Mann-Whitney test. Paired t tests were used to compare within-group mean differences at baseline and at 24 months of follow-up. P < .05 (2-tailed) was considered significant.

Results

Patient Characteristics
Fifty obese (BMI ≥30) patients with relapsing-remitting MS and MetS were selected as the study group and prospectively followed up for a mean (SD) of 26.7 (2.7) months (range, 24-33 months). Twenty patients were treated with metformin, 10 received pioglitazone, and 20 received no treatment (control group). Demographic and clinical data are shown in the Table. No differences in demographics or clinical characteristics (sex ratio, age, disease duration, Expanded Disability Status Scale score, annual relapse rate, percentage of patients receiving disease-modifying therapy, or BMI at baseline) were observed between groups. All patients completed at least 24 months of follow-up.

Lesions as Measured by Brain Magnetic Resonance Imaging
Two years before starting treatment with metformin or pioglitazone, there were no significant differences between the 3 study cohorts in the number of new or enlarging T2 lesions or gadolinium-enhancing lesions as confirmed by brain magnetic resonance imaging (MRI) (Figure 1). After receiving treatment, patients who received metformin and those who received pioglitazone showed a significant decrease in the number of new or enlarging T2 lesions on brain MRI compared with the control group and with MRIs performed 2 years before the start of the study (Figure 1A). The decrease in the number of new or enlarging T2 lesions was evident even after only 6 months of treatment. The lowest mean (SD) number of new or enlarging T2 lesions occurred after 18 months of treatment (metformin, 0.5 [0.7]; pioglitazone, 0.6 [0.8]) and remained low until the end of the 2-year follow-up period. There were no significant differences between the number of new or enlarging T2 lesions between patients receiving metformin and those receiving pioglitazone.

Effects of metformin or pioglitazone treatment on gadolinium-enhancing lesions were similar to those observed with T2 lesions. After beginning treatment with metformin or pioglitazone, the number of gadolinium-enhancing lesions decreased significantly compared with the control group and with the preceding 2 years. The lowest mean (SD) number of gadolinium-enhancing lesions were observed after 24 months of treatment (metformin, 0.1 [0.3]; pioglitazone, 0.3 [0.6]) (Figure 1B).

No significant differences between groups in annualized relapse rate or disability as determined by Expanded Disability Status Scale score were observed at 24 months of follow-up (P = .82 and P = .73, respectively). The study was not powered to detect treatment effects on these outcomes. Nevertheless, fasting glucose levels, fasting insulin levels, insulin resistance as estimated by homeostatic model assessment, glycosylated hemoglobin levels, total cholesterol levels, low-density lipoprotein cholesterol levels, triglyceride levels, and systolic blood pressure all declined significantly (P = .03 to P < .001) in both the metformin- and pioglitazone-treated groups after 12 months of follow-up (eTable in the Supplement). Other measures that improved for both treatment groups included increased high-density lipoprotein
Immunologic Effects of Metformin and Pioglitazone

To assess possible mechanisms through which metformin and pioglitazone reduced inflammatory activity as measured by brain MRI, serum levels of leptin and adiponectin were determined. Leptin and adiponectin are adipokines exerting opposite immunologic effects: while leptin has been shown to be proinflammatory, adiponectin is mainly anti-inflammatory.²¹ The PBMCs in patients receiving metformin showed a marked increase in AMPK messenger RNA expression compared with controls (Figure 2A). Previous studies had shown that activators of AMPK downregulate inflammation in vitro and in vivo in different animal models.²⁰⁻²³ The PBMCs from patients who received pioglitazone showed a significant activation of the transcription factor PPARY (Figure 3A), which has been recognized as playing an important role in immune responses, through its ability to inhibit inflammatory cytokine expression and to redirect immune cell differentiation toward anti-inflammatory phenotypes.²⁴⁻²⁶

In addition, metformin treatment resulted in a significant decrease in mean (SD) serum leptin levels vs controls (5.5 [2.4] vs 10.5 [3.4] ng/mL; P < .001) (Figure 2B). In contrast, mean (SD) adiponectin levels in patients receiving metformin were significantly greater than those of controls (15.4 [5.5] vs 4.5 [2.4] μg/mL; P < .001) (Figure 2C). Likewise, pioglitazone treatment also resulted in marked suppression of mean (SD) leptin levels vs controls (4.1 [0.8] vs 11.0 [2.6] ng/mL; P < .001) (Figure 2B) and increased adiponectin levels compared with the control group (12.6 [3.6] vs 4.8 [0.6] μg/mL; P < .001) (Figure 3C). Notably, serum levels of leptin and adiponectin at baseline did not differ between patient groups (P = .28 to P = .55). Both metformin and pioglitazone treatment led to a significant decrease in leptin serum levels and cholesterol levels (eTable in the Supplement). Adherence to disease-modifying therapy throughout the study was 85% or greater in all groups.

Table. Baseline Characteristics of Patients With Multiple Sclerosis With and Without MetS Treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Valuea</th>
<th>Treated With Metformin Hydrochloride (n = 20)</th>
<th>Treated With Pioglitazone Hydrochloride (n = 10)</th>
<th>No Treatment (n = 20)</th>
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<tr>
<td>Female sex</td>
<td>14 (70.0)</td>
<td>7 (70.0)</td>
<td>14 (70.0)</td>
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<tr>
<td>Age, mean (SD), y</td>
<td>34 (5.8)</td>
<td>32 (5.3)</td>
<td>34 (5.9)</td>
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</tr>
<tr>
<td>Duration of disease, mean (SD), y</td>
<td>6.5 (2.5)</td>
<td>6.3 (2.3)</td>
<td>6.7 (2.5)</td>
<td></td>
</tr>
<tr>
<td>EDSS score, mean (SD)</td>
<td>2.7 (1.2)</td>
<td>2.5 (1.5)</td>
<td>2.6 (1.3)</td>
<td></td>
</tr>
<tr>
<td>Annual relapse rate 2 y before entry, mean (SD)</td>
<td>0.8 (0.49)</td>
<td>0.7 (0.52)</td>
<td>0.8 (0.67)</td>
<td></td>
</tr>
<tr>
<td>Any DMT</td>
<td>16 (80.0)</td>
<td>8 (80.0)</td>
<td>15 (75.0)</td>
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<tr>
<td>Interferon β</td>
<td>10 (62.5)</td>
<td>5 (62.5)</td>
<td>9 (60.0)</td>
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<tr>
<td>Glatiramer acetate</td>
<td>5 (31.3)</td>
<td>3 (37.5)</td>
<td>5 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Natalizumab</td>
<td>1 (6.3)</td>
<td>0</td>
<td>1 (6.7)</td>
<td></td>
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<tr>
<td>BMI, mean (SD)</td>
<td>37.7 (10.8)</td>
<td>36.3 (12.9)</td>
<td>38.9 (15.5)</td>
<td></td>
</tr>
<tr>
<td>Baseline No. of new or enlarging T1-weighted images, mean (SD)</td>
<td>2.5 (0.6)</td>
<td>2.3 (0.5)</td>
<td>2.4 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Baseline No. of Gd-enhancing lesions on T1-weighted images, mean (SD)</td>
<td>1.8 (0.6)</td>
<td>2.2 (0.6)</td>
<td>2.3 (0.8)</td>
<td></td>
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<tr>
<td>Patients with no Gd-enhancing lesions on T1-weighted images, No./Total No.</td>
<td>7 of 20 (35.0)</td>
<td>3 of 10 (30.0)</td>
<td>8 of 20 (40.0)</td>
<td></td>
</tr>
<tr>
<td>Glycosylated hemoglobin, %, mean (SD)</td>
<td>7.3 (1.0)</td>
<td>7.5 (1.8)</td>
<td>7.7 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose, mg/dL, mean (SD)</td>
<td>118 (28)</td>
<td>119 (36)</td>
<td>113 (26)</td>
<td></td>
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<tr>
<td>Fasting insulin, μIU/mL, mean (SD)</td>
<td>25.3 (8.9)</td>
<td>23.8 (7.4)</td>
<td>22.6 (6.3)</td>
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<tr>
<td>HOMA-IR, mean (SD)²¹</td>
<td>7.5 (1.7)</td>
<td>7.3 (2.9)</td>
<td>6.7 (2.8)</td>
<td></td>
</tr>
<tr>
<td>Blood pressure, mm Hg, mean (SD)</td>
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<tr>
<td>Systolic</td>
<td>122 (5.2)</td>
<td>121 (6.1)</td>
<td>119 (6.7)</td>
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</tr>
<tr>
<td>Diastolic</td>
<td>78 (4.4)</td>
<td>77 (5.7)</td>
<td>74 (4.3)</td>
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<tr>
<td>Cholesterol, mg/dL, mean (SD)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>198 (48)</td>
<td>202 (43)</td>
<td>197 (57)</td>
<td></td>
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<tr>
<td>LDL</td>
<td>114 (32)</td>
<td>119 (51)</td>
<td>121 (38)</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>42 (13)</td>
<td>43 (18)</td>
<td>45 (27)</td>
<td></td>
</tr>
<tr>
<td>Triglycerides, mg/dL, mean (SD)</td>
<td>155 (39)</td>
<td>152 (43)</td>
<td>158 (51)</td>
<td></td>
</tr>
<tr>
<td>Dyslipidemia²</td>
<td>8 (40.0)</td>
<td>4 (40.0)</td>
<td>9 (45.0)</td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>9 (45.0)</td>
<td>5 (50.0)</td>
<td>10 (50.0)</td>
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<tr>
<td>Cardiovascular disease²</td>
<td>3 (15.0)</td>
<td>1 (10.0)</td>
<td>2 (10.0)</td>
<td></td>
</tr>
<tr>
<td>Treated with antihypertensive drugs</td>
<td>9 (45.0)</td>
<td>5 (50.0)</td>
<td>9 (45.0)</td>
<td></td>
</tr>
<tr>
<td>Treated with lipid-lowering drugs</td>
<td>8 (40.0)</td>
<td>3 (30.0)</td>
<td>8 (40.0)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); DMT, disease-modifying therapy; EDSS, Expanded Disability Status Scale; Gd, gadolinium diethylenetriamine penta-acetic acid; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment estimation of insulin resistance; LDL, low-density lipoprotein; MetS, metabolic syndrome.

SI conversion factors: to convert glycosylated hemoglobin to convert to proportion of total hemoglobin, multiply by 0.01; glucose to millimoles per liter, multiply by 0.0555; insulin to picomoles per liter, multiply by 6.945; total cholesterol, HDL cholesterol, and LDL cholesterol to millimoles per liter, multiply by 0.0259; and triglycerides to millimoles per liter, multiply by 0.0113.

Data are presented as number (percentage) of patients unless otherwise indicated.

²¹HOMA-IR was calculated as: fasting glucose (millimoles per liter) × fasting insulin (microunits per milliliter)/22.5.

²²Cutoff values for dyslipidemia were an HDL cholesterol level of 40 mg/dL or lower or a triglyceride level of 150 mg/dL or higher.

²³Cutoff values for hypertension were a systolic blood pressure above 140 mm Hg or a diastolic pressure of 90 mm Hg or higher.

²⁴Cardiovascular disease includes ischemic heart disease, cardiac failure, central nervous system hemorrhages, cerebrovascular conditions, and embolic and thrombotic events.
**Figure 1. Disease Activity as Measured by Magnetic Resonance Imaging**

A, Both metformin hydrochloride and pioglitazone hydrochloride were associated with a significant decrease in the number of new or enlarging T2 lesions in comparison with lesions observed 2 years earlier, and with the control group not receiving metformin or pioglitazone. B, Both metformin and pioglitazone were associated with a significant decrease in the number of gadolinium diethylenetriamine penta-acetic acid (Gd)–enhancing lesions in comparison with lesions observed 2 years earlier, and with the control group not receiving metformin or pioglitazone.

**Figure 2. Immunologic Effects of Metformin in Patients With Multiple Sclerosis**

A, Metformin hydrochloride treatment resulted in a robust increase of 5′ adenosine monophosphate–activated protein kinase (AMPK) expression. B, Increased AMPK expression was associated with a significant decrease in leptin levels. C, Increased AMPK expression was associated with a significant increase in adiponectin levels. D, Increased AMPK expression was associated with a decrease in the numbers of cells producing interferon (IFN) γ. E, Increased AMPK expression was associated with a decrease in the numbers of cells producing interleukin (IL)–17. F, Increased AMPK expression was associated with a robust increase in CD4+CD25+FoxP3+ regulatory T (Treg) cell percentages. Data are expressed as AMPK messenger RNA (mRNA) levels relative to glyceraldehyde 3-phosphate dehydrogenase, shown as mean (SE) of mRNA expression in peripheral blood mononuclear cells (PBMCs) from 20 patients treated with metformin and 18 control patients for whom samples were available (A). Results correspond to the number of cytokine-secreting cells specific for myelin basic protein (MBP38-52) per 10⁶ PBMCs as determined by enzyme-linked immunospot assays, from patients treated with metformin and control patients (C and D). The number of antigen-specific cytokine-secreting cells was calculated by subtracting the number of spots obtained in zero antigen background control cultures from the number of spots obtained in cultures exposed to stimulating antigen. Each circle represents values from a single individual. Horizontal lines indicate mean group values. Pioglitazone was given as pioglitazone hydrochloride.
Effects of Metformin and Pioglitazone in Metabolic Syndrome and MS

Figure 3. Immunologic Effects of Pioglitazone in Patients With Multiple Sclerosis

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<thead>
<tr>
<th></th>
<th>Metformin</th>
<th>Pioglitazone</th>
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<tr>
<td>Relative PPAR-γ mRNA Expression</td>
<td><img src="image" alt="" /></td>
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<tr>
<td>Leptin Levels, ng/mL</td>
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<tr>
<td>Adiponectin Levels, μg/mL</td>
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<tr>
<td>IL-6 Producing Cells/10^5 PBMC</td>
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<tr>
<td>TNF-α Producing Cells/10^5 PBMC</td>
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<tr>
<td>CD4+CD25+FoxP3+ Treg Cells, %</td>
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A, Pioglitazone hydrochloride treatment induced significant activation of peroxisome proliferator-activated receptor-γ (PPAR-γ). B, Activation of PPAR-γ was associated with a significant decrease in leptin levels. C, Activation of PPAR-γ was associated with a significant increase in adiponectin levels. D, Activation of PPAR-γ was associated with a decrease in the numbers of cells producing interleukin (IL)-6. E, Activation of PPAR-γ was associated with a decrease in the numbers of cells producing tumor necrosis factor (TNF). F, Activation of PPAR-γ was associated with a robust increase in CD4+CD25+FoxP3+ regulatory T (Treg) cell percentages. Data are expressed as PPAR-γ messenger RNA (mRNA) levels relative to glyceraldehyde 3-phosphate dehydrogenase, shown as mean (SE) of mRNA expression in peripheral blood mononuclear cells (PBMCs) from 10 patients receiving pioglitazone and 14 control patients for whom samples were available (A). Results correspond to the number of cytokine-secreting cells specific for myelin basic protein (MBP)83-102 per 10^5 PBMCs as determined by enzyme-linked immunospot assays, from patients treated with pioglitazone and control patients. The number of antigen-specific cytokine-secreting cells was calculated by subtracting the number of spots obtained in zero antigen background control cultures from the number of spots obtained in cultures exposed to stimulating antigen. Each circle represents values from a single individual. Horizontal lines indicate mean group values. Metformin was given as metformin hydrochloride.

To a marked increase in adiponectin serum levels after 18 months of treatment compared with baseline values (P = .003 and P = .002, respectively).

To test whether metformin and pioglitazone influenced antigen-specific T-cell phenotype during the course of MS, PBMC cytokine production was characterized using ELISPOT assays. In addition, numbers of FoxP3+ Treg cells were evaluated. As shown in Figure 2D and E, numbers of myelin basic protein (MBP) peptide-specific cells secreting IFN-γ and IL-17 were significantly lower in samples collected from metformin-treated patients compared with those from the control group (P < .001). Production of IL-4, IL-6, IL-10, IL-12, and TNF was similar in patients receiving metformin and controls. Furthermore, as illustrated in Figure 3D and E, patients receiving pioglitazone showed significantly lower numbers of MBP peptide-specific cells secreting IL-6 and TNF compared with controls (P < .001). Production of IL-4, IL-10, IL-12, IL-17, and IFN-γ did not differ between patients receiving pioglitazone and controls. Similar results were observed when PBMCs were stimulated with either MBP<sub>83-102</sub> or MBP<sub>143-168</sub>.

In parallel with these findings, both metformin and pioglitazone treatment resulted in a significant increase in the percentage and absolute numbers of CD4+CD25+FoxP3+ Treg cells compared with the control group (P = .001) (Figures 2F and 3F). Regulatory properties of CD4+CD25+ Treg cells isolated from patients treated with metformin or pioglitazone were further investigated by testing their ability to suppress the proliferative responses of CD4+CD25+ MBP peptide-specific T cells (effector T cells). To do this, we stimulated effector T cells with the cognate peptide or with anti-CD3 monoclonal antibody and added increasing numbers of autologous CD4+CD25+ Treg cells. Co-culture of Treg cells with effector cells resulted in dose-dependent inhibition of effector T-cell proliferation. As illustrated in Figure 4A, with a 2:1 co-culture ratio of effector T to Treg cells, 21% inhibition of peptide-specific T-cell proliferation was observed when using Treg cells from controls, compared with 48% and 56% inhibition when using Treg cells from patients receiving metformin or pioglitazone, respectively. Similar results were observed when MBP peptide-specific T-cell lines were stimulated with either the cognate antigen or anti-CD3 monoclonal antibody. Secretion of IL-10 was significantly higher in CD4+CD25+ Treg cells from patients receiving metformin or pioglitazone compared with the control group (Figure 4B). In contrast, transforming growth factor-β secretion was not significantly different between groups. In the next experiments, we aimed to assess whether inhibi-
Moreover, there is evidence of anti-inflammatory properties and levels in inverse correlation with adipose tissue mass and its levels are increased in obese individuals and patients with MetS. It exerts proinflammatory responses by increasing CD4+ T-cell proliferation, promoting cell differentiation to a Th1 lymphocyte profile, inhibiting Treg cell proliferation, stimulating phagocytosis by macrophages, and inducing neutrophil chemotaxis and release of oxygen radicals. leptin-deficient ob/ob mice are resistant to development of both passively and actively induced experimental autoimmune encephalomyelitis (EAE), a resistance reverted by the administration of exogenous leptin. Moreover, administration of leptin to EAE-susceptible mice worsens the clinical course of the disease and antileptin antibodies ameliorate it. Furthermore, leptin levels tend to increase as animals lose weight, preceding onset of EAE, while T lymphocytes secrete leptin in active EAE brain lesions. Likewise, serum leptin levels are increased in patients with MS and inversely correlate with Treg cell numbers.

In contrast, adiponectin has shown anti-inflammatory properties and levels in inverse correlation with adipose tissue mass. Adiponectin inhibits both expression of adhesion molecules induced by TNF and proliferation and phagocytic activity of macrophages and decreases TNF and IL-6 production while increasing IL-10 secretion and Treg cell numbers. Interestingly, stimulation of adiponectin receptors (AdipoR1 and AdipoR2) mediates the inhibitory effects of adiponectin on their target cells, indicating that adiponectin may exert its effects through the activation of these receptors. In addition, adiponectin upregulates the expression of anti-inflammatory cytokines such as IL-10 and downregulates the expression of pro-inflammatory cytokines such as TNF and IL-6. These effects are believed to be mediated by the activation of nuclear factor kappa B (NF-κB) and inhibition of the activation of the transcription factor STAT3.

Discussion

In recent years, the concept that white adipose tissue was exclusively an energy storage site has shifted toward recognition of an endocrine role for white adipose tissue, mediated by production of different adipokines. Obesity leading to dysregulation between proinflammatory and anti-inflammatory adipokines is implicated in the development of metabolic complications of obesity, such as MetS. Leptin and adiponectin have been identified as relevant factors in the relationship between obesity, MetS, and autoimmune diseases.

The prevalence of MetS seems to be higher among patients with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. Interestingly, the presence of MetS is associated with the severity of autoimmune diseases, suggesting a relationship between the chronic inflammatory state in MetS and autoimmune diseases. Furthermore, there is evidence of anti-inflammatory properties of medications commonly used in the treatment of MetS.

Our study showed that both metformin and pioglitazone reduced MS disease activity as measured by brain MRI in patients with MetS. Interestingly, although a large number of patients received disease-modifying therapy, they also presented with highly active disease before enrollment in the study, suggesting a significant effect of MetS on the disease course. Metformin and pioglitazone treatments resulted in decreased leptin serum levels and increased adiponectin levels, mediated by induction of AMPK and PPARγ, respectively. Moreover, both treatments decreased the secretion of proinflammatory cytokines by PBMCs and increased the numbers and regulatory properties of Treg cells.

Leptin is considered a key element in the interplay between metabolism and immunity. Secretion of leptin is proportional to adipose tissue mass and its levels are increased in obese individuals and patients with MetS. It exerts proinflammatory responses by increasing CD4+ T-cell proliferation, promoting cell differentiation to a Th1 lymphocyte profile, inhibiting Treg cell proliferation, stimulating phagocytosis by macrophages, and inducing neutrophil chemotaxis and release of oxygen radicals. In line with these observations, leptin-deficient ob/ob mice are resistant to development of both passively and actively induced experimental autoimmune encephalomyelitis (EAE), a resistance reverted by the administration of exogenous leptin. Moreover, administration of leptin to EAE-susceptible mice worsens the clinical course of the disease and antileptin antibodies ameliorate it. Furthermore, leptin levels tend to increase as animals lose weight, preceding onset of EAE, while T lymphocytes secrete leptin in active EAE brain lesions. Likewise, serum leptin levels are increased in patients with MS and inversely correlate with Treg cell numbers.

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and AdipoR2) leads to activation of AMPK and PPARγ, among other molecules, similar to effects caused by metformin and pioglitazone treatment.27 Adiponectin must therefore limit autoimmune inflammation in the central nervous system in some fashion since mice lacking adiponectin exhibit worse clinical and histologic EAE than do controls, and treatment with adiponectin ameliorates disease.35 Little evidence exists on adiponectin levels in patients with MS. Some authors found decreased adiponectin serum levels in patients with MS.31,36 Our study showed no baseline differences in leptin or adiponectin levels between patients with MS and controls. However, leptin levels were significantly reduced and adiponectin levels increased after metformin or pioglitazone treatment, possibly one of the underlying mechanisms accounting for the reduction in disease activity observed on brain MRI with these treatments.

The PPARγ agonists reduce the incidence of EAE and ameliorate its symptoms by suppressing T-cell activation and decreasing expression of inflammatory genes.24 The PPARγ agonists also exert anti-inflammatory and antiproliferative effects on T lymphocytes from patients with MS.37 In line with these findings, daily treatment with pioglitazone in 2 small cohorts of patients with relapsing-remitting MS showed reduction of lesion burden as measured by fluid-attenuated inversion recovery, fractional anisotropy changes on diffusion-weighted magnetic resonance tensor images, and gray matter atrophy.38,39 Furthermore, in vitro evidence indicates that PPARγ agonists protect neurons and axons from nitric oxide- and hydrogen peroxide–induced damage.25

Meanwhile, metformin treatment attenuates the clinical course of EAE by decreasing Th1 and Th17 lymphocyte cytokine production and increasing IL-10–secreting Treg cell numbers20 and protects oligodendrocytes by enhancing the production of neurotrophic factors.21 Anti-inflammatory effects of metformin have also been shown in murine models of rheumatoid arthritis22 and colitis.23

Despite increasing evidence to support the anti-inflammatory and neuroprotective effects of PPARγ and AMPK agonists, there has not been much research exploring potential beneficial clinical effects of MetS therapies in autoimmune diseases, including MS. A proof-of-concept trial did show reduction of clinical flare-up in patients with systemic lupus erythematosus who were treated with metformin.40 Another small, randomized clinical trial evaluating pioglitazone as add-on therapy in patients with rheumatoid arthritis showed dampening of disease activity.41 In a pilot double-blind randomized clinical trial comparing pioglitazone with placebo in 70 patients with psoriasis, improvement was observed in the patients who received pioglitazone.42

Supporting the anti-inflammatory effects described for MetS treatments, our results show that metformin decreases the number of MBP peptide–specific cells secreting IFN-γ and IL-17 and pioglitazone decreases the number of MBP peptide–specific cells secreting IL-6 and TNF in patients with MS and MetS. Finally, it is well known that Treg cells are crucial for self-tolerance and that their dysregulation is involved in the pathogenesis of several autoimmune diseases.33 A decrease in visceral fat and circulating Treg cells has been observed in obese individuals, probably contributing to a chronic inflammatory state.44 Our data show an increase in the number and function of Treg cells in patients with MS and MetS who were treated with metformin or pioglitazone, further supporting the anti-inflammatory effects of these treatments.

Limitations to this investigation include the small number of patients and the nonrandomized open-label design, making it susceptible to potential sources of confounding and bias.

Conclusions
Collectively, these observations provide both in vitro and in vivo evidence that metformin and pioglitazone treatment regulate immune and inflammatory responses and support recommendation of weight reduction in obese patients with MS. Although these findings need to be interpreted with caution, it is nevertheless a study based on initial exploratory assessments. Further investigation in the field is warranted to improve the management of MS and treatment results.

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