Metformin Does Not Reduce Markers of Cell Proliferation in Esophageal Tissues of Patients With Barrett's Esophagus

Amitabh Chak,[*] Navtej S. Buttar,[‡] Nathan R. Foster,[§] Drew K. Seisler,[§] Norman E. Marcon,[¶] Robert Schoen,[#] Marcia R. Cruz-Correa,[**] Gary W. Falk,[‡‡] Prateek Sharma,[§§] Chin Hur,[|||] David A. Katzka,[‡] Luz M. Rodriguez,[¶¶] Ellen Richmond,[¶¶] Anamay N. Sharma,[‡] Thomas C. Smyrk,[||] Sumithra J. Mandrekar,[§] and Paul J. Limburg,[‡] for the Cancer Prevention Network

[*]Department of Gastroenterology and Hepatology, University Hospitals Case Medical Center, Cleveland, Ohio; [‡]Department of Gastroenterology and Hepatology, [§]Biomedical Statistics and Informatics, and [||]Anatomic Pathology, Mayo Clinic, Rochester, Minnesota; [¶]Department of Medicine, Gastroenterology, St Michael's Hospital, Toronto, Ontario, Canada; [#]Department of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh, Pittsburgh, Pennsylvania; [**]Department of Gastroenterology Oncology at UPR Cancer Center, University of Puerto Rico, San Juan, Puerto Rico; [‡‡]Department of Gastroenterology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania; [§§]Department of Gastroenterology, Hepatology and Motility, Kansas City VA, Kansas City, Missouri; [|||]Gastroenterology, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts; and [¶¶]Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

BACKGROUND & AIMS: **Obesity is associated with neoplasia, possibly via insulin-mediated cell pathways that affect cell proliferation. Metformin has been proposed to protect against obesity-associated cancers by decreasing serum insulin. We conducted a randomized, double-blind, placebo-controlled, phase 2 study of patients with Barrett's esophagus (BE) to assess the effect of metformin on phosphorylated S6 kinase (pS6K1), a biomarker of insulin pathway activation.**

METHODS: **Seventy-four subjects with BE (mean age, 58.7 years; 58 men [78%; 52 with BE >2 cm [70%]) were recruited through 8 participating organizations of the Cancer Prevention Network. Participants were randomly assigned to groups given metformin daily (increasing to 2000 mg/day by week 4, n = 38) or placebo (n = 36) for 12 weeks. Biopsy specimens were collected at baseline and at week 12 via esophagogastroduodenoscopy. We calculated and compared percent changes in median levels of pS6K1 between subjects given metformin vs placebo as the primary end point.**

RESULTS: **The percent change in median level of pS6K1 did not differ significantly between groups (1.4% among subjects given metformin vs −14.7% among subjects given placebo; 1-sided $P = .80$). Metformin was associated with an almost significant reduction in serum levels of insulin (median −4.7% among subjects given metformin vs 23.6% increase among those given placebo, $P = .08$) as well as in homeostatic model assessments of insulin resistance (median −7.2% among subjects given metformin vs 38% increase among those given placebo, $P = .06$). Metformin had no effects on cell proliferation (on the basis of assays for KI67) or apoptosis (on the basis of levels of caspase 3).**

CONCLUSIONS: **In a chemoprevention trial of patients with BE, daily administration of metformin for 12 weeks, compared with placebo, did not cause major reductions in esophageal levels of pS6K1. Although metformin reduced serum levels of insulin and insulin resistance, it did not discernibly alter epithelial proliferation or apoptosis in esophageal tissues. These findings do not support metformin as a chemopreventive agent for BE-associated carcinogenesis. ClinicalTrials.gov number, NCT01447927.**

Keywords: HOMA-IR; Diabetes Drug; Cancer Development; Tumorigenesis.

Abbreviations used in this paper: **AE, adverse event; AMP, adenosine monophosphate; AMPK, adenosine monophosphate–activated protein kinase; BE, Barrett's esophagus; BMI, body mass index; EAC, esophageal adenocarcinoma; EGD, esophagogastroduodenoscopy; HOMA-IR, homeostatic model of insulin resistance; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IGF-1R, insulin-like growth factor-1 receptor; IR, insulin receptor; mTOR, mammalian target of rapamycin; NSAID, nonsteroidal anti-inflammatory drug; pS6K1, phosphorylated S6 kinase 1.**

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Obesity has been linked to a variety of malignancies.[1–4] Recent studies suggest that one explanation for the role of obesity in the development of cancer is activation of the insulin/insulin-like growth factor (IGF) pathway.[5–7] A diet high in energy, high in animal fat, and low in fiber in combination with physical inactivity contributes to insulin resistance and resulting hyperinsulinemia. Complex interactions of increased levels of insulin, IGF-1, and members of the serum IGF binding protein (IGFBP) family (IGFBP1 through IGFBP6) determine the levels of insulin and IGF that are available to mediate effects at the cellular level through the insulin receptor (IR) and the IGF-1 receptor (IGF-1R).[3,6–8] Activation of IR and IGF-1R stimulates cellular proliferation and inhibits apoptosis via molecular pathways that are mediated by PI3K, AKT, mammalian target of rapamycin (mTOR), S6K1, and other signaling molecules.

Central adiposity is a risk factor that is independently and consistently associated with Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC).[9] Activation of the insulin/IGF pathway is associated with Barrett's-mediated carcinogenesis.[10,11] Metformin is an insulin sensitizer commonly used to treat diabetes mellitus. It lowers serum insulin levels and directly inhibits cell growth. Besides inhibiting gluconeogenesis, this biguanide derivative activates adenosine monophosphate (AMP)–activated protein kinase (AMPK) in epithelial cells by an LKB-dependent mechanism. AMPK appears to be a key target for cancers associated with diabetes mellitus and obesity.[6,12] Activation of AMPK by metformin increases insulin-stimulated glucose uptake and inhibits mTOR via TSC2/1, resulting in decreased protein synthesis mediated by the down-regulation of ribomosomal protein S6 kinase 1 (S6K1). This decrease in phosphorylated S6K1 (pS6K1) inhibits cell proliferation. Metformin also has AMPK-independent, indirect antiproliferative effects related to lower systemic levels of insulin. Recent studies have shown its potential as a cancer prevention drug in other common obesity-associated cancers.[13–18]

The prognosis for EAC patients has remained poor, with the large majority dying of cancer-related causes within 5 years.[19] Novel interventions, such as chemoprevention in BE, are a high research priority. The goal of this study was to investigate the potential for metformin as a chemoprevention agent by determining its effect on phosphorylated ribosomal S6K in Barrett's epithelium.

Methods

All aspects of the study protocol were reviewed and approved by the appropriate Institutional Review Board for human research at each participating site. Mayo Clinic in Rochester, MN served as the coordinating research base. The Data and Safety Monitoring Board of the Mayo Clinic Cancer Center reviewed safety data every 6 months. All authors had access to the study data and reviewed and approved the final manuscript.

Recruiting Sites

Participants were recruited at 8 Cancer Prevention Network member organizations: University Hospitals Case Medical Center, Cleveland, OH; Kansas City VA Medical Center, Kansas City, MO; Massachusetts General Hospital, Boston, MA; Mayo Clinic, Rochester, MN; St Michael's Hospital, Toronto, ON, Canada; University of Pittsburgh, Pittsburgh, PA; University of Pennsylvania, Philadelphia, PA; and the University of Puerto Rico, San Juan, PR.

Study Participants

Seventy-five eligible participants were enrolled between February 2012 and January 2013. The target population included participants ($\geq$18 years) with histologically confirmed BE, defined as the presence of specialized columnar epithelium in the tubular esophagus with $\geq$2 cm of involvement and no evidence of high-grade dysplasia or cancer on the basis of both clinical surveillance and additional research biopsies. Participants were required to have documented intestinal metaplasia with goblet cells in $\geq$1 of 4 research biopsy samples ($\geq$50% intestinal metaplasia), use of a proton pump inhibitor before enrollment, and no history of diabetes mellitus. Women of childbearing potential were required to document a negative pregnancy test before enrollment. General exclusion criteria were history of confirmed esophageal high-grade dysplasia, esophageal carcinoma, or any cancer; vitamin B_{12} deficiency; history of lactic acidosis; medication for weight loss $\leq$2 months before enrollment; treatment with other oral hypoglycemia agents or biguanides; receipt of other investigational agents $\leq$3 months before enrollment; history of allergic reactions attributed to compounds of similar composition to the study agent; elective surgery during the study period; genetic disorders such as family history of hereditary gastrointestinal polyp disorder; or comorbidities that might limit adherence to the study protocol.

Baseline Evaluation

After informed consent, participants completed a focused interview, physical exam, peripheral blood draw, anthropometric measurements (height, weight, calculated body mass index [BMI], and waist-hip ratio), and esophagogastroduodenoscopy (EGD) with biopsies for eligibility testing.

Baseline Endoscopy

Endoscopic landmarks including the diaphragmatic hiatus, end of the tubular esophagus as marked by the proximal margin of gastric folds, and squamocolumnar junction were recorded. Extent of the circumferentially involved BE segment was determined by using the

Prague classification system.[20] Hiatal hernia size was measured by linear distance between the end of the tubular esophagus and the diaphragmatic hiatus. Short- and long-segment BE were defined as specialized columnar epithelium lining <3 cm and ≥3 cm of the distal esophagus, respectively.

Four-quadrant endoscopic surveillance biopsies were obtained at 2-cm intervals along the length of the Barrett's epithelium. Additional specimens were obtained from any irregularities. Endoscopic research biopsies (up to 8) were obtained with the same forceps from a 1-cm zone of Barrett's epithelium 1–2 cm above the proximal margin of the gastric folds. Research biopsies were washed for 5 seconds in phosphate-buffered saline. Half of the biopsies were snap frozen in liquid nitrogen. Half were placed in 10% neutral buffered formalin at room temperature.

Findings of irregularities within the Barrett's segment, erosive esophagitis >Los Angeles class A, high-grade dysplasia or cancer, or inadequate Barrett's mucosa to satisfy the study end points (defined as <1 of 4 research samples with ≥50% intestinal metaplasia by central pathology review) excluded further study participation.

Study Design

Willing, eligible participants were randomly assigned in double-blind fashion to receive a 12-week intervention with 1 of 2 study agent combinations (metformin vs placebo) by using a 1:1 randomization scheme based on the Pocock–Simon dynamic allocation procedure,[21] which stratified by regular nonsteroidal anti-inflammatory drug (NSAID) use (regular use vs no regular use), obesity (BMI ≥30 vs < 30 kg/m^2), gender, length of Barrett's segment (<5 vs ≥5 cm circumferential involvement), and participating site. Extended release metformin/placebo was self-administered according to a defined escalation schema: week 1: 500 mg/day, one 500-mg tablet taken orally daily; week 2: 1000 mg/day, one 500-mg tablet taken twice a day orally; week 3: 1500 mg/day, 2 tablets taken orally each morning and 1 tablet taken orally each evening; and weeks 4–12 (±7 days): 2000 mg/day, two 500-mg tablets taken orally twice a day. The primary objective of this trial was to compare the percent change in mean pS6K1 value, which was based on esophageal biopsies obtained at the baseline and post-intervention EGD procedures, between participants randomly assigned to the metformin and placebo arms. Phosphorylation of S6K1 was selected as the primary effect biomarker because this primary substrate of mTOR is a common downstream mediator of both the AMPK and insulin pathways. Thus, pS6K1 assays both direct and indirect effects of metformin.

Adverse events (AEs) were classified and graded by using National Cancer Institute Common Terminology Criteria for Adverse Events, version 4 (available at www.ctep.cancer.gov). Attribution of agent-related AEs was performed by the site investigator who was blinded to the intervention assignments.

Post-intervention Evaluation

Participants returned at week 12 (±7 days) after randomization to assess adherence, concomitant medication use, and AEs. Physical exam and peripheral blood draw were performed. The post-intervention blood draw was not mandated. Post-intervention endoscopy, research biopsies, and tissue handling were performed according to the standardized protocol applied at baseline. Additional details on processing of serum and tissue biomarkers are included in Supplementary Materials.

Statistical Analyses

The primary objective was to compare the percent change in mean pS6K1 level in BE mucosal biopsies between the active versus placebo intervention arms. The null hypothesis for this study was that the percent change (%Δ) in mean pS6K1 values (from baseline to post-intervention) would be the same or increased for the metformin arm as compared with the placebo arm. The alternative hypothesis was that the percent change in mean pS6K1 values would be decreased in the metformin arm as compared with placebo. We estimated that the standard deviation for the distribution of %Δ in pS6K1 would be approximately the range of possible values (ie, −1 to 1) divided by 4 (ie, 0.50 or 50%) because of a lack of prior data in this disease setting. Assuming equal standard deviations (ie, 50%) across the metformin and placebo groups, 30 participants per arm yielded 84% power to detect at least a 35% decrease in the metformin arm as compared with placebo, by using a 1-sided t test with a significance level of .05. If the primary end point data were not normally distributed, the Wilcoxon rank sum test would be used for this analysis to compare the medians instead. Study participants were considered evaluable for the primary end point if pS6K1 data were available from both the baseline and post-intervention evaluations based on the modified intent-to-treat principle. Analysis details for secondary endpoints are included in Supplementary Materials.

Results

Cohort Description

A CONSORT overview of participant recruitment is shown in Supplementary Figure 1. Ninety-three unique participants provided informed consent and were preregistered for the baseline evaluation. A total of 18 participants were deemed screen failures, and 1 participant

Table 1. Baseline Characteristics, Adherence, and AEs by Randomized Arm

	Placebo (N = 36)	Metformin (N = 38)	*P* value
Age (*y*)			.43[a]
Mean (SD)	58.1 (8.4)	59.2 (11.0)	
Median	60.5	60.5	
Quartile 1, quartile 3	52.0, 63.0	53.0, 66.0	
Range	(39.0–79.0)	(20.0–81.0)	
Sex, n (%)			1.00[b]
Female	8 (22.2)	8 (21.1)	
Male	28 (77.8)	30 (78.9)	
Performance score, n (%)			1.00[b]
0	35 (97.2)	37 (97.4)	
1	1 (2.8)	1 (2.6)	
BMI (kg/m^2)			.58[a]
Mean (SD)	30.4 (6.4)	30.9 (5.1)	
Median	29.9	30.1	
Quartile 1, quartile 3	26.6, 32.9	28.0, 33.8	
Range	(20.2–52.0)	(22.3–44.5)	
Waist-to-hip ratio			.61[a]
N	36	37	
Mean (SD)	1.0 (0.1)	1.0 (0.1)	
Median	1.0	1.0	
Quartile 1, quartile 3	1.0, 1.0	0.9, 1.0	
Range	(0.7–1.1)	(0.8–1.1)	
Length of Barrett's segment, n (%)			1.00[b]
<5 cm circumferential involvement	11 (30.6)	11 (28.9)	
≥5 cm circumferential involvement	25 (69.4)	27 (71.1)	
Dysplasia status at pre-intervention, n (%)			.57[b]
No dysplasia	30 (83.3)	29 (76.3)	
Low-grade or indefinite for dysplasia	6 (16.7)	9 (23.7)	
Smoking history, n (%)			.73[b]
Current smoker	2 (5.6)	4 (10.5)	
Never smoked	20 (55.6)	18 (47.4)	
Quit/former smoker	14 (38.9)	16 (42.1)	
NSAID use, n (%)			1.00[b]
Regular use	8 (22.2)	9 (23.7)	
No regular use	28 (77.8)	29 (76.3)	
No. of pills taken, median (range)			.09[a]
N	35	38	
Mean (SD)	253.9 (73.4)	235.8 (73.6)	
Median	280.0	256.5	
Quartile 1, quartile 3	249.0, 300.0	232.0, 288.0	
Range	(1.0–300.0)	(68.0–320.0)	
Adherence (%), median (range)			.18[a]
N	35	38	
Mean (SD)	93.7 (11.8)	91.3 (13.8)	
Median	98.0	96.7	
Quartile 1, quartile 3	92.0, 99.7	89.6, 99.2	
Range	(54.5–104.9)	(32.9–100.7)	
Any AE regardless of treatment or grade			.81[b]
No	13 (36.1)	12 (31.6)	
Yes	23 (63.9)	26 (68.4)	
AEs related to study treatment			.25[b]
No	23 (63.9)	19 (50.0)	
Yes	13 (36.1)	19 (50.0)	
Grade 1 AEs			.82[b]
No	18 (50.0)	17 (44.7)	
Yes	18 (50.0)	21 (55.3)	
Grade 2 AEs			.31[b]
No	28 (77.8)	25 (65.8)	
Yes	8 (22.2)	13 (34.2)	
Grade 3 AEs			.49[b]
No	35 (97.2)	38 (100.0)	
Yes	1 (2.8)	0 (0.0)	

SD, standard deviation.
[a]Wilcoxon rank sum test.
[b]Fisher exact test.

Table 2. pS6K1 Results by Treatment Arm

	Placebo (N = 33)	Metformin (N = 36)	*P* value
pS6K1 (pre-intervention)			.58[a]
Mean (SD)	163.0 (221.6)	188.5 (306.9)	
Median	99.7	73.9	
Range	(2.5–1218.6)	(4.2–1566.5)	
pS6K1 (post-intervention)			.38[a]
Mean (SD)	122.9 (133.8)	123.6 (114.0)	
Median	71.4	102.8	
Range	(5.9–530.8)	(13.0–627.9)	
pS6K1 (absolute change)			.72[b]
Mean (SD)	−40.1 (166.5)	−64.9 (252.4)	
Median	−9.9	0.9	
Range	(−687.7 to 283.2)	(–1379.8 to 132.6)	
pS6K1 (percent change)			.80[b]
Mean (SD)	60.6 (301.0)	42.5 (157.1)	
Median	−14.7	1.4	
Range	(−97.7 to 1646.1)	(−88.1 to 694.0)	
pS6K1 (percent change [no outliers[c]])			.87[b]
N	29	32	
Mean (SD)	−14.1 (62.5)	−3.3 (51.1)	
Median	−24.4	−4.0	
Range	(−97.7 to 134.2)	(−88.1 to 143.6)	

SD, standard deviation.
[a]Wilcoxon rank sum test.
[b]Wilcoxon rank sum test (1-sided per protocol).
[c]Excludes percent change values >200%.

withdrew before receiving any study drug for a total of 74 participants in the intervention cohort (38 metformin, 36 placebo). Reasons for screen failure included out of range lab values (n = 8), high-grade dysplasia, esophagitis, or esophageal stricture (n = 4), intestinal metaplasia on <25% of biopsies (n = 2), and other (n = 4). Preregistered screen failures (n = 18) and the 1 participant who withdrew before receiving study drug were similar to intervention cohort participants with respect to age, sex, smoking history, BMI, and length of Barrett's segment ($P \geq .15$). Within the intervention cohort (n = 74), 5 participants were not evaluable for the pS6K1 analyses because they dropped out of the study because of AEs before the post-EGD evaluation, leaving 69 evaluable participants for the primary end point.

Intervention Arms, Adverse Events, and Agent Adherence

Intervention arms were evenly balanced at baseline with respect to all factors (Table 1) including sex ($P = 1.00$), length of BE segment ($P = .00$), age ($P = .43$), BMI ($P = .58$), smoking history ($P = .73$), NSAID use ($P = 1.00$), and dysplasia status ($P = .57$) in the 74 randomized participants who also received study drug (38 metformin, 36 placebo).

AEs were reported by 49 of 74 trial participants (66%) after starting the assigned study intervention. The observed overall AE rates were higher for the metformin participants, but none of the differences reached statistical significance (Table 1). Specifically, 19 of 38 metformin participants (50%) had a treatment-related AE (possibly, probably, or definitely related) compared with 36% for the placebo participants ($P = .25$). The incidences of grade 1 events were similar between arms ($P = .82$), whereas grade 2 events were reported at a higher rate for metformin vs placebo participants (34% vs 22%, $P = .31$). Only 1 subject had a grade 3 AE (dyspepsia), which occurred in the placebo arm.

Commonly occurring maximum grade AEs (3 or more events) were also compared between arms. Metformin-treated subjects had a higher rate of abdominal pain (grade 1, 8% vs 0%; grade 2, 8% vs 0%), diarrhea (grade 1, 21% vs 11%; grade 2, 5% vs 3%), fatigue (grade 1, 8% vs 3%), headache (grade 1, 8% vs 3%), and nausea (grade 1, 13% vs 6%), but none of these observed differences reached statistical significance for each AE grade. When pooling across grades because of the small numbers, metformin-treated subjects had a significantly higher rate of grade 1/2 abdominal pain compared with placebo (16% vs 0%, $P = .025$). None of the other pooled comparisons were significant between arms. The most common AEs overall were diarrhea and abdominal pain

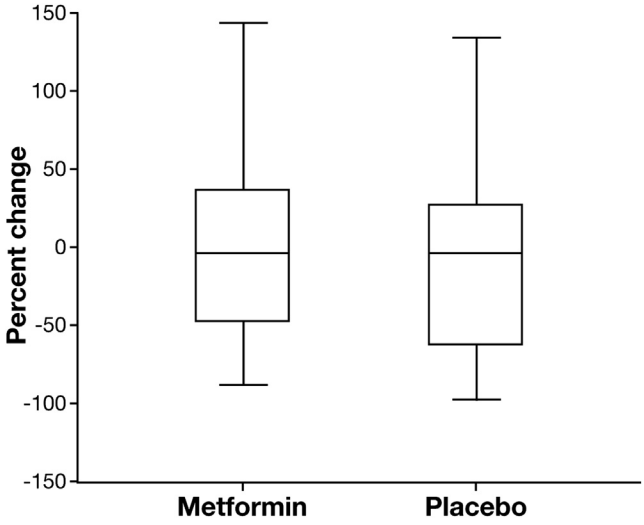

Figure 1. Box plots for percent change in pS6K1values by each treatment arm (metformin and placebo) after excluding 8 outliers (4/arm), where percent change from baseline exceeded 200%.

for metformin-treated subjects, where 10 of 38 subjects (26%) had a grade 1 or 2 diarrhea, and 6 of 38 (16%) had a grade 1 or 2 abdominal pain. There was a serious AE for a subject treated with metformin. This serious AE was a grade 2 amnesia that started on August 24, 2012 and resolved the next day. The number of subjects who went off study early because of an AE was similar for metformin (5 of 38, 13%) and placebo (3 of 36, 8%).

Agent adherence was excellent (median of 97% and 98% for metformin and placebo arms, respectively), with nearly all participants receiving the majority of the assigned study doses, and was similar across the randomization arms ($P = .18$) (Table 1). The placebo participants did take more pills on the average (median 280 vs 257, $P = .09$), but this was due to being on treatment longer on the average.

Primary End Point: Tissue Phosphorylated S6 Kinase 1 Concentration

Of the 74 participants in the intervention cohort, 69 were evaluable for the primary end point based on the modified intent-to-treat principle (n = 36 and 33 for metformin vs placebo, respectively). There was no statistically significant difference between the baseline or post-intervention pS6K1values across the 2 intervention arms (Table 2). There was no significant change (percent and absolute) in the pS6K1 values from pre-intervention to post-intervention within each arm (Wilcoxon signed rank, $P \geq .61$). The metformin and placebo arms were similar with respect to median percent change from baseline for pS6K1values (1-sided $P = .80$, Table 2). Specifically, the median percent change from baseline in pS6K1 values was 1.4% for metformin compared with a decrease of 14.7% in placebo-treated participants (Table 2). These primary end point results were also similar when excluding the 8 outliers (4/arm), where the percent change from baseline exceeded 200% (1-sided $P = .87$, Figure 1). Finally, the 2 arms were also similar with respect to absolute change from baseline (1-sided $P = .72$, Table 2), even after excluding the 2 major outliers (1/arm), where the absolute change from baseline was −500 or less (1-sided $P = .72$). The results for tissue studies of proliferation and apoptosis are included in Supplementary Table 1.

Discussion

We report a double-blind, randomized, controlled prospective chemoprevention trial of metformin in BE patients. Mean fasting serum insulin levels and insulin resistance assessed by homeostasis model assessment (HOMA)-IR in the BE patients were similar to those reported in a previous study,[10] and 2000 mg per day metformin for 3 months was associated with a borderline reduction in insulin as well as IR. However, metformin had no discernible effects on levels of phosphorylation of S6K1, the intracellular mediator of insulin and IGF activation in Barrett's epithelium, compared with placebo. Metformin 2000 mg per day also did not alter proliferation or apoptosis in Barrett's epithelium significantly as assayed by Ki-67 and caspase-3, indicating no effect on insulin/AMPK independent proliferation pathways. This study suggests that metformin at tolerable doses will not be effective as a single agent in preventing the progression of BE in the general population to EAC.

The current strategy of screening for BE in older patients with chronic gastroesophageal reflux disease followed by periodic endoscopic surveillance is based on the premise that BE is the primary precursor of EAC, and EAC can be most effectively cured when detected early.[22,23] However, because the 5-year survival for EAC remains below 20%,[24] it is clear that we need to develop improved, less invasive approaches. Chemoprevention in the early metaplastic stage is an attractive strategy, and discovery of an effective and tolerable chemopreventive agent would avoid the need for repeated endoscopic surveillance. Unfortunately, data from our study do not support a chemopreventive role for metformin in BE-associated carcinogenesis.

To date, most BE chemoprevention trials have focused on reducing inflammation with proton pump inhibitors and/or cyclooxygenase inhibitors.[25–27] The role of central obesity in esophageal carcinogenesis is of growing interest.[9] Molecular mechanisms by which central obesity could lead to cancer include inflammatory mediators, changes in immune function, oxidative stress/DNA damage, hormones/growth factors, and metabolic detoxification factors. One mechanism that has been implicated in obesity-associated cancers is the insulin pathway.[3,28] Epidemiologic studies suggest that obesity-mediated IR and diabetes contribute to BE and

its progression to EAC.[29,30] Some cross-sectional studies have found an association between hyperinsulinemia and BE, whereas others have not.[10,31,32] Furthermore, epidemiologic studies suggest that metformin might be protective against certain cancers.[13–18] Because of the null findings from the currently reported trial, future intervention trials may be more appropriately focused on modulating adipokines such as leptin and adiponectin, alterations of which have also been postulated to be involved in esophageal carcinogenesis.[32–38] Recent observational studies propose a chemopreventive role for statins in patients with BE that should also be pursued prospectively.[39] It is also possible that metformin could be chemopreventive in the subset of BE subjects who are centrally obese or those with elevated insulin levels, although trials much larger than ours would be required to address this possibility.

Metformin 2000 mg for 12 weeks was found to be generally well-tolerated by our study participants, none of whom had clinically diagnosed diabetes mellitus. A few subjects were unable to tolerate metformin because of gastrointestinal side effects, but these side effects ceased once metformin was discontinued. These findings suggest that metformin may be reasonably considered for chemoprevention trials involving subjects with other premalignant conditions, regardless of diabetes mellitus status.

Strengths of the present trial include the rigorous definition of BE and the double-blind, randomized, placebo-controlled, multicenter study design. To maximize the efficiency of accrual, participants were allowed to take aspirin or NSAIDs during the intervention period. Trial limitations include the fact that the sample size was powered to detect a fairly large, 35% relative decrease in pS6K1. Thus, we could have missed a smaller but clinically significant chemopreventive effect of metformin. Although the pS6K1 assay has previously been used to determine the effects of bile acids on signaling pathways in BE and EAC cell lines,[40] the variation of this assay in assessing BE tissue has not been assessed. A large variance in the assay would also make it difficult to detect a metformin effect. Also, metformin was given for a period of only 12 weeks to improve adherence to the study protocol. It remains conceivable that longer exposure may have yielded more pronounced effects on the end points of interest. Phosphorylated S6K1 was selected as our primary effect biomarker because it is the common downstream molecular mediator of PI3K as well as AMPK pathways. There are other carcinogenic pathways that were not assayed in this study. It is possible that metformin could affect BE without affecting S6K1. Our study did not mandate a blood draw at the post-intervention endoscopy. Thus, secondary end points such as change in serum insulin and HOMA-IR were only measured in half the study subjects. Finally, markers of proliferation and apoptosis such as Ki-67 and caspase-3 are only indirect markers of carcinogenesis. Long-term studies would be required to determine whether an agent truly does or does not prevent cancer.

In summary, metformin 2000 mg per day in BE patients on proton pump inhibitor, although tolerable and safe, was not effective in altering pS6K1, proliferation, or apoptosis pathways in non-dysplastic BE. Although this study could have missed moderate effects of metformin on pS6K1 or effects of metformin on alternate carcinogenic pathways, we suggest that future chemoprevention trials consider targeting alternate carcinogenic pathways, perhaps involving adiponectin and/or leptin, in BE-associated carcinogenesis.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at http://dx.doi.org/10.1016/j.cgh.2014.08.040.

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Reprint requests
Address requests for reprints to: Amitabh Chak, MD, University Hospitals/Case Medical Center, 11100 Euclid Avenue, Cleveland, Ohio 44106. e-mail: amitabh.chak@UHhospitals.org; fax: (216) 844-8011.

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Conflicts of interest
This author discloses the following: Dr Limburg served as a consultant for Genomic Health, Inc from August 12, 2008 through April 19, 2010. Mayo Clinic has licensed Dr Limburg's intellectual property to Exact Sciences, and Mayo Clinic and he have contractual rights to receive royalties through this agreement. The remaining authors disclose no conflicts.

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Supplementary Materials

Methods

Serum Markers

Fasting glucose concentrations were measured by the glucose-oxidase method, and serum insulin levels were measured with a double antibody radioimmunoassay. IR was calculated by using HOMA-IR = [Fasting serum insulin (μU/mL) × fasting glucose (mg/dL)]/405.

Tissue Biomarkers

Western blot for pS6K protein expression. Protein lysates from esophageal biopsies were prepared as previously described.[1,2] The protein concentration was determined for each sample by using a protein assay based on the Bradford method (Biorad, Dublin, CA). Twenty to 40 μL of protein lysate was boiled in β-mercaptoethanol for 5 minutes and then loaded on 10% resolving Tris-HCL sodium dodecylsulfate–polyacrylamide precast gels for electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked and then incubated with the primary antibody against pS6K (Anti-phospho-S6K1 Tyr 389; Cell Signaling, Beverly, MA) at a final dilution of 1:250 for 12 hours. The membranes were washed and incubated for 1 hour with the secondary antibody of horseradish peroxidase–linked donkey anti-rabbit immunoglobulin G diluted 1:2000. Immunodetection was performed by using enhanced chemiluminescence. After a 1-minute exposure to the enhanced chemiluminescence reagents, an x-ray film was exposed to the membrane for 1 minute, and then the film was processed. Image J software was used to quantitate protein expression, which was corrected by using actin as loading control.

Immunohistochemistry for proliferation and apoptosis. To determine the effect of metformin on cell survival, a Ki-67–cleaved caspase-3 dual immunostaining approach was used, as previously reported.[3] Briefly, deparaffinization was processed in 3 changes of xylene and rehydration in a series of ethanol (100%, 95%, and 70% EtOH) to a running distilled water rinse. Heat inactivated epitope retrieval was performed in a preheated 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0 buffer. After the slides were cooled in HIER buffer and rinsed well in running distilled water, they were placed on a DAKO Cytomation Autostainer (at room temperature). Sections were incubated with an endogenous peroxidase block (Biocare Medical, Walnut Creek, CA) for 5 minutes to inactivate endogenous peroxides. Nonspecific antibody binding was blocked by using a protein block Background Sniper (Biocare Medical) for 5 minutes. Sections were then incubated in a prediluted monoclonal and polyclonal antibody double-stain cocktail, Ki-67(M) +Caspase-3 (R) (Biocare Medical) for 60 minutes. The MACH 2 (HRP (ms) + ALP(rb)) secondary antibody cocktail (Biocare Medical) was incubated for 30 minutes. Sections were incubated in Cardassian DAB (HRP) (Biocare) for 5 minutes, which visualizes the Ki67. Sections were then incubated in Vulcan Fast Red (ALP) (Biocare) for 15 minutes, which visualizes the cleaved caspase-3. Sections were then counterstained for 5 minutes with a modified Schmidt's hematoxylin and rinsed well in running tap water to blue. Ki67 and caspase-3 expression was semiquantitatively graded by an experienced gastrointestinal pathologist (T.R.S.).

Statistical Analyses

Secondary analyses. Secondary analyses compared baseline participant characteristics, agent adherence, and AEs between intervention arms. All eligible participants who were randomized and started treatment were evaluable for these end points. Summary statistics and frequency tables were used to describe baseline subject characteristics, agent adherence, and AES. The Fisher exact and Wilcoxon rank sum tests were used to test for associations between intervention arms and categorical and continuous data, respectively.

For the translational end points, the sample size varied from 29 participants (16 metformin; 13 placebo) for assessment of glucose, insulin, and HOMA-IR values to 65 subjects (34 metformin; 31 placebo) for assessing caspase-3. All intervention cohort participants with available data for these translational end points were included on the basis of the modified intent-to-treat principle.

Translational end points consisted of comparing changes in proliferation (Ki-67) and apoptosis (cleaved caspase-3) as determined from Barrett's mucosal biopsy samples obtained at baseline and post-intervention by intervention assignment. In addition, changes in glucose, insulin, and HOMA-IR serum marker values were compared between intervention arms. The Wilcoxon rank sum test was used for comparisons related to the translational end points. All randomized participants with data available from both the baseline and post-intervention evaluations were included on the basis of the modified intent-to-treat principle. All statistical tests were 2-sided, except for the primary end point results, where 1-sided P values were reported. Statistical analyses were performed by using SAS version 9.2 (SAS Institute, Inc, Cary, NC).

Discussion

Translational End Points

All available data were analyzed for the translational end points on the basis of the modified intent-to-treat principle. Baseline median fasting serum insulin levels were similar between metformin and placebo (10.1 vs 10.4, $P = .68$, Supplementary Table 1). In addition, baseline median HOMA-IR values were also similar in the metformin and placebo groups (2.2 vs 2.4, $P = .65$,

Supplementary Table 1). There was a borderline significant reduction from baseline for insulin median percent change values in the metformin arm vs placebo arm (–4.7% vs 23.6%, $P = .08$, Supplementary Table 1). A similar result was also seen for median HOMA-IR for metformin vs placebo (–7.2% vs 38%, $P = .06$, Supplementary Table 1). We found no significant differences between the intervention arms for fasting glucose concentrations (data not shown). In addition, we found no significant differences for Ki-67 or caspase-3 between the intervention arms (Supplementary Table 1).

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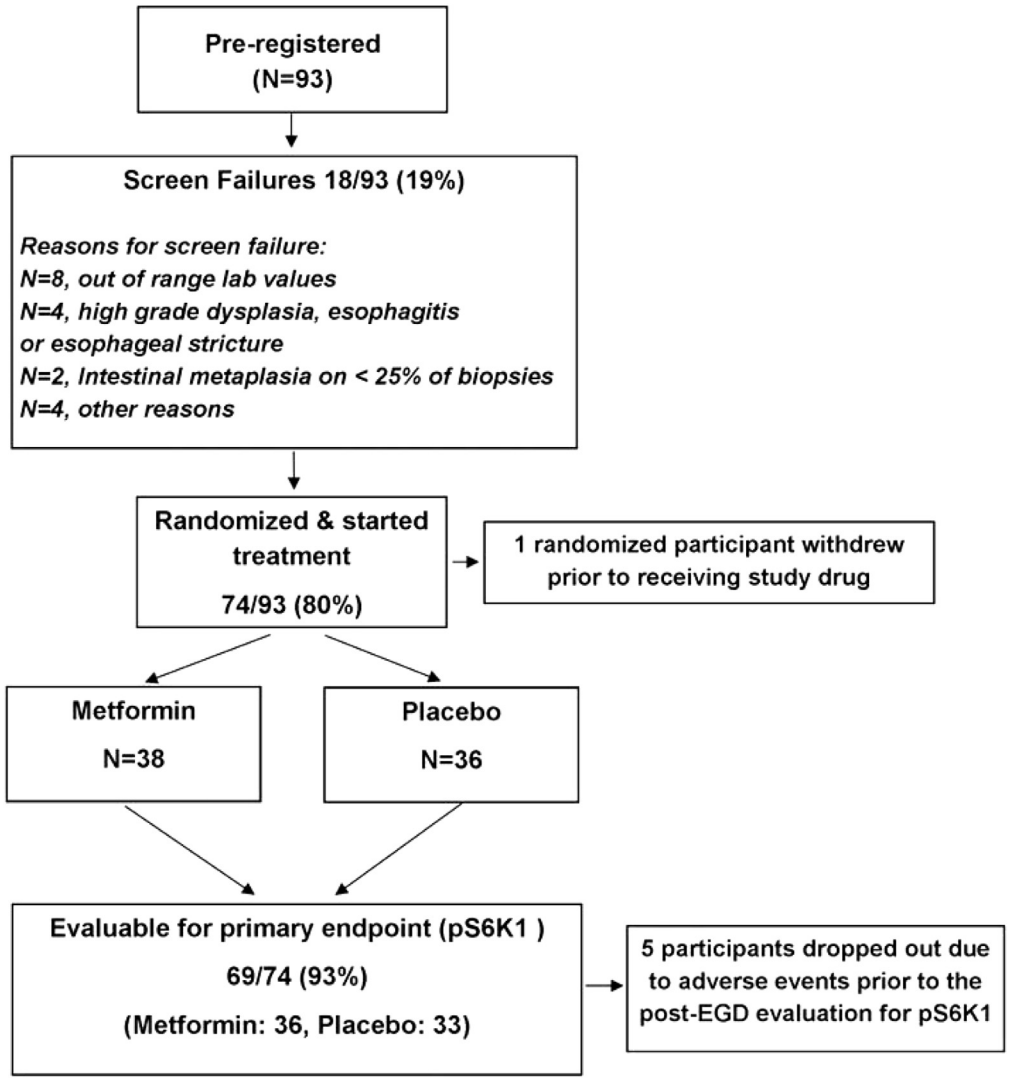

Supplementary Figure 1. CONSORT diagram that shows number of participants who were preregistered, number of screen failures, along with number of participants who were randomized and evaluable for the primary end point.

Supplementary Table 1. Translational Endpoint Results by Treatment Arm

	Placebo (N = 13)[a]	Metformin (N = 16)[a]	*P* value
Insulin Baseline			0.68[b]
Mean (SD)	13.6 (9.7)	11.4 (6.7)	
Median (Range)	10.4 (2.7-40.9)	10.1 (3.6-29.9)	
Insulin Post-Baseline			0.14[b]
Mean (SD)	16.9 (13.3)	11.2 (8.6)	
Median (Range)	13.6 (7.3-59.0)	9.1 (0.8-32.8)	
Insulin Percent Change			**0.08**[1]
Mean (SD)	175.5 (575.8)	-10.1 (37.6)	
Median (Range)	23.6 (-81.4-2085.2)	-4.7 (-77.8-57.0)	
HOMA-IR Baseline			0.65[b]
Mean (SD)	3.2 (2.5)	2.7 (1.8)	
Median (Range)	2.4 (0.6-10.1)	2.2 (0.7-7.5)	
HOMA-IR Post-Baseline			0.13[b]
Mean (SD)	3.9 (2.8)	2.6 (2.3)	
Median (Range)	3.2 (1.6-12.5)	2.0 (0.2-8.8)	
HOMA-IR percent change			**0.06**[b]
Mean (SD)	179.8 (582.6)	-10.7 (39.9)	
Median (Range)	38.0 (-82.0-2110.9)	-7.2 (-79.5-46.0)	
Ki67 percent change[c]			0.55[b]
N	31	32	
Mean (SD)	-5.1 (22.9)	5.7 (57.1)	
Median (Range)	0.0 (-40.0-50.0)	0.0 (-57.1-300.0)	
Caspase-3 percent change[c]			0.43[b]
N	31	31	
Mean (SD)	96.5 (262.5)	192.5 (393.4)	
Median (Range)	25.0 (-75.0-1400.0)	50.0 (-83.3-1400.0)	

[a]Sample size, unless otherwise noted.
[b]Wilcoxon Rank-Sum test.
[c]Changes from baseline to post-baseline.