ORIGINAL ARTICLE

Basic Study

Sex differences and effects of oestrogen in rat gastric mucosal defence

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Abstract

AIM
To evaluate sex differences and the effects of oestrogen

administration in rat gastric mucosal defence.

METHODS
Sex differences in gastric mucus thickness and accumulation rate, absolute gastric mucosal blood flow using microspheres, the integrity of the gastric mucosal epithelium in response to a chemical irritant and the effects of oestrogen administration on relative gastric mucosal blood flow in an acute setting was assessed in an *in vivo* rat experimental model. Subsequently, sex differences in the distribution of oestrogen receptors and calcitonin gene related peptide in the gastric mucosa of animals exposed to oestrogen in the above experiments was evaluated using immunohistochemistry.

RESULTS
The absolute blood flow in the GI-tract was generally higher in males, but only significantly different in the corpus part of the stomach (1.12 ± 0.12 mL/min·g in males and 0.51 ± 0.03 mL/min·g in females) ($P =$ 0.002). After removal of the loosely adherent mucus layer the thickness of the firmly adherent mucus layer in males and females was 79 ± 1 μm and 80 ± 3 μm respectively. After 60 min the mucus thickness increased to 113 ± 3 μm in males and 121 ± 3 μm in females with no statistically significant difference seen between the sexes. Following oestrogen administration (0.1 followed by 1 μg/kg·min), mean blood flow in the gastric mucosa decreased by 31% [68 ± 13 perfusion units (PFU)] in males which was significantly different compared to baseline ($P =$ 0.02). In females however, mean blood flow remained largely unchanged with a 4% (5 ± 33 PFU) reduction. The permeability of the gastric mucosa increased to a higher level in females than in males ($P =$ 0.01) after taurocholate challenge. However, the calculated mean clearance increase did not significantly differ between the sexes [0.1 ± 0.04 to 1.1 ± 0.1 mL/min·100 g in males and 0.4 ± 0.3 to 2.1 ± 0.3 mL/min·100 g in females ($P =$ 0.065)]. There were no significant differences between 17β-Estradiol treated males (mean ratio of positive staining ± SEM) (0.06 ± 0.07) and females (0.11 ± 0.11) in the staining of ERα ($P =$ 0.24). Also, there were no significant differences between 17β-Estradiol treated males (0.18 ± 0.21) and females (0.06 ± 0.12) in the staining of ERβ ($P =$ 0.11). Finally, there were no significant differences between 17β-Estradiol treated males (0.04 ± 0.05) and females (0.11 ± 0.10) in the staining of CGRP ($P =$ 0.14).

CONCLUSION
Gastric mucosal blood flow is higher in male than in female rats and is reduced in male rats by oestrogen administration.

Key words: Sex differences; Gastric mucosal defence; Blood flow; Oestrogen; Gastric physiology; Mucus

Core tip: We report a sex difference in absolute gastric mucosal blood flow using an *in vivo* rat experimental model. Male rats had approximately twice as high blood flow in the gastric corpus mucosa compared to females. Moreover, relative gastric mucosal blood flow decreased during oestrogen administration in males but not in females and the permeability of the gastric mucosa increased to a higher level in females than in males after taurocholate challenge. However, mean clearance increase, mucus thickness and accumulation rate and the expression of ERα, ERβ or calcitonin gene related peptide in the gastric mucosa did not differ significantly between the sexes.

INTRODUCTION

There is an unexplained difference between the sexes regarding the incidence of several gastric diseases. For instance, there is a male predominance (2-3:1) in the incidence of gastric adenocarcinoma[1] and a hypothesis that these sex differences could be due to a protective effects of female sex-hormones, mainly oestrogens[2]. Moreover, there is a similar male predominance in the incidence of peptic ulcer disease; although this difference has been declining over time[3] and oestrogens are hypothesised to play a role in the protection against gastro-duodenal injury[4,5].

In light of previous epidemiological and experimental studies we hypothesised that a potentially protective effect of oestrogen would be exerted by influencing the mechanisms of gastric mucosal defence. The gastric mucosal integrity is maintained by several defence mechanisms that can be divided into three levels: A pre-epithelial level (mucus-bicarbonate), the epithelial level including tight junctions and fast cell turnover, and a sub-epithelial level, mainly blood flow[6-10].

We first evaluated basal sex differences in gastric mucosal blood flow, using the microspheres technique. Secondly, we evaluated the effects of oestrogen on relative gastric mucosal blood flow, using Laser Doppler Flowmetry in a unique *in vivo* experimental model developed in our laboratory[11,12]. Further, gastric mucus thickness and accumulation rate, and the integrity of the gastric mucosal epithelium in response to a challenge with the nonsteroidal anti-inflammatory drug (NSAID) diclofenac and the bile acid taurocholate were studied in the same *in vivo* animal model. Finally, we evaluated sex differences in the distribution of oestrogen receptors (ERs) and

calcitonin gene related peptide (CGRP), an oestrogen sensitive potent vasodilator[13], in the gastric mucosa using immunohistochemistry.

MATERIALS AND METHODS

Animal preparation
All experiments were approved by the Regional Ethical Committee for Laboratory Animal Experiments in Uppsala. The animal protocol was designed to minimise pain or discomfort to the animals. Male and female 9 wk old Sprague-Dawley rats (both sexes are sexually mature at 8-10 wk) (Taconic M&B A/S, Lille Skensved, Denmark) weighing 250-350 g and 180-225 g respectively were kept in standardised conditions of temperature (21-22 ℃) and ambient lighting using a 12 h night and day cycle. Rats were allowed to acclimatise in wide cages with a mesh bottom and free access to pelleted food and tap water for at least seven days before the experiments started. Before each experiment they were fasted for 18-20 h with free access to water. To avoid additional stress the animals were handled as little as possible outside their cages and administered 120 mg/kg of thiobutabarbital sodium (Inactin; Sigma-Aldrich, St Louis, Missouri, United States) intraperitoneally to induce anaesthesia before being returned to the cage. After an appropriate length of time the level of anaesthesia was evaluated by the reactions of the eye-lid and foot to tactile and painful stimulus respectively. If anaesthesia was deemed non-sufficient an additional bolus of thiobutabarbital sodium was administered when necessary during the experimental procedure. No opioids or other analgesics were administered to the animals. Core body temperature was kept at 37-38 ℃ using a heating pad connected to a rectal thermistor. A PE-200 cannula was inserted into the trachea to facilitate spontaneous breathing. After the end of each experiment all animals were euthanised with an intravenous injection of saturated potassium chloride solution.

Measurement of gastric mucosal blood flow using microspheres
Animal preparation specific for this experiment: A PE-50 cannula containing Heparin (Leo Pharma, Malmö, Sweden; 12.5 IU/mL) dissolved in 0.9% saline was inserted into the ascending aorta *via* the right carotid artery as well as the right and left femoral artery. The mean systemic arterial blood pressure (MAP) was under continuous measurement by connecting the catheter in the right femoral artery to a pressure transducer whereas the catheter in the left femoral artery was connected to a constant rate withdrawal pump. The position of the carotid catheter just below or above the aortic valve for injection of the microspheres was confirmed at autopsy at the end of the experiments.

Absolute blood flow measurements using microspheres - experimental protocol: After stable

blood pressure had been established (less than 10% variation during 10 min) approximately 300000 non-radioactive black microspheres with a mean diameter of 15 μm (E-Z Trac Ultraspheres®; IMT, Stason Labs., Irvine, CA, United States), were suspended in 0.15 mL saline containing 0.01% (v/v) Tween 80 and 0.002% (w/v) Thimerosal, sonicated for 5 min and injected through the carotid artery catheter within 10 s. The catheter was then flushed with 0.3 mL saline for 20 s.

The reference blood sample was withdrawn into a Heparin-containing (Leo Pharma, Malmö, Sweden; 5000 IU/mL) syringe at a constant rate of 0.5 mL/min from the catheter in the left femoral artery starting 10 s before the injection of the microspheres and continuing for 60 s after the injection. Blood pressure was continuously monitored to ensure that this procedure did not affect MAP. Thereafter the animals were euthanised and a piece of the right and left kidney, duodenum, colon, antrum and corpus of the stomach were quickly removed, blotted and weighed. In a pilot study (*n* = 5), muscularis and mucosa/submucosa of stomach and gastrointestinal tract specimens were dissected and analysed separately. In all these experiments, the flow to the muscularis layer was not possible to measure with this technique due to low number of microspheres (⩽ 400). The weight and the blood flow of the muscularis were low compared with the mucosa/submucosa. Therefore, a micro-dissection to remove the muscularis from the mucosa/submucosa layer was not performed in subsequent experiments. The specimens were placed on objective glass and subjected to quick freezing in liquid nitrogen before thawing to help visualise the microspheres. The number of microspheres present in the organs was counted in a light microscope. The microsphere content in the arterial blood reference samples was counted in the same manner after transferring the blood to glass microfiber filters. The organ blood flow was calculated according to the formula:

$Q_{org} = (N_{org} \times Q_{ref})/N_{ref}$

Where Q_{org} = organ blood flow (mL/min), Q_{ref} = flow of the reference sample (mL/min), N_{org} = number of microspheres present in the organ and N_{ref} = number of microspheres present in the reference sample. The value was then converted into blood flow in mL/min·g of tissue by dividing the calculated flow above with the total weight of the tissue in grams. The blood flow values calculated from the microsphere content of the kidneys were used to reassert that the microspheres were sufficiently mixed in the circulation. A difference of less than 10% in the blood flow values was taken to indicate adequate mixing. Only animals with adequate mixing were included in the statistical calculations.

Measurement of mucus thickness and accumulation rate
Animal and tissue preparation specific for this experiment: The femoral artery was catheterised

with a PE-50 cannula containing Heparin (12.5 IU/mL) dissolved in 0.9% saline and connected to a pressure transducer for continuous measurement of MAP. The femoral vein was cannulated for drug administration and a continuous infusion of a modified Ringer's solution (25 mmol/L NaHCO₃, 120 mmol/L NaCl, 2.5 mmol/L KCl and 0.75 mmol/L CaCl₂) at a rate of 1 mL per hour.

The gastric preparation for mucus thickness measurements has been described previously in detail[11,14]. In brief, the abdomen was opened through a midline incision and the stomach was exteriorised. The fore-stomach was opened along the greater curvature and the rat was placed on its left side on an in-house built Lucite microscope stage. The corpus of the stomach was everted and draped over a truncated cone with the luminal side up. A mucosal chamber with a hole in the bottom was placed over the stomach, exposing approximately 1.2 cm² of the mucosal surface and the junction was sealed with silicone grease. The mucosal chamber was filled with un-buffered 0.9% saline (5 mL) and kept at 37-38 ℃ by warm water perfusing the bottom of the chamber. The saline was changed every 10 min and pH was measured. The animal was allowed to recover for at least 1 h after surgical preparation until systemic blood pressure stabilised.

Mucus thickness measurements - experimental protocol: Mucus thickness and accumulation rate was measured using micropipettes connected to a micro-manipulator as described previously in detail[12,14,15]. In brief the epithelial cell surface of the mucus gel was visualised using carbon particles (extra pure activated charcoal; Merck Inc., Darmstadt, Germany). A micro-pipette was pushed into the mucus gel at an angle (α) of 25-35° to the epithelial surface and the distance (D) travelled by the micropipette from the luminal surface of the mucus gel to the epithelial cell surface was measured by a digimatic indicator (IDC Series 543; Mitutoyo Corp., Tokyo, Japan) connected to the micro-manipulator. Mucus gel thickness (T) was calculated using the formula $T = D(\sin \alpha)$. A mean value from four or five measurements at different locations was used. Removal of the outer loosely adherent mucus layer was performed by suction with a thin catheter. The inner firmly adherent mucus layer remained and the thickness of this was measured. The accumulation of mucus was studied by measuring the mucus thickness every 20 min for 60 min at which point the loosely adherent mucus layer was removed again and the firmly adherent mucus layer was measured a second time.

Measurements of gastric mucosal blood flow using Laser Doppler Flowmetry before and after oestrogen administration
Animal and tissue preparation specific for this experiment: The preparations in this experiment are identical to those described briefly in section B and in detail elsewhere[11].

Blood flow measurement - laser doppler flow-metry: Laser doppler flowmetry (LDF) (PeriFlux 4001 Master and PeriFlux PF3; PeriMed AB, Stockholm, Sweden) was used to measure gastric mucosal blood flow. The helium neon laser (wavelength 635 nm) was guided to the gastric mucosa by an optical fibre and back-scattered light was detected by a pair of fibres separated by 0.25-0.5 mm. The Doppler shift from an illuminated tissue depends on the velocity and the number of moving red blood cells[16]. The accuracy of the LDF method for gastrointestinal applications has been described previously[17,18]. The laser probe was mounted on a micromanipulator and maintained at a distance of about 0.5 mm from the gastric mucosa in the chamber solution. Because the recorded signal is known to decrease exponentially with distance from the probe and 70% of the total blood flow in the gastric wall is mucosal[6] the recorded signal was considered to be mainly mucosal in origin. Using this technique blood flow was determined as a voltage output and expressed as perfusion units (PFU). Changes in blood flow were expressed as a percentage of baseline values and mean blood flow changes were calculated from the area under the curve during 10 min periods.

Oestrogen administration and experimental protocol: 17β-Estradiol (Sigma Chemical, St Louis, MO, United States) was dissolved in 100% ethanol to form a stock solution which was initially diluted to 2% ethanol in saline with a concentration of 1 mmol/L that could be stored at -20 ℃. Subsequently this stock was further diluted to a final concentration of 0.1 mmol/L which was stored at +4 ℃ without precipitation. On the day of the experiment these solutions were further diluted in phosphate-buffered saline (PBS) to 10 and 1 nmol/L respectively as described by Binko *et al*[19]. Previous studies have shown that maximal 17β-Estradiol levels achieved in cycling rodents is approximately 0.1 nmol/L[20]. A two-step dose regimen for intravenous administration was then adopted influenced by Philp *et al*[21]. However, based on the fact that this group only found antiarrhythmic effects in the higher dose regimens and in order to elucidate if any response would be seen at all as well as to reduce the number of animals required we decided to adopt a regimen with a higher starting dose and a steeper dose-response curve corresponding to approximately 30 and 300 times the physiologic serum concentration seen in female rats. This was done using an initial intravenous bolus dose of 1 and 10 μg/kg (0.17 mL) respectively, administered to both intact males and females, followed by a continuous one hour long infusion of first 0.1 (E1) and finally 1 (E2) μg/kg·min (6 and 60 μg/kg·h) at a rate of 1 mL per hour. Moreover, a few pilot experiments confirmed that an initial bolus dose of 0.1 μg/kg followed by a continuous one hour long infusion of 0.01 μg/kg·min (0.6 μg/kg·h), did not
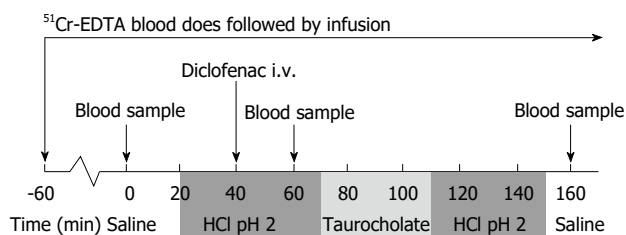
Figure 1 Gastric mucosal permeability; experimental protocol. Time in minutes is given on the X-axis. At -60 (1 h before the start of the experiment but after the animal has been allowed to stabilise after surgery) a bolus dose and a continuous intravenous infusion of ^{51}CrEDTA in Ringer's solution is started. Time 0 marks the start of the experimental procedure. Interventions intravenously are given above the X-axis. Changes in luminal solutions covering the gastric mucosa are given below the X-axis.

cause any significant changes in blood flow (LDF) in either of the sexes (data not shown). Each animal is its own control and all results in this study are thus based on differences in mean changes from the baseline. During the course of the 2 h protocol, MAP and gastric mucosal blood flow were measured continuously.

Data management: Each hour of infusion was divided into six 10 min periods and the changes in MAP and LDF during each period was calculated as a mean value. To compare the different doses of oestrogen a mean value was calculated using the last 30 min (*i.e.,* three 10 min periods) of each infusion period. In one of the female rats the experiment was stopped before the entire last period was finished due to a reduction in blood pressure. In that case the last 10 min period was used as an approximation.

Measurements of gastric mucosal permeability
Animal and tissue preparation specific for this experiment: The preparations in these experiments are identical to those described briefly in section B.

Mucosal permeability: The blood-to-lumen clearance of ^{51}chromium-labeled EDTA (^{51}CrEDTA; DuPont-NEN, Boston, MA) was studied before, during and after a chemical challenge to the gastric mucosa in both male and female rats to evaluate any sex differences in gastric mucosal permeability. These experiments were conducted as described in detail by Petersson *et al*[22] and presented in Figure 1. In brief, after the animal had been allowed to stabilise after surgery and 60 min before the start of the experiment an intravenous bolus dose of 50-75 μCi ^{51}CrEDTA in Ringer's solution was given followed by an infusion of 10-30 μCi per hour (1 mL per hour) during the entire experimental procedure. At the start of the experiment a blood sample of 0.2 mL was drawn and compensated for by an equal volume of 7% BSA (Sigma-Aldrich Chemie, Steinheim, Germany). Blood samples were also taken at 60 min into the experiment and at 160 min at the end of the experimental protocol. All blood samples were centrifuged

and 50 μL of plasma was removed for measurement of radioactivity in counts per minute (cpm). During the experiments the gastric mucosa was covered with 5 mL of isotonic saline or 5 mL of 10 mmol/L HCl. This luminal solution and blood samples were analysed for activity in a gamma counter (1282 Compugamma Cs; Pharmacia, Uppsala, Sweden). Each clearance value was calculated by dividing the luminal cpm value by the corresponding plasma cpm value according to the formula: Lumen sample (cpm/mL) × Sample volume (mL) × 100/Plasma (cpm/mL) × Tissue weight (g) × Time (min). Clearance was calculated in this way every ten minutes during the experiment. If there was a deviation of less than 10% between the three different blood sample counts a mean plasma cpm/mL value was calculated and used for all clearance calculations. If there was a deviation of more than 10% the activity was plotted against time and a straight line was drawn between the two nearest values.

Mucosal blood flow: Gastric mucosal blood flow (LDF) and systemic mean arterial pressure were measured and recorded as described under section C only to ensure the stability of the experimental setup. The recorded values were not used in the analysis.

Experimental protocol: The experimental protocol was adapted from Petersson *et al*[22] (Figure 1). MAP and gastric mucosal blood flow were allowed to remain at steady state for 20-30 min at which point the first blood sample was drawn as described above. Every 10 min during the 160 min of the experimental protocol the 5 mL solution to which the mucosa was exposed to was withdrawn for analysis and instantly replaced. At the start of the experiment the mucosa was exposed to saline for the first 20 min followed by 130 min of 10 mmol/L isotonic HCl and finally to 10 min of saline. Forty minutes into the experiment the COX inhibitor diclofenac (Voltaren; Novartis, Täby, Sweden) was given as an intravenous bolus dose of 5 mg/kg as a "systemic" form of challenge as studies have shown that NSAIDs increase gastric mucosal permeability[23]. Seventy minutes after the start of the experiment the bile salt taurocholate 20 mmol/L (Sigma-Aldrich Chemie) was added to the acidic luminal solution for 40 min as a topical challenge.

In each animal the mean clearance during the last 20 min before the administration of taurocholate was chosen as a control value and the mean clearance during the last 20 min of the taurocholate exposure was chosen as the effect value.

Detection of ERα, ERβ and CGRP in the gastric mucosa by immunohistochemistry
Animal and tissue preparation specific for this experiment: The tissues used in these experiments were collected from the stomachs from all animals that were exposed to oestrogen. The animal and tissue preparation as well as the experimental protocol is

given under section C. After each animal was eutha-nised the stomach was removed and divided into an anterior and posterior half along the greater curvature. One of the halves was fixed in formaldehyde, dehy-drated and embedded in 4% wax. Each wax block was cut into 4 μm thick sections and every 10th section was collected, deparaffinized, and rehydrated.

Antibodies: The antibodies used were rabbit poly-clonal anti-ERα (MC-20; Santa Cruz Biotechnology), chicken polyclonal anti-ERβ503 (produced at the Department of Biosciences and Nutrition, Karolinska Institutet at Novum Huddinge, Sweden)[24] and rabbit anti-Calcitonin Gene Related Peptide (anti-CGRPC8198) (Sigma). Biotinylated anti-rabbit, anti-chicken antibod-ies were from Vector Laboratories (Burlingame, CA).

Immunohistochemical staining: Antigens were retrieved by microwave 650 W in 10 mmol/L citrate buffer (pH 7.0) for 15 min. The sections were incubated in 0.5% H_2O_2 in PBS for 30 min at room temperature to quench endogenous peroxidase, then incubated in 0.5% Triton X-100 in PBS for 15 min. To block the nonspecific binding, sections were incubated in BlockAce (Dai-Nippon Pharmaceutical, Japan) for 40 min at room temperature. Sections were incubated with the following antibodies and dilutions: anti-ERα (1:250), anti-ERβ (1:500) and anti-CGRP (1:5000) in PBS overnight at 4 ℃. After washing, sections were incubated with biotinylated corresponding secondary antibodies (all in 1:200 dilutions) for 1 h at room tem-perature. The Vectastain avidin-biotin complex (ABC) kit (Vector) was used for the ABC method according to the manufacturers' instructions. Peroxidase activity was visualized with 3,3'-diaminobenzidine (DAKO). The sections were mounted on slides and lightly coun-terstained with haematoxylin. Negative controls were incubated without primary antibody.

Immunohistochemical assessment: Immunohisto-chemical slides were evaluated by YO, blinded to animal sex status, using light microscopy. A total of 16 male and 6 female rats were investigated after exposure to 17β-Estradiol. As our focus was on the changes in blood flow in the stomach, we hypothesised that endothelial cells would be the most important targets of oestrogen treatment. Therefore, all further assessment was made on the basis of this hypothesis.

All endothelial cells of arteries in the samples were investigated and the number of stained and non-stained cells on the slides was counted. To examine the effects of 17β-Estradiol treatment, the ratio of stained cells to the total number of endothelial cells were calculated and applied to statistical analysis. This was initially done by using proportion scores and inten-sity scores as described by Allred *et al*[25]. However, after viewing all the slides the staining intensity in all samples was found to be week (intensity score = 1) or absent (intensity score = 0) and a negligible number

of cells showed intermediate staining (intensity score = 2). In the light of this observation the intensity scoring was scrapped and a scoring of either negative (0) or positive (1) staining was adopted and the percentage of staining was calculated as the number of stained cells in one sample divided by the total number of such cells in the sample.

Statistical analysis
All data are expressed as means ± SE. Data was checked for normality using the Shapiro-Wilk test. In microsphere experiments (A) unpaired *t*-tests with Welch's correction was used for comparisons between groups. Two-way repeated measurements ANOVA with Dunnett's multiple comparisons test were used in mucus and blood flow experiments (B&C). Both paired (before and after challenge) and unpaired (between the sexes) *t*-tests were used in permeability experi-ments (D). Unpaired *t*-test was used in immunohisto-chemistry experiments (E). All tests were two-tailed and a *P* value of < 0.05 was considered statistically significant. GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, CA, United States was used for statistical calculations.

RESULTS

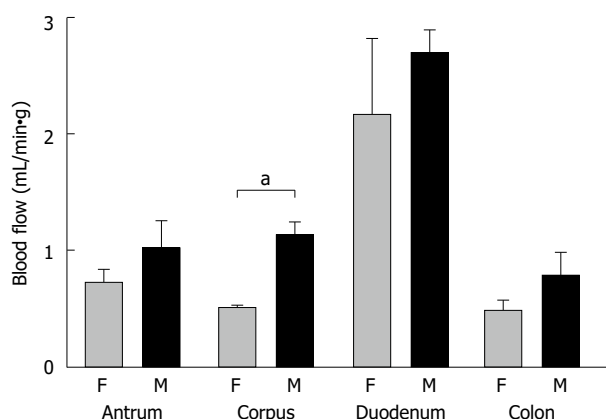### Gastric mucosal blood flow using microspheres
The absolute blood flow in the GI-tract was generally higher in males than in females, but only significantly different in the corpus part of the stomach [1.12 ± 0.12 mL/min·g in males (*n* = 8) and 0.51 ± 0.03 mL/min·g in females (*n* = 5)] (*P* = 0.002) (Figure 2).

Mucus thickness and accumulation rate
After removal of the loosely adherent mucus layer the thickness of the firmly adherent mucus layer in males and females was 79 ± 1 μm (*n* = 11) and 80 ± 3 μm (*n* = 8) respectively. After 60 min the mucus thickness increased to 113 ± 3 μm in males and 121 ± 3 μm in females with no statistically significant difference seen between the sexes (*P* = 0.12). After a second removal of the loosely adherent mucus layer the thickness of the firmly adherent layer was 80 ± 1 μm in males and 78 ± 4 μm in females indicating that the increase in mucus thickness was due to accumulation of loosely adherent mucus (Figure 3).

Gastric mucosal blood flow before and after oestrogen administration
Following oestrogen administration (0.1 followed by 1 μg/kg·min), mean blood flow in the gastric mucosa decreased by 31% (68 ± 13 PFU) in males (*n* = 7) which was significantly different compared to baseline (*P* = 0.02). In females (*n* = 5) however, blood flow remained largely unchanged with a 4% (5 ± 33 PFU) decrease. MAP remained unchanged during the experi-mental protocol with no significant changes either within each group or between the sexes (Figure 4).

Figure 2 Absolute blood flow in the GI-tract of the rat. Absolute (means ± SE) blood flow (mL/min·g) in the GI-tract of male (M) (*n* = 32 for all experiments) and female (F) (*n* = 24 for all experiments) animals, measured with the microspheres technique. The absolute blood flow in the GI-tract was generally higher in males, but only significantly different (a) in the corpus part of the stomach.
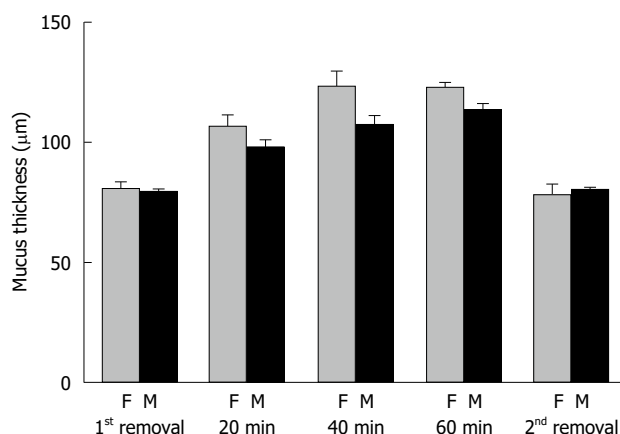
Figure 3 Mucus thickness and accumulation rate. Mucus thickness and accumulation rate (means ± SE) in male (M) (*n* = 8) and female (F) (*n* = 11) animals. Time in minutes is given on the X-axis. 1st removal marks the mucus thickness of the firmly adherent mucus layer after removal of the loosely adherent mucus layer at the start of the experiment. Subsequent time periods show the accumulation rate of the loosely adherent mucus layer which is again removed at the second removal.

Gastric mucosal permeability

No effect on clearance was seen after an intravenous bolus dose of diclofenac (data not shown). The calculated mean clearance increased from 0.1 ± 0.04 to 1.1 ± 0.1 mL/min·100 g (*P* = 0.01) in males (*n* = 6) and from 0.4 ± 0.3 to 2.1 ± 0.3 mL/min·100 g (*P* = 0.001) in females (*n* = 6). There was no statistical significance between the sexes at baseline (*P* = 0.2). The absolute permeability of the gastric mucosa was greater after taurocholate challenge in females compared to males (*P* = 0.01). However, the difference in mean clearance increase between the sexes did not reach significance (*P* = 0.065) (Figure 5A and B). This increase in permeability was completely reversible to 0.1 ± 0.07 mL/min·100 g in males and 0.6 ± 0.2 mL/min·100 g in females within 60 min of removal of taurocholate.
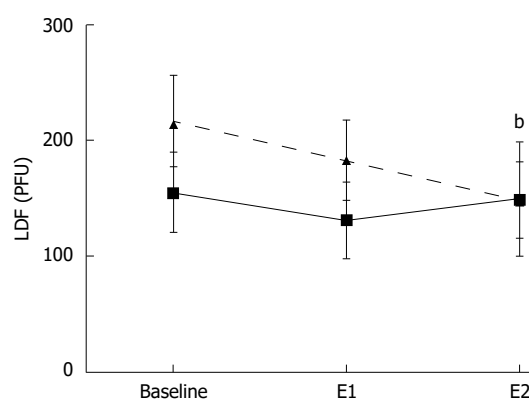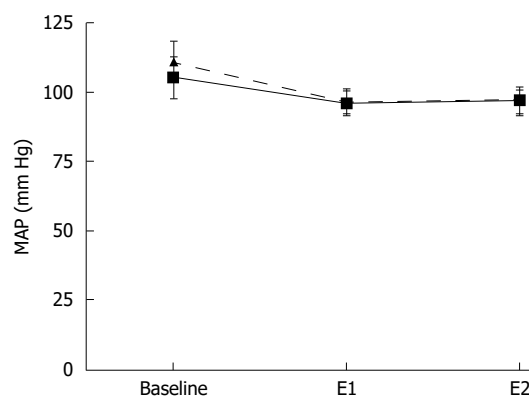

Figure 4 Gastric mucosal blood flow before and after oestrogen administration. Gastric mucosal blood flow [laser doppler flow (LDF)] presented as perfusion units (PFU) and mean arterial pressure (MAP) in male (dashed line) and female (solid line) animals. The X-axis shows values at baseline and following oestrogen administration at 0.1 μg/kg·min (E1) as well as 1 μg/kg·min (E2) respectively. Mean blood flow in the gastric mucosa decreased by 31% (68 ± 13 PFU) in males (*n* = 7) which was significantly different compared to baseline (b).

ERα, ERβ and CGRP in the gastric mucosa

The ratio of positive staining was calculated in every sample and applied to the statistical analysis. Table 1 shows immunostaining of ERα, ERβ and CGRP in endothelial cells of the stomach wall. Values are calculated as mean ratios of positive staining ± SE.

Staining for ERα after exposure to oestrogen could be observed in gastric epithelial cells, glandular cells (both chief cells and parietal cells), infiltrating cells, myenteric neurons near the serosa in the outer longitudinal muscle of the muscularis and in endothelial cells of blood vessels. The staining (proportion of stained cells in relation to the total number of observable cells) of ERα in endothelial cells was low and the intensity was weak. There were no significant differences between 17β-Estradiol treated males and females in the staining of ERα (*P* = 0.24).

Staining for ERβ was seen in chief cells, at the base of the gastric glands, infiltrating cells, adipocytes and in endothelial cells of blood vessels. The staining of ERβ was seen in a larger number of endothelial cells and of a greater intensity than for ERα. There were no significant differences between 17β-Estradiol treated

A

B
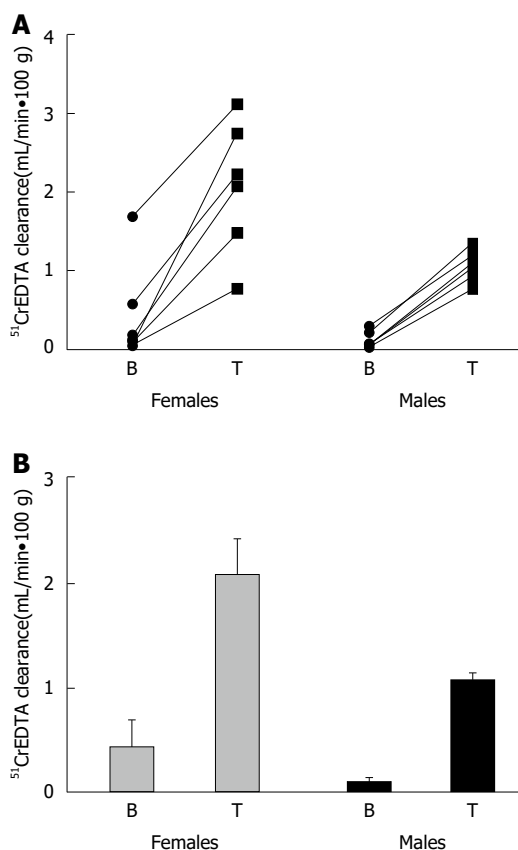
Figure 5 Difference in mean clearance increase between the sexes did not reach significance. A: Absolute clearance increase. Absolute ^{51}CrEDTA clearance increase (mL/min•100 g) in female (*n* = 6) and male (*n* = 6) animals from baseline (B) to after exposure to an NSAID and Taurocholate (T) challenge. B: Calculated mean clearance increase. Calculated mean ± SE ^{51}CrEDTA clearance increase (mL/min•100 g) in female (*n* = 6) and male (*n* = 6) animals from baseline (B) to after exposure to an NSAID and Taurocholate (T) challenge. NSAID: Nonsteroidal anti-inflammatory drug.

Table 1 Immunostaining of oestrogen receptors and calcitonin gene related peptide

	Male (*n* = 16)	Female (*n* = 6)	*P* value
ERα	0.06 ± 0.07	0.11 ± 0.11	0.24
ERβ	0.18 ± 0.21	0.06 ± 0.12	0.11
CGRP	0.04 ± 0.05	0.11 ± 0.10	0.14

Immunostaining of ERα, ERβ and CGRP in endothelial cells of the stomach wall expressed as mean ratios ± SE of positive staining. CGRP: Calcitonin gene related peptide; ERs: Oestrogen receptors.

males and females in the staining of ERβ (*P* = 0.11).

CGRP staining was present in gastric glands, infiltrating cells, myenteric neurons of the muscularis and in endothelial cells of blood vessels. CGRP staining in endothelial cells was less than for ERα and ERβ. There were no significant differences between 17β-Estradiol treated males and females in the staining of CGRP (*P* =0.14) (Figure 6).

DISCUSSION

The main finding in this experimental study of the rat is that there was a difference in the gastric mucosal blood flow between the sexes. The absolute blood flow in the corpus of the stomach was substantially lower in female than in male rats and during oestrogen administration the gastric mucosal blood flow decreased in males but not in females. Moreover, the permeability of the mucosal epithelial barrier was significantly higher in females than in males after exposure to a combination of an NSAID and bile acid. There were no sex differences regarding mucus thickness and accumulation rate. Also, there were no sex differences

in the expression of endothelial ERα, ERβ or CGRP in the stomach.

To our knowledge a sex difference in absolute gastric mucosal blood flow has not been previously reported. Since a higher blood flow has been suggested to protect the gastric mucosa[26,27] and females have a lower incidence of several gastric diseases such as adenocarcinoma, our hypothesis was that gastric mucosal blood flow would be higher in females than in males. However, there are several other studies in both humans and animals, which demonstrate similar sex differences in blood flow albeit not in the gastric mucosa. In a clinical study by Cooke *et al*[28] it was shown that skin blood flow as well as blood flow in the hands and fingers of females were significantly lower than in males. In another study in dogs, Pontari *et al*[29] demonstrated that mucosal blood flow in the bladder was significantly lower in females than in males.

Oestrogen has been shown to cause activation of endothelial nitric oxide synthase (eNOS) leading to production of NO, local vasodilation and an increase in blood flow through non-genomic (within seconds to minutes) stimulation of primarily membrane-bound ERα - but also ERβ receptors as well as through direct effects that are independent of the endothelium and ERs[30]. Therefore, the finding that oestrogen decreased relative gastric mucosal blood flow in males was unexpected. However, in light of our own results that absolute gastric mucosal blood flow is lower in female than in male rats, it is not at all that surprising. In humans oestrogen was seen to induce vasodilation in a major artery in females but not in males, concluding that there may be gender differences in the effects of oestrogen therapy on endothelial functions and NO production/release[31]. Oestrogen has been shown to increase levels of CGRP in sensory nerve-endings leading to increased blood flow through endothelial production of NO, COX-1 and prostacyclin which in turn inhibits neutrophil activation reducing inflammatory response[32]. Although these factors explain in part why females are more resistant to gastric mucosal injury they do not explain the mechanisms behind lower gastric mucosal blood flow in females at baseline or the decrease in blood flow in males during oestrogen administration. Further research to elucidate these mechanisms is warranted.

We did not observe any changes in gastric mucosal

ERα	ERβ
CGRP	

Arrow showing staining of endothelial cells.
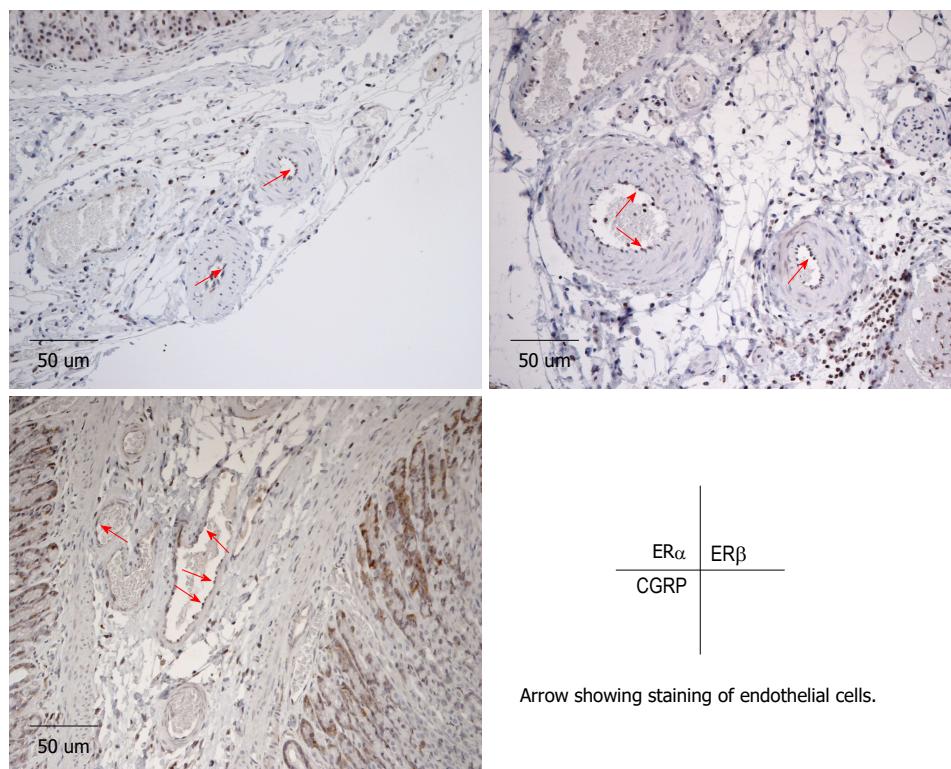
Figure 6 Immunostaining of ERα, ERβ and calcitonin gene related peptide in the male rat stomach. Haematoxylin-stained slides depicting arteries in the male rat stomach. Arrows indicate immunostaining of ERα (top left), ERβ (top right) and CGRP (bottom left) in the endothelium after exposure to oestrogen. Immunostaining is also visible in many other cell-types surrounding the blood vessels. CGRP: Calcitonin gene related peptide.

blood flow during 17β-Estradiol administration in females. This response agrees with a previous study by Zhang *et al*[33] in which blood flow was measured using the H_2-gas clearance technique and a much lower dose of oestrogen was used. Of interest from that study is also that the gastric mucosal blood flow level recorded in the corpus part of the stomach is virtually identical to our absolute values in females recorded using the microsphere method.

The differences found here between males and females in absolute values of gastric mucosal blood flow, as well as the influence of oestrogen cannot be explained by differences in the conductions of the experiments. The male and female animals were subject to the exact same protocols and experiments were carried out interchangeably between the sexes overall during the same period of calendar time minimising the influence of different batches of animals or seasonal variations. The reason to why we chose not to perform bilateral ovariectomy, which is conducted in most other studies of this nature, was that our aim was to study the response in the intact animal with a normal and preserved oestrogen cycle. One limitation is that we did not measure in which phase of the oestrous cycle the female animal was before the start of the experiment. This could potentially affect the response to exogenous oestrogen administration. However, since the dose of oestrogen administered should result in between 30 and 300 times the normal physiological serum concentration the impact of the oestrous phase in these animals should be minimal.

After exposure to a combination of NSAID and bile acid, not resulting in permanent damage, the permeability of the gastric mucosa was significantly higher in females than in males. Moreover, the basal clearance levels agree with earlier results in male rats presented by our laboratory[22]. The finding of a difference in gastric permeability between the sexes during challenge of the mucosa point to a greater ability in females to increase removal of potentially toxic substances from the gastric mucosa. However, since this method can only evaluate clearance from blood to lumen and not from the lumen to the cell surface we cannot draw any conclusions about sex differences in the function of the mucosal epithelial barrier.

Our measurements of mucus thickness and accumulation rate are in accordance with previous work by Atuma *et al*[12] in which, however, only male rats were investigated. To our knowledge sex differences in mucus thickness and accumulation rate as well as the integrity of the mucosal epithelial barrier after exposure to NSAID and bile acid have not been previously evaluated. Even though we did not observe any basal physiological sex differences in mucus thickness and accumulation rate the effects of sex hormones on gastric mucosal pathology and especially drug-induced damage and ulcer healing have been extensively studied with a tendency to sex dependent results. A

study in female rats demonstrated that 17β-Estradiol aggravated ulcer healing[34] whereas another study in male rats presented conflicting results showing an attenuating effect of both progesterone and oestrogen on drug-induced gastric damage[35].

Oestrogen acts on intracellular ERs that regulate gene expression and are found in both cancerous and non-cancerous gastric tissue, although their purpose is not fully understood[36]. We did not find any differences in ER or CGRP receptor expression between the sexes. However, oestrogen effects could still be dissimilar without any differences in receptor expression why further analysis on this matter is warranted.

We found a sex difference in absolute gastric mucosal blood flow not previously reported. Male rats had approximately twice as high blood flow in the gastric corpus mucosa compared to females and the blood flow decreased during oestrogen administration in males but not in females. The significance and mechanisms of these findings need to be further eluci-dated. The observed lack of sex differences regarding the properties and integrity of the mucosal epithelial barrier and the distribution of ERα, ERβ or CGRP in this study are tentative and warrant further investigation. Thus, our results do not explain why human females seem to be protected against gastric diseases such as peptic ulcer disease and adenocarcinoma, but increase our knowledge of sex differences in basic gastric mucosal physiology in the rat.

ACKNOWLEDGMENTS

COMMENTS

Background
There is a male predominance in the incidence of gastric diseases such as adenocarcinoma (2-3:1) unexplained by sex differences in the prevalence of known risk factors. This implies a basic physiological difference between the sexes and supports the hypothesis that sex differences in the incidence of several gastric diseases could be due to a protective effect of female sex hormones, mainly oestrogens. The authors therefore hypothesised that a potentially protective effect of oestrogen would be exerted by influencing the mechanisms of gastric mucosal defence.

Research frontiers
The relationship between gastric disease, oestrogen and gastric mucosal defence has been suggested in a number of epidemiological and experimental studies. However, the mechanisms of these interactions need to be further elucidated.

Innovations and breakthroughs
The authors found a sex difference in absolute gastric mucosal blood flow of the rat not previously reported. Male rats had approximately twice as high blood flow in the gastric corpus mucosa compared to females and the blood flow decreased during oestrogen administration in males but not in females.

Applications
These results do not explain why human females seem to be protected against

gastric diseases such as adenocarcinoma, but increase our knowledge of sex differences in basic gastric mucosal physiology in the rat. The significance and mechanisms of our findings need to be further elucidated.

Terminology
Gastric mucosal defence refers to pre-epithelial (the mucus-bicarbonate barrier), epithelial and sub-epithelial (mucosal blood flow) mechanisms, which protect the stomach against hydrochloric acid and potentially noxious agents.

Peer-review
The paper is interesting but has a major problem. In the introduction and conclusions the authors made extensive references to gastric cancer and hypothesized that a potentially protective effect of estrogen could be exerted by influencing the mechanisms of gastric mucosal defense including blood flow. Since they did not study gastric cancer model in rat (*e.g.*, nitrosoguanidine-induced gastric cancer in rats) nor the effect of estrogens in this model they could not test their hypothesis.

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ORIGINAL ARTICLE

Basic Study

α2-Heremans-schmid glycoprotein (fetuin A) downregulation and its utility in inflammatory bowel disease

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Informed consent statement: Informed consent was obtained from all study participants, along with a verbal permission for the use of the acquired samples for scientific research.

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Abstract

AIM

To investigate the impact of inflammatory bowel disease (IBD) on α2-Heremans-Schmid Glycoprotein (AHSG/fetuin A) and potential associations with disease and patient characteristics.

METHODS

AHSG serum levels were determined in treatment-naïve newly-diagnosed patients, 96 with ulcerative colitis (UC), 84 with Crohn's disease (CD), 62 with diarrhea-predominant or mixed irritable bowel syndrome (IBS, D- and M- types) and 180 healthy controls (HC), by an enzyme linked immunosorbent assay (ELISA).

All patients were followed for a minimum period of 3 years at the Gastroenterology Department of the University Hospital of Larissa, Greece. C-reactive protein (CRP), anti-glycan antibodies, anti-*Saccharomyces cerevisiae* mannan antibodies IgG, anti-mannobioside carbohydrate antibodies IgG, anti-laminariobioside carbohydrate antibodies IgG and anti-chitobioside carbohydrate antibodies IgA were also determined *via* immunonephelometry and ELISA, respectively.

RESULTS
The mean ± SE of serum AHSG, following adjustment for confounders, was 0.32 ± 0.02 g/L in IBD, 0.32 ± 0.03 g/L in CD and 0.34 ± 0.03 g/L in UC patients, significantly lower than in IBS patients (0.7 ± 0.018 g/L) and HC (0.71 ± 0.02 g/L) ($P < 0.0001$, in all cases). AHSG levels were comparable between the CD and UC groups. Based on AHSG levels IBD patients could be distinguished from HC with about 90% sensitivity and specificity. Further adjusted analysis verified the inverse association between AHSG and penetrating, as well as stricturing CD (partial correlation coefficient: -0.45 and -0.33, respectively) ($P < 0.05$). After adjusting for confounding factors, inverse correlations between AHSG and CRP and the need for anti-TNFα therapy or surgery, were found (partial correlation coefficients: -0.31, -0.33, -0.41, respectively, $P < 0.05$, in all cases). Finally, IBD individuals who were seropositive, for at least one marker, had AHSG levels falling within the two lower quartiles (OR = 2.86, 95%CI: 1.5-5.44, $P < 0.001$) while those with at least two serological markers positive exhibited AHSG concentrations within the lowest quartile (OR = 5.03, 95%CI: 2.07-12.21, $P < 0.001$), after adjusting for age, sex and smoking.

CONCLUSION
AHSG can be used to distinguish between IBD and IBS patients or HC while at the same time "predicting" complicated disease behavior, need for therapy escalation and surgery. Moreover, AHSG may offer new insights into the pathogenesis of IBD, since it is involved in key processes.

Key words: Inflammatory bowel disease; Irritable bowel syndrome; Fetuin A

Core tip: α2-Heremans-Schmid Glycoprotein (AHSG/fetuin A) a multi-task negative acute-phase protein is downregulated in patients with inflammatory bowel disease (IBD). Based on this significant decrease a discrimination between IBD patients with either Crohn's disease or ulcerative colitis and those with irritable bowel syndrome or healthy controls can be made. A more robust AHSG decrease correlates well with and predicts complicated disease behavior, need for biological therapy and surgery, within three years from diagnosis. These associations are supported by additional links between AHSG and acute-phase markers *i.e.*, C-reactive protein or serological markers linked to IBD course *i.e.*, anti-glycan antibodies.

INTRODUCTION

Inflammatory bowel disease (IBD) has been long ago recognized as a systemic inflammatory entity and as such, it is anticipated to induce changes exceeding the boundaries of bowel mucosa, being reflected in a broader spectrum of tissues, including blood[1-7]. Examples of such changes are the fluctuations in the levels of C-reactive protein (CRP), tumour necrosis factor alpha (TNF-α), interleukins, S100 proteins, metalloproteinases, angiogenins, *etc*[4-7]. Since a different task is carried out by each of these biomarkers, it has been proposed that IBD induces multifarious responses which in turn, may affect the levels of different compounds *via* feedback mechanisms, consumption or reprioritisation of synthesis[4-8]. These observations along with the inflammatory nature of IBD itself seem to encourage the study of novel proteins that tend to become affected by the establishment of systemic inflammation[3,8]. Such a candidate is fetuin A or α2-Heremans-Schmid glycoprotein (AHSG), a substance synthesized in the liver, bone marrow and fetal organs, exhibiting properties similar to those possessed by negative acute-phase proteins[9]. AHSG has been shown to carry out various immunologic tasks, including regulation of macrophage-related lipopolysaccharide-triggered opsonisation, TNF-α and transforming growth factor beta (TGF-β) levels[10-13]. AHSG is also an inhibitor of ectopic tissue calcification such as that taking place in coronary arteries and heart valves, while at the same time promoting mineralization within fibrils and subsequently, proper bone formation[13-15]. Other recently discovered properties of AHSG include a binding ability to insulin receptor, modifying its sensitivity, participation in wound healing as well as tumor growth processes, through the induction of a more effective cell migration[8,9]. Increased AHSG levels have been recorded in fetal organs (probably reflecting an additional role for AHSG in normal organ development), in adults with either metabolic syndrome or increased insulin resistance alone, patients infected with the severe acute respiratory syndrome-coronavirus as well as individuals at high risk for future cardiovascular events: stroke and acute myocardial infarction[8,9,16]. Low levels of AHSG

Table 1 Patient/control and disease characteristics

	UC	CD	IBS	HC
No	96	84	62	180
Age, yr (mean ± SD)	50.2 ± 13.4	42.3 ± 15.6	49 ± 19.4	46.7 ± 12.3
Age at onset				
≥ 40 yr	-	15	-	-
< 40 yr	-	69	-	-
Sex				
Male	60	45	36	114
Female	36	39	26	66
Current smoking				
Yes	24	57	39	93
No	72	27	23	87
Disease extent (UC)				
Proctitis	16	-	-	-
Left-sided colitis	28	-	-	-
Pancolitis	52	-	-	-
Disease location (CD)				
Upper GI involvement	-	11	-	-
Ileum	-	32	-	-
Colon	-	10	-	-
Ileocolon	-	41	-	-
Disease behavior (CD)				
NS/NP	-	49	-	-
Stricturing	-	20	-	-
Penetrating	-	15	-	-
Extraintestinal manifestations				
None	69	33	-	-
≥1	27	51	-	-
Treatment for remission				
5-ASA	94	82	-	-
Corticosteroids	45	60	-	-
Immunosuppressants	18	30	-	-
Anti- TNFα	3	24	-	-
Surgery	3	12	-	-

UC: Ulcerative colitis; CD: Crohn's disease; IBS: Irritable Bowel Syndrome; HC: Healthy controls; GI: Gastrointestinal; NS/NP: Non stricturing/non penetrating; ASA: Aminosalicylates; TNFα: Tumor necrosis factor-alpha.

have been recorded in patients on hemodialysis, those with cirrhosis, hepatoma or rheumatoid arthritis (RA), an entity sharing common inflammatory pathways with IBD, and have been linked to vascular - excessive valvular and coronary artery calcification, ischemic events- and skeletal disorders - osteopenia[14,17,18]. Moreover, similar phenomena: upregulation of TNF-α and TGF-β, remodelling in intestinal microvessels, both on an acute and chronic basis, as well as manifestations linked to mineral homeostasis *i.e.,* osteoporosis and urolithiasis, are all exerted in IBD. When bearing in mind these characteristics, the idea of examining AHSG levels in IBD, seems more than tempting[4,19-23].

MATERIALS AND METHODS

Patients

Among several patients visiting ER wards, outpatient clinics or being hospitalised for chronic diarrhea, a total of 242 patients were recruited: 96 diagnosed with ulcerative colitis (UC), 84 with Crohn's disease (CD) and 62 with irritable bowel syndrome (IBS). All patients were followed for a minimum period of 3 years at the Department of Gastroenterology at the University Hospital of Larissa, Greece. Another group consisting of 180 healthy individuals was also formed (HC). Study groups were age and sex matched ($P >$ 0.05) with one exception: the CD and UC groups, differed significantly ($P = 0.002$) as anticipated, since CD is often associated with younger age. All individuals participating in the study lacked any known disease *i.e.,* end-stage renal disease, RA, cirrhosis, hepatoma, liver metastases potentially affecting AHSG levels, with the exception of diabetes mellitus (DM), which was treated as a confounding factor and was embedded in the models used for multivariate testing. The demographic and clinical characteristics of patients and HC are presented in Table 1.

The diagnosis of IBD and IBS was established upon the co-evaluation of findings originating from clinical and endoscopic procedures, imaging studies, histopathology and laboratory analyses. IBS patients were also diagnosed and classified as D-IBS or M-IBS (40 and 22 individuals, respectively) according to the Rome Ⅲ criteria[24]. Disease activity in the IBD group was documented using conventional indices: Crohn's Disease Activity Index (CDAI) and the Clinical Activity Index (CAI), for UC[25,26]. A CDAI score greater than

150 and a CAI score exceeding 4, on a 0-16 scale, were considered as active CD and active UC, respectively. Disease location and behavior, in CD, were determined using the Vienna classification whereas for disease extent, in UC, the Montreal classification was used[27,28]. No animals were used for the present study.

Sample collection and preparation
Blood samples were collected upon presentation of patients in our hospital, in serum separator tubes and were allowed to clot for 30 min. All samples were then centrifuged and the obtained serum was stored at -25 ℃ for later analysis. The pre-analytical phase, including sampling and handling methods (sampling tubes, storage conditions *etc.*) was identical in all cases.

Laboratory assays
AHSG assay: For AHSG determinations, a two-site "sandwich" enzyme-linked immunosorbent assay (ELISA) was performed, using a commercially available human Fetuin A ELISA kit (BioSource Europe SA, Belgium). Assay calibrators, controls and prediluted patient serum samples (10 μL initially) containing human AHSG were added to microplate wells, coated with a high affinity polyclonal goat anti-human AHSG antibody. During incubation period, the antibody could capture human AHSG in the sample. Unbound proteins were then washed away and a horseradish peroxidase (HRP) conjugated polyclonal anti-human AHSG antibody was added to each well, so that a "sandwich" of "capture antibody-human AHSG-HRP conjugated detecting antibody" could be formed. After additional washing, incubation with a substrate solution took place, the reaction was stopped and the developed colour was quantified spectrophotometrically. The enzymatic activity of the detecting antibodies, bound to the AHSG on the wall of the microwells, was directly proportional to the amount of AHSG in the sample. A calibration curve which was generated by plotting the absorbance *vs* the respective human AHSG concentration for each calibrator, allowed sample AHSG determination.

C-reactive protein assay: For the determination of C-reactive protein (CRP), immunonephelometry was performed using the Behring Nephelometer Analyzer Ⅱ, as well as the N High Sensitivity commercially available kit (Dade Behring Gmbh, Germany). The control and standard sera were provided by the same company and used according to the manufacturer's instructions.

Anti-glycan antibodies assay: Serum levels of anti-*Saccharomyces cerevisiae* mannan antibodies (gASCA) IgG, anti-mannobioside carbohydrate antibodies (AMCA) IgG, anti-laminariobioside carbohydrate antibodies (ALCA) IgG and anti-chitobioside carbohydrate antibodies (ACCA) IgA were also determined in 108 IBD (56 CD and 52 UC) patients, using commercially available ELISA kits (IBDX, Glycominds Ltd., Israel). Cut-off levels for positivity were set at 50, 100, 60, and 90 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, and ACCA IgA, respectively, as instructed by the manufacturer.

Statistical analysis
Normality (Kolmogorov-Smirnov) test was initially carried out and since the normality assumption was satisfied for the comparison of means between two groups, Student's *t*-tests were used. For comparisons between multiple groups, one-way ANOVA and Tukey's post-hoc tests were applied. Variables are expressed as mean ± SD or mean ± SE. For variables without comparable variations, Welch's correction has been applied. AHSG was tested for its ability to predict IBD, UC and CD, separately, using receiver operating characteristic (ROC) curves, while area under the curve (AUC) and cut-off values, with the optimal sensitivity and specificity, were also calculated. For the simple correlation studies, Pearson's rank test was used. Statistical significance was set at $P < 0.05$. Whenever statistical significance or trend ($0.05 < P < 0.1$) was recorded in univariate analysis, multivariate testing was also performed. Using multiple linear regression and a backward selection process independent variables affecting AHSG levels - confounders - were identified. As candidate confounding factors were initially considered age, sex, smoking, DM, treatment modalities, disease duration and behaviour, age at onset. For the associations originating from multiple linear regression, partial correlation coefficients - quantifying the relationship between two variables while controlling for other factors- are reported. Whenever a categorical parameter was treated as dependent variable, logistic regression analyses, simple and multiple, were applied and odds ratios (ORs) as well as 95%CIs, unadjusted/adjusted for confounding, were calculated. Adjusted means were also calculated using analysis of covariance. Statistical analyses were conducted using GraphPad Prism (4.0 and 7.0) and the MedCalc 10.2.0.0 statistical softwares. Statistical review of the study was performed by a biostatistician.

Ethical considerations
The study was approved by the University of Thessaly Medical School Ethics Committee. Informed consent was obtained from all study participants, along with a verbal permission for the use of the acquired samples for scientific research.

RESULTS

AHSG levels with regard to disease characteristics
The mean ± SE of AHSG in serum was 0.33 ± 0.01 g/L for IBD, 0.73 ± 0.02 g/L for IBS patients and 0.7 ± 0.02 g/L for HC. The recorded difference between
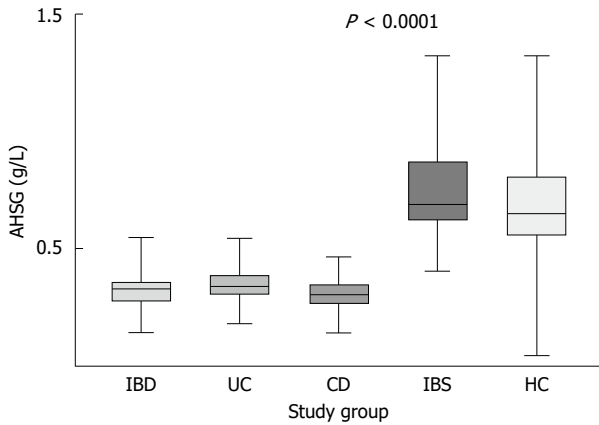
Figure 1 α2-Heremans-schmid glycoprotein levels in patients with inflammatory bowel disease, ulcerative colitis, Crohn's disease, irritable bowel syndrome patients and healthy controls. AHSG: α2-Heremans-schmid glycoprotein; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; IBS: Irritable bowel syndrome; HC: Healthy controls.

the IBD and control groups was statistically significant ($P < 0.0001$) and this was also the case when CD and UC were compared separately with IBS patients and HC. AHSG levels in the CD group were 0.31 ± 0.01 g/L, significantly lower than those of IBS patients and HC ($P < 0.0001$). Likewise, UC patients also exhibited lower AHSG levels (0.34 ± 0.01 g/L) compared to IBS patients and HC ($P < 0.001$). These differences remained significant between IBD (0.32 ± 0.02 g/L), CD (0.32 ± 0.03 g/L) or UC patients (0.34 ± 0.03 g/L), IBS patients (0.7 ± 0.018 g/L) and HC (0.71 ± 0.02 g/L), after adjustment for age and sex ($P < 0.001$, for all comparisons). When AHSG levels were compared between UC and CD patients or between IBS patients and HC, no significant differences were observed ($P > 0.05$, in both cases) (Figure 1).

ROC curve analysis showed that the optimal cut-off of AHSG for the prediction of IBD was 0.44 g/L (90% sensitivity and specificity). Similarly, an AHSG value of 0.44 g/L could distinguish CD patients from non-IBD individuals (IBS and HC) with a sensitivity of 90% and a specificity of 90.6%, while a value of 0.42 g/L could discriminate UC patients and non-IBD subjects with 91.7% sensitivity and 89.3% specificity. The AUC was 0.94 (95%CI: 0.91-0.97), 0.94 (95%CI: 0.91-0.97) and 0.95 (95%CI: 0.92-0.98) for the prediction of IBD, UC and CD, respectively ($P < 0.0001$, in all cases) (Figure 2).

AHSG levels with regard to disease characteristics

All IBD patients exhibited active disease when blood was drawn for later analysis. In our study, in patients with active disease the AHSG levels were marginally associated with CDAI and CAI scores ($r = -0.24$, $P = 0.08$). When disease location (CD) and extent (UC) were taken under consideration no statistically significant differences were observed ($P > 0.05$ in all cases).

Another part of the present study included the
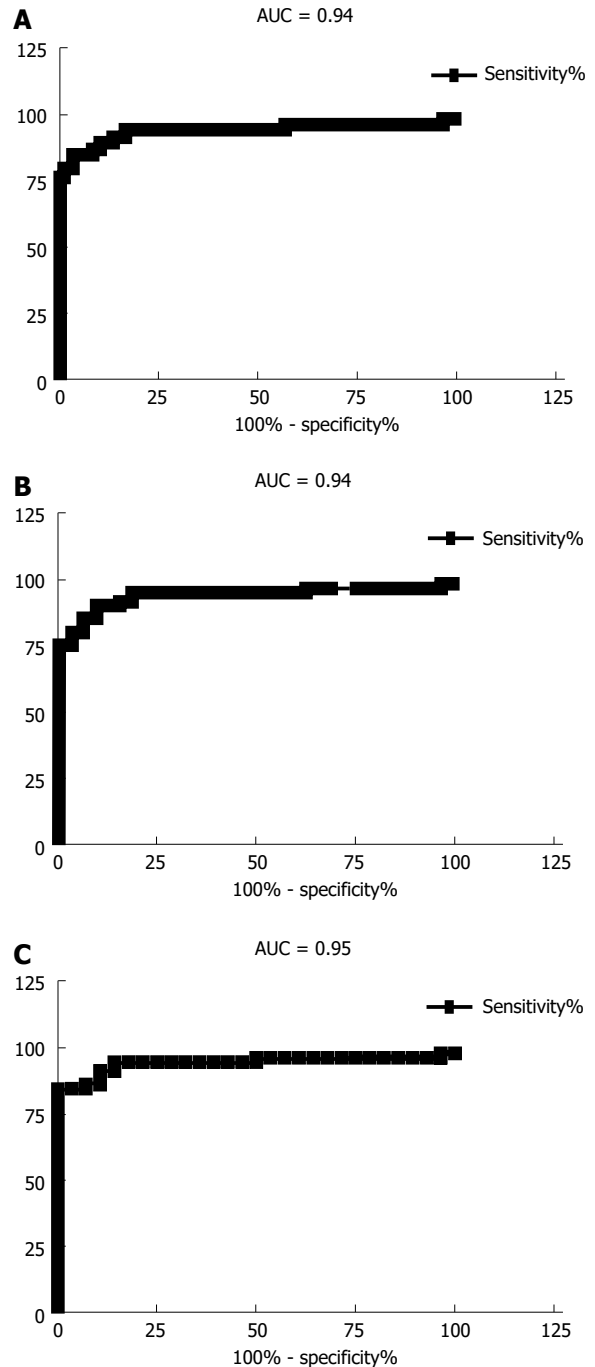
Figure 2 Receiver operating characteristic curves for the discrimination. A: inflammatory bowel disease (IBD) and non-IBD group -irritable bowel syndrome and healthy controls; B: ulcerative colitis; C: Crohn's disease and non-IBD group. AUC: Area under curve.

comparison of AHSG serum concentrations among CD patients with diverse disease behavior: stricturing, penetrating and non-stricturing non-penetrating (ns/np). The performed analysis showed that patients with stricturing or penetrating disease had lower AHSG levels (0.26 ± 0.07 g/L and 0.26 ± 0.06 g/L, respectively), compared to patients in the ns/np subgroup (0.33 ± 0.07 g/L). These differences were statistically significant between CD patients with stricturing and ns/np ($P < 0.01$) or between the
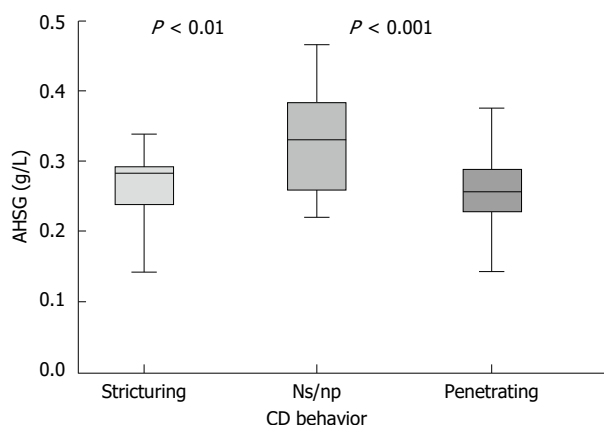
Figure 3 α2-Heremans-schmid glycoprotein levels in Crohn's disease patients exhibiting distinct disease behavior patterns: stricturing, penetrating or non stricturing non penetrating (ns/np). AHSG: α2-Heremans-schmid glycoprotein; CD: Crohn's disease.

penetrating and ns/np disease subgroup ($P < 0.001$) but not between the stricturing and penetrating subgroups ($P > 0.05$) (Figure 3). Further analysis verified the inverse association between AHSG and penetrating, as well as stricturing CD, both before ($r = -0.44$ and -0.32, respectively, $P < 0.05$), as well as after adjustment (partial correlation coefficient: -0.45 and -0.33, respectively, $P < 0.05$) for age, sex and smoking status. In order to perform additional testing of the link between lower AHSG levels and complicated disease behavior, logistic regression was applied, while considering penetrating or stricturing CD, as dependent, and AHSG concentrations, in quartiles - lowest, low, high, highest - as independent variables. The results originating from this analysis showed that AHSG levels, in the lowest quartile, were associated with both penetrating as well as stricturing disease, before (OR = 4.25, 95%CI: 1.54-11.8 and OR = 1.23, 95%CI: 1.1-8.31, respectively) and after adjustment (OR = 8.36, 95%CI: 2.57-27.17 and OR = 3.5, 95%CI: 1.47-12.9, respectively) for age, sex and smoking status ($P < 0.01$, in all cases).

AHSG levels were also examined with respect to the presence of one or more IBD-related extraintestinal manifestations. IBD patients exhibiting extraintestinal manifestations had comparable AHSG levels (0.32 ± 0.01 g/L) to the IBD subgroup without such disorders (0.33 ± 0.01 g/L, $P = 0.55$). Interestingly, 11 IBD patients with a history of recurrent urolithiasis had AHSG levels at the lowest quartile, this result, however, did not reach statistical significance. All data on AHSG variations according to the already described disease characteristics are presented in Table 2.

AHSG levels with focus on patients' characteristics

The levels of serum AHSG were studied with respect to gender of IBD patients, so that potential differences could be highlighted. Both male and female IBD patients exhibited comparable AHSG levels (0.32

± 0.01 g/L and 0.33 ± 0.01 g/L, respectively) ($P = 0.53$). Likewise, AHSG levels were similar between IBD patients, while taking into account age at onset and smoking habits (Table 3).

Serum AHSG concentrations were examined with regard to treatment modalities adequate for inducing and maintaining remission during the 3-year follow up period: 5-aminosalicylates (5-ASA), corticosteroids, immunosuppressants, anti-TNFα agents or surgery. During comparison, IBD patients requiring surgical intervention or the use of anti-TNFα therapy exhibited lower AHSG concentrations (0.26 ± 0.07 g/L and 0.3 ± 0.05 g/L), compared to those adequately treated with 5-ASA (0.33 ± 0.05 g/L) or corticosteroids (0.35 ± 0.1 g/L) ($P < 0.05$, in both cases). Further evaluation of the recorded associations, using simple linear regression analysis, showed that AHSG levels were inversely associated with the need for anti-TNFα treatment ($r = -0.31$, $P < 0.05$) and surgery ($r = -0.36$, $P < 0.05$). After multivariate analysis - also considering age, sex, activity, duration, smoking status- the inverse association between AHSG and need for anti-TNFα therapy or surgery remained statistically significant (partial correlation coefficients: -0.33 and -0.41, respectively - $P < 0.05$, in both cases). Since these results are suggestive of a link between a more profound downregulation of AHSG levels and the need for anti-TNFα treatment or surgical intervention, AHSG concentrations were classified into quartiles. Using logistic regression, AHSG levels in the lowest quartile were found to be an independent predictor of the need for anti-TNFα treatment, in a model adjusted for other treatment modalities, age, sex, smoking status and disease duration (OR = 5.22, 95%CI: 1.58-17.3, $P < 0.01$). Similarly, by applying the same adjusted model, it was shown that need for surgery could be independently predicted by the presence of AHSG levels within the lowest quartile (OR = 5.51, 95%CI: 1.11-27.3, $P < 0.01$).

AHSG and its association with IBD markers

IBD patients had higher CRP levels (median: 3.2 mg/dL, range: 0.9-29.3 mg/dL) compared to IBS (median: 1.05 mg/dL, range: 0-4.3 mg/dL) and HC groups (median: 0.9 mg/dL, range: 0-2.8 mg/dL) ($P < 0.001$). A correlation study of AHSG with the levels of the inflammatory marker CRP was performed revealing marginal association ($r = -0.28$, $P = 0.07$). Multivariate analysis, considering as confounding variables age, sex and smoking on the other hand, revealed a closer association between the two substances with a -0.31 partial correlation coefficient and a $P = 0.02$ level of significance.

Positivity rates for serological markers were 18% for gASCA, 25.6% for ALCA, 5% for AMCA and ALCA while actual median concentrations are presented in Table 4. Additional associations were investigated by examining positivity for these serological markers and

Table 2 α2-Heremans-schmid glycoprotein levels (g/L) with respect to disease characteristics

	IBD	UC	CD	*P* value
Extent (UC)				
Proctitis	-	0.35 ± 0.1	-	> 0.05
Left-sided colitis	-	0.34 ± 0.12	-	
Pancolitis	-	0.35 ± 0.10	-	
Location (CD)				
Upper GI	-	-	0.29 ± 0.09	> 0.05
Ileum	-	-	0.32 ± 0.08	
Colon	-	-	0.31 ± 0.09	
Ileocolon	-	-	0.29 ± 0.07	
Behavior (CD)				
NS/NP (a)	-	-	0.33 ± 0.07	a *vs* b: < 0.01
Stricturing (b)	-	-	0.26 ± 0.07	a *vs* c: < 0.001
Penetrating (c)	-	-	0.26 ± 0.06	b *vs* c: > 0.05
Extraintestinal manifestations				
None	0.33 ± 0.01	0.33 ± 0.09	0.33 ± 0.07	> 0.05
≥ 1	0.32 ± 0.01	0.36 ± 0.1	0.30 ± 0.08	

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; GI: Gastrointestinal; NS/NP: Non stricturing/Non penetrating.

Table 3 Variations of α2-Heremans-schmid glycoprotein (g/L) according to patient's characteristics

	UC	*P*	CD	*P* value
Sex				
Male	0.34 ± 0.07	> 0.05	0.30 ± 0.08	> 0.05
Female	0.34 ± 0.12		0.33 ± 0.07	
Current smoking				
Yes	0.31 ± 0.02	> 0.05	0.30 ± 0.01	> 0.05
No	0.32 ± 0.01		0.28 ± 0.01	
Age at onset				
≥ 40	NA	-	0.29 ± 0.01	> 0.05
< 40	NA		0.32 ± 0.02	

UC: Ulcerative colitis; CD: Crohn's disease; NA: Not applicable.

Table 4 Concentrations of anti-glycan antibodies in inflammatory bowel disease patients

	Median(range)		*P* value
	UC	CD	
gASCA	25.5 (18-27.2)	49.5 (28.5-111)	< 0.0001
ALCA	46.9 (31-77.5)	66 (30.4-94.5)	> 0.05
AMCA	67 (50.1-114.6)	45.8 (37.7-75)	0.0001
ACCA	65 (48-102)	63 (51.1-105.3)	> 0.05

UC: Ulcerative colitis; CD: Crohn's disease; ASCA: Anti-*Saccharomyces cerevisiae* mannan antibodies; AMCA: Anti-mannobioside carbohydrate antibodies; ALCA: Anti-laminariobioside carbohydrate antibodies; ACCA: Anti-chitobioside carbohydrate antibodies.

AHSG (in quartiles). Interestingly, an inverse association between serology and AHSG levels was recorded. IBD individuals who were seropositive, for at least one marker, had AHSG levels falling within the two lower quartiles (OR = 2.87, 95%CI: 1.51-5.45, *P* < 0.001) while those with at least two serological markers positive exhibited AHSG concentrations within the lowest quartile (OR = 5.12, 95%CI: 2.17-12.08, *P* < 0.001). Further analysis also considering potential confounding factors such as age, sex and smoking did not alter the reported associations significantly: OR = 2.86 (95%CI: 1.5-5.44) for single and OR = 5.03 (95%CI: 2.07-12.21) for multiple seropositivity (*P* < 0.001, in both cases).

DISCUSSION

In accordance with a preliminary report from our team in 2010, AHSG levels were downregulated in patients with IBD thus, allowing discrimination from IBS and HC individuals[29]. Furthermore, a more profound downregulation of AHSG was very well associated with complicated disease behavior and the need for biological anti-TNFα treatment or surgery. Additional associations with CRP and anti-glycan antibodies offered better insight into AHSG's link with acute-phase response and

IBD course.

The finding of an IBD-induced downregulation of AHSG is nothing but surprising since, AHSG levels decrease in the presence of robust inflammation[9,11,18]. Moreover, an interplay had already been documented between AHSG and the "notorious", in IBD, TNF-α and TGF-β[11-13]. An additional finding further confirming the tight link of AHSG with TNFα, was the discovery of a binding site for TNFα, within the AHSG gene. As a consequence of TNFα's binding on this region, the expression of AHSG gene is suppressed, leading to decreased AHSG production while other substances are favored-repriorisation of liver synthesis[8,11-13]. Due to the magnitude and rather IBD-selective character of this suppression, however, an evaluation of AHSG's ability to differentiate between entities has been performed. When a cut-off of 0.44 g/L was used, AHSG levels were shown to discriminate IBD from IBS and HC with a sensitivity and specificity of 90%.

When magnitude of disease activity, as expressed through CDAI and CAI scores, was taken into consideration marginal differences were detected. According to the study by Ma *et al*[30] significant differences in AHSG levels exist between IBD patients with active or inactive disease. On the other hand, a correlation between AHSG and CRP was confirmed, in our study. This may be, at least in part, due to limitations of the indices themselves although one should bear in mind that apart from the negative acute phase protein properties attributed to AHSG, its downregulation is also a part of a more complex liver deregulation, resulting from an excessive uptake and processing of signals[8-18]. Perhaps, possible associations of AHSG with other indices should also be evaluated[31-32]. At this point, however, an asset of the present study has to be underlined: the AHSG levels recorded in IBD patients correspond to a prior-to-therapy status and therefore, could not have been altered by any of the known drugs used to treat IBD.

A different set of results was obtained while examining AHSG levels with respect to UC extent and CD location. The recorded AHSG levels in this study could not predict disease location or extent and therefore, could not be used for this purpose.

This was not the case when disease behavior was taken into account. A further downregulation of AHSG was observed, in CD patients with stricturing or penetrating disease, compared to those with the ns/np subtype. This is not surprising as a more diffuse transmural inflammation would exert a stronger proinflammatory effect[33]. Moreover, in many organs inflammatory stress leads to fibrosis and subsequently tissue calcification[34]. From another pathogenetic perspective, a reason for the observed link between AHSG and stricturing disease could also be the reduced AHSG-induced counteraction of TGF-β's fibrogenic and antiproliferative potential[12,13,19]. Likewise, microvascular calcification, reduced blood flow and microthrombosis, exerted phenomena in the presence

of low AHSG levels are also major findings during a chronic phase in the intestinal vasculature of IBD patients, leading to diminished intestinal perfusion and in turn to ulceration and fibrosis[21,35].

Another scopus of the present study was to test whether there is a link between AHSG levels and the presence of extraintestinal manifestations. The performed analysis did not reveal any associations between AHSG serum concentration and the presence of any extraintestinal manifestations. In this case, a limitation exists: the rather small number of IBD patients manifesting specific disorders *i.e.*, urolithiasis.

A study focusing on the characteristics of IBD patients and AHSG levels was also performed. AHSG concentrations were compared among patients of different gender, smoking status and age at onset. None of these characteristics - male or female sex, current smoking or non-smoking habit and age at onset- could be linked with significant AHSG variations.

This was not the case when AHSG levels were evaluated with respect to treatment modalities adequately inducing and sustaining disease remission during the three-year follow-up period. A robust downregulation of AHSG levels correlated with the need for anti-TNFα therapy. This finding is within reason since, a more profound and persisting upregulation of TNFα, requiring subsequently the use of an anti-TNFα agent, would result in a greater AHSG decrease. As far as the link between AHSG levels and need for surgery, is concerned, one should also bear in mind that this subgroup predominantly consisted of CD patients with stricturing and penetrating disease. In the case of a refractory UC, requiring colectomy, safe conclusions could not be drawn due to the small number of colectomised patients, in our study.

The evidence mentioned above seems to make rather appealing the idea of AHSG's use as a diagnostic or even "predictive" tool rather tightly linked to IBD behavior as well as with markers related to it, such as the anti-glycan antibodies[36]. A prerequisite for this type of use is that its lack of a disease-specific nature, with similar fluctuations also reported in other disorders, is taken into account[9,14,17,18]. A combined clinical and AHSG-based algorithm for the diagnosis of IBD, on the other hand, could help overcome this limitation.

Setting aside the already-performed diagnostically-oriented interpretation of current results, a careful examination of AHSG's potential implication in the pathogenesis and complications of IBD, should also be performed. As already mentioned, AHSG is an inhibitor of unwanted, ectopic, tissue calcification and its decreased levels, as in this case, have been linked with excessive valvular and arterial calcification[4]. Although, initial reports originated from animal studies and those recruiting dialysis patients, similar results were obtained in patients with ischemic heart disease, alone[37]. Since a decrease in circulating AHSG is present in IBD, it would be logical to hypothesize that an accompanying reduced inhibition of vascular and valvular calcification

would also be present. As this study was not designed to "tackle" these matters, the actual contribution of low AHSG levels in vascular changes and related acute or chronic ischemic events in IBD[38], remains to be clarified.

Apart from the implication of AHSG in vascular pathology, another role, that of bone mineralisation has been identified[13-15]. Under the influence of AHSG, a "reliable", stress-resistant bone structure is formed, while in the absence of AHSG, minerals deposit outside fibrils, leading to defective osseous formation and finally osteoporosis[15]. As IBD patients are at high risk for osteoporosis, resulting from corticosteroids, malnutrition and inflammation-induced osteopenia[22] the examination of possible contribution of AHSG suppression for the onset of osteopenic manifestations might prove fruitful. In a rather similar manner, a defective coordination in mineral use, predominantly that of calcium, due to decreased AHSG levels, may lead to urolithiasis. Indeed, in the study of Stejskal *et al*[39] lower levels of AHSG in urine have been associated with the presence of urolithiasis. Although, in the case of IBD, well-established mechanisms- *i.e.,* hyperoxaluria, reduced citric acid *etc*[18] - for urinary compications exist, when bearing in mind that still 80% of urinary stones consist of calcium salts it is within reason to test for additional candidates involved in calcium homeostasis and urinary manifestations, such as AHSG[40].

Conclusively, AHSG seems to emerge as a molecule of potential diagnostic and perhaps predictive value in IBD, as its downregulation shows a tight link with acute-phase. As well as with chronic inflammatory responses in both CD and UC. This link also encompasses the more challenging cases, those with complicated disease behavior, as well as those requiring advanced treatment strategies such as biological agents or surgery. As far as the pathogenetic role of AHSG in IBD is concerned, further studies designed to assess the impact and the association of low AHSG levels with respect to micro- and macro-vascular changes, skeletal and urinary complications recorded in patients with CD and UC, are needed.

ACKNOWLEDGMENTS

COMMENTS

Background
α2-Heremans-schmid glycoprotein (AHSG/fetuin A), a negative acute-phase protein with multiple functions, becomes downregulated in the presence of inflammation.

Research frontiers
An AHSG downregulation in inflammatory bowel disease (IBD) patients has been initially reported by our research team in 2010. Another independent study, later verified this result. A major confounding factor, that of IBD-specific treatment existed in both studies.

Innovations and breakthroughs
This is the first study assessing AHSG levels in treatment-naive patients. The low levels of AHSG found in IBD patients are not recorded in control subjects- IBS patients and healthy controls- and are well-associated with complicated disease behavior, as well as the need for anti-TNFα treatment or surgery. Additional links with the acute phase protein C-reactive protein and serological markers linked to IBD course, the anti-glycan antibodies, have been found.

Applications
AHSG could serve as an additional marker for IBD diagnosis and prediction of a more challenging course requiring treatment with biological agents or surgery, during the three-year period following initial diagnosis.

Peer-review
The paper is valuable. It finds AHSG can be used to distinguish between IBD and irritable bowel syndrome, and may predict complicated disease behavior, and foretell the need for intensified therapy and surgery. Moreover, AHSG may offer new insights into the pathogenesis of IBD.

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ORIGINAL ARTICLE

Basic Study

IL23R single nucleotide polymorphisms could be either beneficial or harmful in ulcerative colitis

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Abstract

AIM
To investigate the association of seven single nucleotide polymorphisms (SNPs) of the *IL23R* gene with the clinical picture of ulcerative colitis (UC).

METHODS
Genomic DNA samples of 131 patients (66 males, 65

females, mean age 55.4 ± 15.8 years) with Caucasian origin, diagnosed with UC were investigated. The diagnosis of UC was based on the established clinical, endoscopic, radiological, and histopathological guidelines. DNA was extracted from peripheral blood leukocytes by routine salting out method. Polymerase chain reaction and restriction fragment length polymorphism were used to identify the alleles of seven SNPs of *IL23R* gene (rs11209026, rs10889677, rs1004819, rs2201841, rs7517847, rs10489629, rs7530511).

RESULTS
Four out of seven analyzed SNPs had statistically significant influence on the clinical picture of UC. Two SNPs were associated with greater colonic extension (rs2201841 P = 0.0084; rs10489629 P = 0.0405). For two of the SNPs, there was more frequently need for operations (rs2201841 P = 0.0348, OR = 8.0; rs10889677 P = 0.0347, OR = 8.0). The rs2201841 showed to be a risk factor for the development of iron deficiency (P = 0.0388, OR = 6.1837). For patients with the rs10889677, a therapy with azathioprine was more frequently necessary (P = 0.0116, OR = 6.1707). Patients with rs10489629 SNP had a lower risk for weight loss (P = 0.0169, OR = 0.3394). Carriers of the heterozygous variant had a higher risk for an extended disease (P = 0.0284). The rs7517847 showed a protective character leading to mild bowel movements. Three SNPs demonstrated no statistically significant influence on any examined clinical features of UC.

CONCLUSION
We demonstrated susceptible or protective character of the investigated *IL23R* SNPs on the phenotype of UC, confirming the genetic association.

Key words: *IL23R* gene; Ulcerative colitis; Phenotype; Polymorphism; Hungarian

Core tip: *IL23R* gene plays important role in the development and influences the phenotype of inflammatory bowel diseases. We investigated the association of seven single nucleotide polymorphisms (SNPs) of *IL23R* gene with the clinical picture of ulcerative colitis (UC). Two SNPs were associated with greater colonic extension. At two SNPs, there was more frequently need for operations. Rs2201841 was found as a risk factor for the development of iron deficiency. Patients with rs10889677, therapy with azathioprine was more frequently necessary. Patients with rs10489629 SNP had lower risk for weight loss. This study demonstrated the influence of the investigated SNPs of *IL23R* on the phenotype of UC, confirming genetic association.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) represent the two most common forms of inflammatory bowel diseases (IBD). IBDs are complex diseases with suspected genetic and environmental etiology. Several IBD-associated genes were identified. Interleukin-23 receptor (*IL23R*) is one of those genetic factors found by genome-wide association studies (GWAS)[1]. Various studies suggest that the IL23-Th17 cell-axis plays an important role in the pathogenesis of IBDs.

IL23R is the human gene of the IL23-receptor located on chromosome 1p31[2]. IL23R is expressed by CD4+ T cells, monocytes/macrophages, and CD11c+ dendritic cells (DCs)[3]. IL23 is a pro-inflammatory cytokine belonging to the Interleukin 12-family[4]. It is a heterodimer consisting of subunit p19 (IL23A), and p40, a subunit of IL12B[5]. IL23 is mainly produced by activated macrophages and DCs[4].

Th17 cells differentiate from naive CD4+ Th cells under the influence of transforming growth factor beta (TGFβ) and IL6[6]. This can happen in a concentration-dependent manner. In a low concentration, TGFβ has synergistic effects with IL6 and IL21, which results in the induction of retinoid orphan nuclear receptor gamma t (Rorγt) and an increase of IL23, as well as promoting the differentiation into Th17 cells. In a high concentration, as well as in combination with IL2, TGFβ decreases the IL23-level, and raises the forkhead box protein transcription factor 3-level (Foxp-3), which is a master regulator in the differentiation of regulatory T cells (Treg). This leads to the conclusion that there might exist a fluid balance between Th17 and Treg cells[7]. Pathogenic Th17 cells seem to play an important role in autoimmunity. Overexpression of Th17-associated cytokines in the bowel tissue of IBD patients was proven[8].

IL17, a pro-inflammatory cytokine produced by Th17 cells, is produced under the influence of IL23[6,7]. It activates stromal and epithelial cells, which results in the excretion of cytokines and chemokines for chemotaxis of neutrophil leukocytes[9].

These pathways make IL23R a potential target in the treatment of various autoimmune inflammatory diseases. Ustekinumab and briakinumab, antibodies against the p40 subunit (IL12 and IL23) already demonstrated therapeutic efficiency in psoriasis, as well as in CD[10-12]. An IL12 antibody was able to induce remission in CD[13,14].

Duerr *et al*[1] were the first who identified *IL23R* as a gene associated with IBDs. They were able to provide evidence for an association in non-Jewish UC population.

In a big German cohort Glas *et al*[4] investigated interactions of IBD genes and the influence of *IL23R* on the phenotype. All of observed *IL23R* gene variants, showed a strong association to CD and a weaker association to UC. Nevertheless, eight out of ten single nucleotide polymorphisms (SNPs) showed significant association with UC. The fact that these SNPs were either protective or susceptible in both CD and UC suggests similar disease-modifying effects. Rs7517847 was the SNP with the strongest association to, and the only independent risk factor of UC. The SNP with the strongest association to CD (rs1004819) was analyzed for phenotypic correlation. Though the TT homozygous carriers showed more frequently ileal involvement and stenosis, it did not reach significance. Rs7517847 did not show any specific influence on the phenotype of UC. There was no evidence for epistasis between the *IL23R,* and three other IBD genes *CARD15/NOD2*, *SLC22A4* and *SLC22A5*. Gene-gene interactions seemed to influence the phenotype but didn't reach statistical significance[4].

Hayatbakhsh *et al*[15] were able to connect the presence of rs7517847 in UC patients with two main clinical manifestations: blood in the stool and bowel movements. In a Jiangsu Han population the SNP rs17375018 with the G allele was correlated with mild activity in UC[16]. Recent studies also suggest the SNPs of *IL23R* to be a predictive factor of response to the therapy, *e.g.*, non-response to mesalazine and corticosteroids as well as higher response rate for azathioprine and infliximab[17,18].

The aim of our present study was to investigate the association of seven SNPs of *IL23R* (rs11209026, rs10889677, rs1004819, rs2201841, rs7517847, rs10489629, rs7530511) with the clinical picture of UC in Hungarian patients diagnosed with UC.

MATERIALS AND METHODS

Patients
We examined 131 Hungarian patients of Caucasian origin (66 males, 65 females, mean age 55.4 ± 15.8 years) diagnosed with UC. The origin of the DNA samples was the central Biobank, governed by the University of Pecs, as part of the National Biobank Network of Hungary (www.biobanks.hu), which belongs to the pan-European Biobanking and Bio-molecular Resources Research Infrastructure preparatory phase project (http://bbmri.eu/bbmri/). The governance, maintenance and management principles of the Biobank had been approved by the National Scientific Research Ethics Committee (ETT TUKEB). Clinical data guidelines and regulations of the local Ethics Committee and Helsinki Declaration in 1975 were followed during collection and use of DNA samples. At blood collection patients gave their informed consent for the future use of their anonymized DNA.

The diagnosis of UC was based on the established clinical, endoscopic, radiological, and histopathological guidelines. Patients with indeterminate colitis were excluded from the study.

Genotyping
DNA was extracted from peripheral blood leukocytes by routine salting out method. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to identify the alleles of the *IL23R* gene. The PCR amplifications were performed on MJ Research PTC-200 thermal cyclers (Bio-Rad LTD., Budapest, Hungary). Amplification included an initial denaturation step (96 ℃ for 2 min) followed by 35 cycles of denaturation (95 ℃ for 30 s), annealing for 30 s at 54 ℃ (rs1004819); 60 ℃ for 45 s (rs10889677 and rs7530511); 55 ℃ for 45 s (rs7517847, rs11209026 and rs10489629); 72 ℃ for 30 s (rs2201841), primer extension at 72 ℃ for 45 s and final extension at 72 ℃ for 5 min. Each polymerase chain reaction contained 200 μmol/L of each dNTP, 1 unit of Taq polymerase, 5 μL of reaction buffer (100 mmol/L Tris HCl, pH = 9.0; containing 500 mmol/L KCl, 15 mmol/L MgCl$_2$), 0.2 μmol/L of each primer and 1 μL DNA to be amplified in a final volume of 50 μL. The amplicons were digested by allelespecific restriction endonucleases *TaaI* (rs1004819), *HpyF3I* (rs2201841), *MnlI* (rs10889677), *BseMII* (rs7517847), *HphI* (rs7530511), *Hpy188I* (rs11209026) and *SspI* (rs10489629). The amplicon contained an obligate cleavage site of the restriction enzyme for the suitable visual control of the efficacy of the digestion. The restriction fragments were separated by electrophoresis on 3% agarose gels containing ethidium bromide, and visualized by UV transillumination.

Statistical analysis
The investigated phenotypic parameters included severity (age of manifestation, extension, frequency of relapse, blood in stool, bowel movements), complications (fever, weight loss, anemia, iron deficiency, hypoalbuminemia, need for operation), extraintestinal manifestation (in eyes, joints, skin), medication (aminosalicylate, azathioprine, corticosteroids), as well as familial cases of IBDs and colorectal cancer.

For the age of manifestation, three groups were formed analogous to the Montreal classification of CD (group 1: younger than 17 years, group 2: age between 17 and 40 years, group 3: older than 40 years). The extension of the disease along the colon was defined accordingly to the E-stages of the Montreal classification for UC (E1: involvement limited to the rectum, E2: involvement limited to distal of splenic flexure, E3: involvement extends to proximal to the splenic flexure)[19]. For analyzing the frequency of bowel movements, the patients were divided in three groups (group 1: one up to three a day, group 2: four up to ten a day, group 3: more than ten a day).

Data were examined for independence. Null hypothesis was formulated as follows: the phenotype of UC is independent of the genotype of *IL23R*.

Table 1 Genotypes of the investigated *IL23R* single nucleotide polymorphisms in Hungarian population *n* (%)

SNP	Genotype	UC	Allele	UC
rs11209026	GG	109 (95.6)	G	223 (97.8)
	AG	5 (4.4)	A	5 (2.2)
	AA	0 (0.0)		
	total	114		
	RAF	0.0219		
rs10889677	CC	51 (45.5)	C	155 (69.2)
	AC	53 (47.3)	A	69 (30.8)
	AA	8 (7.1)		
	total	112		
	RAF	0.308		
rs1004819	GG	52 (40.3)	G	172 (66.7)
	AG	68 (52.7)	A	86 (33.3)
	AA	9 (7.0)		
	total	129		
	RAF	0.333		
rs2201841	TT	58 (45.0)	T	176 (68.2)
	CT	60 (46.5)	C	82 (31.8)
	CC	11 (8.5)		
	Total	129		
	RAF	0.317		
rs7517847	TT	40 (35.7)	T	136 (60.7)
	GT	56 (50.0)	G	88 (39.3)
	GG	16 (14.3)		
	Total	112		
	RAF	0.392		
rs10489629	GG	44 (34.6)	G	155 (61.0)
	AG	67 (52.8)	A	99 (39.0)
	AA	16 (12.6)		
	Total	127		
	RAF	0.389		
rs7530511	CC	94 (73.4)	C	220 (85.9)
	TC	32 (25.0)	T	36 (14.1)
	TT	2 (1.6)		
	Total	128		
	RAF	0.140		

RAF: Risk allele frequency; SNP: Single nucleotide polymorphism; UC: Ulcerative colitis.

2 × 3 or 3 × 3 contingency tables were created depending on the attribute. For characteristics that were tested for presence or lack of presence, 2 × 3 contingency tables were created. For characteristics divided in three subsets such as age of onset, Montreal-classification, frequency of relapse and bowel movements, 3 × 3 contingency tables were generated. Genotype was partitioned into wild type (Wt), heterozygous (Hz) and homozygous (Ho) susceptible SNP.

The distribution was tested in total (wild type + heterozygous + homozygous) as well as separated and regrouped. χ^2-test was performed if the expected value was not lower than five in at least 80% of the cells. In any other case Fishers exact test was performed. For χ^2-test SPSS Statistics 22.0 was used (SPSS Inc., Chicago, IL, United States). SPSS performs Fishers exact test for 2 × 2 contingency tables. Two other calculators were used, available on vassarstat.net and in-silico.net. Results were verified using Kruskal-Wallis-Test. Odds Ratios were determined only for 2 × 3 contingency tables.

χ^2 test was accomplished using SPSS Statistics

RESULTS

The frequencies of the genotypes and alleles are shown in Table 1.

At the investigation of rs11209026, out of 114 subjects 109 (95.6%) carried the wild type (GG), while 5 (4.4%) were heterozygous (GA). The AA homozygous variant did not appear in this population. That is not surprising, considering a mean allele frequency of A = 0.0219. Regarding the low number of the SNP no significant results could have been expected.

For rs1004819, out of 129 subjects, 52 (40.3%) were wild type carriers (GG), while 68 (52.7%) carried the heterozygous (GA) and 9 (7%) the homozygous (AA) variant. For this polymorphisms no significant results were observed.

Out of 129 patients, 58 (40.0%) were carrier of the wild type (TT) of rs2201841, 60 (46.5%) carried the heterozygous (TC) and 11 (8.5%) the homozygous (CC) variant. Three characteristics reached level of significance: Montreal-Classification, appearance of iron deficiency and need for surgery (Figure 1). The Montreal-Classification and iron deficiency reached significance for the total distribution (Wt + Hz + Ho). Heterozygous carriers had significant higher risk to require surgery compared to the wild type (*P* = 0.0348, OR = 8.0). The distribution of the Montreal-Classification is shifted to greater extension for carriers of the heterozygous variant. Total distribution reached level of significance (*P* = 0.0084). This was verified by comparison of the obtained and expected values. The significance was mainly caused by the heterozygous TC variant (*P* = 0.0429).

Carriers of the rs2201841 had significant higher risk for iron deficiency (*P* = 0.0299). When Hz and Ho SNPs were combined and compared with the Wt, no significance was observed (*P* = 0.7476). Statistically significance was found when Wt and Hz SNPs were merged and compared with the Ho variant (*P* = 0.0388, OR = 6.1837). Surgery was more frequently needed for heterozygous carriers of rs2201841 (*P* = 0.0348, OR = 8.0). The homozygous form seemed to have no influence on the need for operations.

Out of 112 patients, 40 (35.7%) carried the wild type (TT), 56 (50%) the heterozygous (TG) and 16 (14.3%) the homozygous (GG) rs7517847 SNP. Frequency of bowel movements was significant (*P* = 0.0078). If instead of three (< 4, 4-10, > 10) just two groups were used (< 3 and > 3 defecations per day), the distribution was still significant (*P* = 0.0358). If homozygous and heterozygous SNP were added together and compared to the wild type, no significant difference was found (*P* = 0.0634). Significance was caused by the heterozygous variant compared to the wild type (*P* = 0.0050). The rs7517847 seems to have
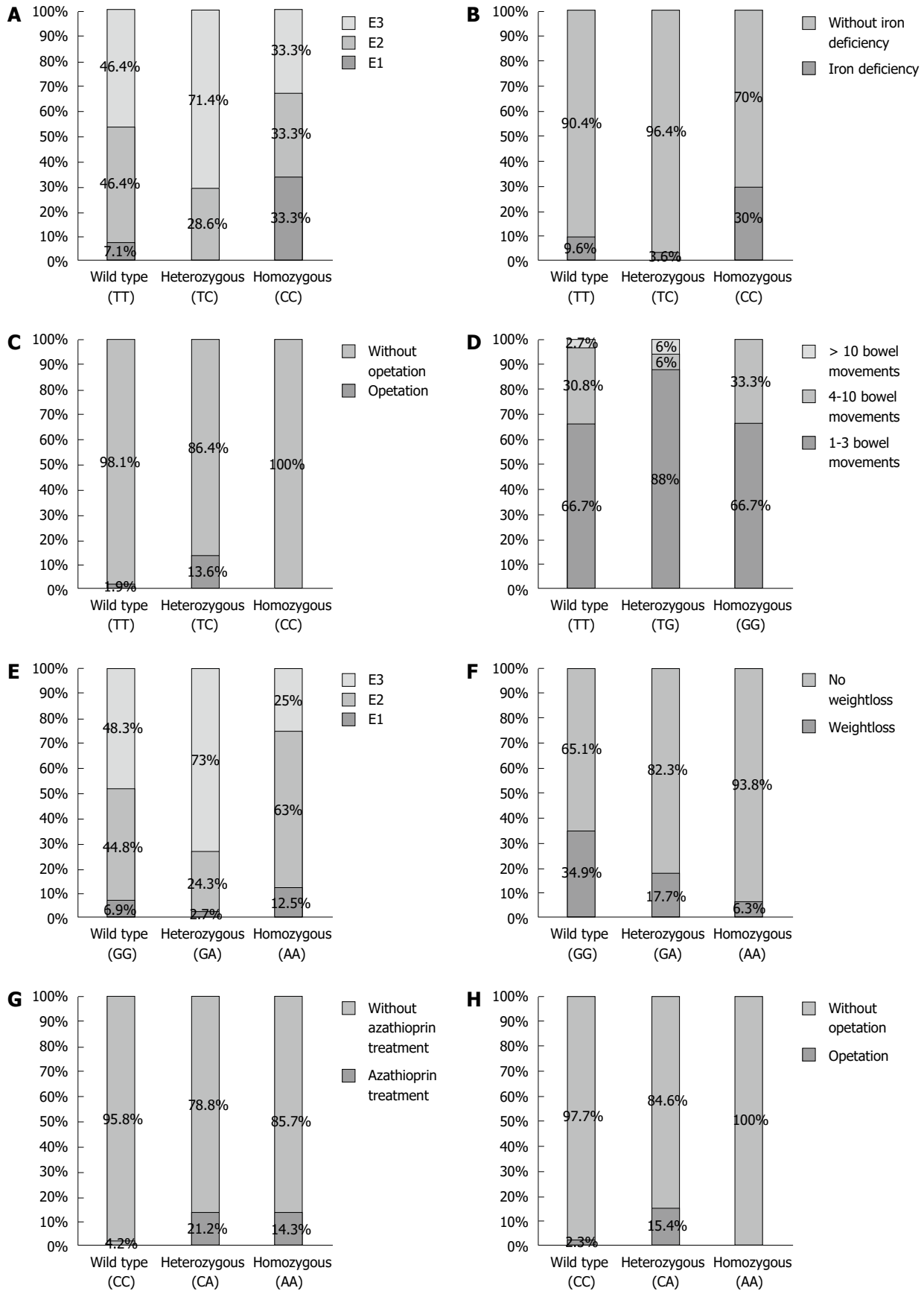
Figure 1 Distribution of the phenotype characteristics for the *IL23R* single nucleotide polymorphisms. A: Distribution of Montreal-classification (E1-3) for rs2201841; B: Summary of the occurrence and non-occurrence of iron deficiency for rs2201841; C: Representation of the number of operations regarding to rs2201841; D: Representation of stool frequency in three categories for rs7517847; E: Distribution of Montreal-classification (E1-3) on the alleles of rs10489629; F: Distribution of patients with and without weight loss to the alleles of rs10489629; G: Need to azathioprine therapy in respect to rs10889677; H: Representation of the number of operations regarding to rs10889677.

Table 2 *IL23R* single nucleotide polymorphisms with statistically significant influence on the clinical picture of ulcerative colitis

SNP	Wt *vs* Hz	Wt *vs* Ho	Wt *vs* Hz + Ho	Wt + Hz *vs* Ho	Total distribution
rs2201841					
Montreal-classification	0.0429[a]	0.2270	0.1921	0.0217[a]	0.0084[a]
Iron deficiency	0.1892	0.1108	0.7476	0.0388[a]	0.0299[a]
Need for operation	0.0348[a]	1.0000	0.0766	0.6017	0.0564
rs7517847					
Bowel movements	0.0050[a]	0.8183	0.0634	0.2979	0.0078[a]
rs10489629					
Montreal classification	0.1017	0.3906	0.4445	0.1170	0.0405[a]
Weight loss	0.0652	0.0457[a]	0.0169[a]	0.1191	0.0350[a]
rs10889677					
Azathioprine	0.0116[a]	0.3407	0.0136[a]	0.6113	0.0285[a]
Need for operation	0.0347[a]	0.8491	0.0746	0.6153	0.0595

Indicates significant difference ([a]$P < 0.05$). Hz: Heterozygous; Ho: Homozygous; Wt: Wild type; Total distribution: Wt + Hz + Ho; SNP: Single nucleotide polymorphism.

a protective character and prevent its carrier from severe bowel movements.

Analyzing the rs7530511 SNP, 94 (73.4%) of 129 patients carried the wild type (CC), 32 (25.0%) the heterozygous (CT) and 2 (1.6%) the homozygous (TT) variant. No significant results were observed.

Out of 127 patients 44 (34.6%) carried the wild type (GG), 67 (52.8%) the heterozygous (GA) and 16 (12.6%) the homozygous (AA) variant of rs10489629 SNP. Total distribution (Wt + Hz + Ho) of the Montreal-Classification was significant ($P = 0.0405$). Carrying the Hz and Ho SNP alone didn't reach level of significance. Carriers of the heterozygous variant had a higher risk for an extended disease ($P = 0.0284$). For weight loss significance was observed ($P = 0.035$). If wild type was compared with heterozygous and homozygous SNP together (Hz + Ho) significance was reached ($P = 0.0169$, OR = 0.3394). The homozygous variant showed significance ($P = 0.0457$, OR = 0.1244). Patients who carried the heterozygous susceptible SNP had numerous but statistically not significant lower risk of weight loss ($P = 0.0652$).

In the case of rs10889677 SNP, out of 112 patients, 51 (45.5%) carried the wild type (CC), 53 (47.3%) the heterozygous (CA) and 8 (7.1%) the homozygous (AA) variant. Carriers of the susceptible SNP needed more frequently azathioprine treatment ($P = 0.0285$). If wild type was compared with the heterozygous and homozygous SNP, level of significance was found ($P = 0.0136$, OR = 5.8732). The heterozygous variant also leads to a higher risk for need for surgery ($P = 0.0347$, OR = 8.0).

Summarized four out of seven SNPs had a statistically significant influence on the phenotype of UC. The results are shown in Table 2.

The rs2201841 showed higher stage of extension along the colon ($P = 0.0084$). The CC homozygous carriers had a higher risk for iron deficiency ($P = 0.0388$, OR = 6.183). Patients with the heterozygous genotype needed more often operation ($P = 0.0348$, OR = 8.0).

Patients with the rs7517847 GT heterozygous variant suffered less from bowel movements ($P = 0.005$).

The rs10489629 SNP in heterozygous form was connected to greater colonic extension ($P = 0.0405$). Both the heterozygous and homozygous from this variant were associated with lower risk of weight loss ($P = 0.0169$, OR = 0.3394). In case of rs10889677, therapy with azathioprine was more often necessary for patients carrying the heterozygous and the homozygous SNP ($P = 0.0116$, OR = 6.1707). Heterozygous carriers showed higher risk for the need of operations ($P = 0.0347$, OR = 8.0).

The reason for the statistical significance only to appear for the heterozygous genotype in some cases can be attributable to low numbers of homozygous carriers.

The variants rs11209026, rs1004819, and rs7530511 had no statistical significant influence on the phenotype of UC.

DISCUSSION

Several recent studies suggest *IL23R* to be a suspect in the pathogenesis of diverse autoimmune diseases such as IBDs[1,4,20-25], psoriasis[11,22,26], Graves disease[27], ankylosing spondylitis[28,29], and rheumatoid arthritis[26,29-31]. Previous investigations in Hungarian populations verified *IL23R* to play a role in the development of UC[25,32]. The present study demonstrated the correlation between the SNPs of *IL23R,* and the phenotype of UC.

Two of the susceptible SNPs for the development of UC (rs2201841, rs10889677) seem to shift the clinical picture from mild into more severe. The rs10889677 was associated with azathioprine-therapy, suggesting the patients to be refractory to 5-aminosalicylic-acid (5-ASA). These results are analogous to the findings of Cravo *et al*[18]. Interestingly one risk-polymorphism (rs10489629) had risk-conferring (greater extension), and protective features (lower risk of weight loss).

The reason for the protective and harming character of rs10489629 remains unclear. A possible connection could be a more frequent need for steroids because of the greater extension of the disease along the colon, leading to a lower risk for weight loss. This study did investigate the need for corticosteroids but not the administration frequency. No significant higher need for steroids could been shown. The rs7517847 variant showed a protective character (less bowel movements). Rs7517847 has shown to protect the individual from acquiring the disease[4], assuming a general protective character of this polymorphism.

An Iranian study (Hayatbakhsh *et al*[15], 2012) demonstrated the protective influence of rs7517847 on bowel movements, and blood in stool, the two important features of UC. We were partly able to reproduce these results for the examined Hungarian population. The rs1004819 showed no influence on these characteristics, neither in the Iranian nor the present study. Chinese research detected similar results suggesting the rs17375018 SNP to have a protective character[20]. There are studies that suggest *IL23R* to be a risk factor for the development of extraintestinal manifestations[18]. This was not reproducible in our study with Hungarian UC patients.

The data of the present study are in contrast to the results of Duerr *et al*[1] and Glas *et al*[4], who did find an association between *IL23R* and inflammatory bowel diseases but no influence on the phenotype of ulcerative colitis.

More studies are necessary to clarify the exact role of *IL23R* in the development of IBDs, as well as other autoimmune diseases, to improve the knowledge about their pathogenesis and pathophysiology. This may allow individual risk stratification, individual pharmacotherapy, and new approaches for medication with targeted therapy.

ACKNOWLEDGMENTS

COMMENTS

Background

Inflammatory bowel diseases (IBD) are complex diseases with suspected genetic and environmental etiology. Interleukin-23 receptor (*IL23R*) is one of those genetic factors found by genome-wide association studies. IL23R is expressed by CD4+ T cells, monocytes/macrophages, and CD11c+ dendritic cells. IL23 is a pro-inflammatory cytokine which has potential target in the treatment of various autoimmune inflammatory diseases. Duerr *et al* were the first who identified *IL23R* as a gene associated with IBDs. They were able to provide evidence for an association in non-Jewish ulcerative colitis (UC) population. In a big German cohort Glas *et al* (2007) investigated interactions of IBD genes and the influence of *IL23R* on the phenotype. Eight out of ten single nucleotide polymorphisms (SNPs) showed significant association with UC. The fact that these SNPs were either protective or susceptible in both Crohn's disease and UC suggests similar disease-modifying effects. Recent studies also suggest the SNPs of *IL23R* to be a predictive factor of response to the therapy, *e.g.*, non-response to mesalazine and corticosteroids as well as higher response rate for azathioprine and infliximab.

Research frontiers

Previous investigations in Hungarian populations verified *IL23R* to play a role in the development of UC. The present study demonstrated the correlation between the SNPs of *IL23R*, and the phenotype of UC.

Innovations and breakthroughs

Two of the susceptible SNPs for the development of UC (rs2201841, rs10889677) seem to shift the clinical picture from mild into more severe. The rs10889677 was associated with azathioprine-therapy, suggesting the patients to be refractory to 5-aminosalicylic-acid. The rs7517847 variant showed a protective character (less bowel movements). Rs7517847 has shown to protect the individual from acquiring the disease, assuming a general protective character of this polymorphism.

Applications

These results may allow individual risk stratification, individual pharmacotherapy, and new approaches for medication with targeted therapy. However, more studies are necessary to clarify the exact role of *IL23R* in the development of IBDs.

Peer-review

The study is well conducted, the results are interesting but, as the number of patients is not very high, the data should be validated in another cohort in a future study.

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ORIGINAL ARTICLE

Basic Study

Role of LAP+CD4+ T cells in the tumor microenvironment of colorectal cancer

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Abstract

AIM

To investigate the abundance and potential functions of LAP+CD4+ T cells in colorectal cancer (CRC).

METHODS

Proportions of LAP+CD4+ T cells were examined in peripheral blood and tumor/paratumor tissues of CRC patients and healthy controls using flow cytometry. Expression of phenotypic markers such as forkhead box (Fox)p3, cytotoxic T-lymphocyte-associated protein (CTLA)-4, chemokine CC receptor (CCR)4 and CCR5 was measured using flow cytometry. LAP-CD4+ and LAP+CD4+ T cells were isolated using a magnetic cell-sorting system and cell purity was analyzed by flow cytometry. Real-time quantitative polymerase chain reaction was used to measure expression of cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β.

RESULTS

The proportion of LAP+CD4+ T cells was significantly higher in peripheral blood from patients (9.44% ± 3.18%) than healthy controls (1.49% ± 1.00%, $P < 0.001$). Among patients, the proportion of LAP+CD4+ T cells was significantly higher in tumor tissues (11.76% ± 3.74%) compared with paratumor tissues (3.87% ± 1.64%, $P < 0.001$). We also observed positive correlations between the proportion of LAP+CD4+ T cells and TNM stage ($P < 0.001$), distant metastasis ($P < 0.001$) and serum level of carcinoembryonic antigen ($P < 0.05$). Magnetic-activated cell sorting gave an overall enrichment of LAP+CD4+ T cells (95.02% ± 2.87%), which was similar for LAP-CD4+ T cells (94.75% ± 2.76%). In contrast to LAP-CD4+ T cells, LAP+CD4+ T cells showed lower Foxp3 expression but significantly higher levels of CTLA-4, CCR4 and CCR5 ($P < 0.01$). LAP+CD4+ T cells expressed significantly larger amounts of IL-10 and TGF-β but lower levels of IL-2, IL-4, IL-17 and interferon-γ, compared with LAP-CD4+ T cells.

CONCLUSION

LAP+CD4+ T cells accumulated in the tumor microenvironment of CRC patients and were involved in immune evasion mediated by IL-10 and TGF-β.

Key words: LAP+CD4+ T cells; Colorectal cancer; Tumor microenvironment; Interleukin-10; Transforming growth factor-β

Core tip: Many carcinomas, including colorectal cancer, gastric and nasopharyngeal cancer, are associated with elevated numbers of T regulatory (Treg) cells. It is suggested that Treg cells promote tumor development and metastasis by inhibiting the proliferation of effector T lymphocytes. LAP+CD4+ T cells, a recently identified subset of CD4+ Treg cells, have 50-fold more potent immunosuppressive ability than traditional CD4+CD25+ T cells. Here, we present several lines of evidence correlating LAP+CD4+ T cells with colorectal cancer progression.

INTRODUCTION

Colorectal cancer (CRC) is the third most common carcinoma in men and second most common in women, with > 1 million new cases and > 500000 deaths every year worldwide[1,2]. CRC progression is a complex process involving interactions between host cellular immunity factors and the tumor, which take place in the so-called tumor microenvironment[3,4]. This environment includes numerous factors that promote tumor growth, such as energy and nutrients in blood vessels, growth factors from immune cells and stromal cells, and proinflammatory mediators secreted by tumor cells[5]. The environment also contains numerous factors that can limit tumor growth, such as tumor-infiltrating immune cells and tertiary lymphoid structures[6]. This complex mixture of factors largely determines patient prognosis and serves as an attractive therapeutic target[6,7].

Several studies have suggested that during CRC progression, peripheral regulatory T (Treg) cells and myeloid suppressor cells increase in the tumor microenvironment, which is associated with worse prognosis[8-10]. Part of the reason appears to be that these cell populations counteract the host's antitumor immune response[11]. Downregulating Treg cells can render antitumor responses more effective, which may improve prognosis in patients with CRC and other malignant carcinomas[12].

LAP+CD4+ T cells are a newly identified subset of Treg cells that express latent-associated peptide (LAP), and function within the latent transforming growth factor (TGF)-β complex to block interaction between TGF-β and receptors on immune cells[13]. Among the various Treg cell populations, LAP+CD4+ T cells are endowed with more potent immunosuppressive function than traditional CD4+CD25+Foxp3+ Treg cells[14], and they are associated with autoimmune disease progression[13,15-17]. However, we are unaware of studies examining whether LAP+CD4+ T cells contribute to CRC progression. Thus, we analyzed the abundance, phenotype and cytokine secretion of LAP+CD4+ T cells in the tumor microenvironment in patients with CRC.

MATERIALS AND METHODS

Ethics statement

All patients enrolled in this study provided written informed consent. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (Fortaleza, Brazil; October 2013), and it was approved by the Research Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, China.

Study participants and samples

This study involved 50 patients who underwent primary tumor resection for colorectal adenocarcinoma at the First Affiliated Hospital of Guangxi Medical University from January to August 2014. Samples of peripheral blood were obtained preoperatively, and colorectal tumor and paratumor tissues were obtained postoperatively from each patient. Paratumor tissue samples were taken from tissue near the

Table 1 Clinical characteristics of patients with colorectal cancer

Characteristics	Value[1]
Male	31
Female	19
Age, yr	57.4 (37-76)
Location of primary tumor	
Colon	22
Rectum	28
TNM stage	
I / II	23
III / IV	27

[1]Values are n or mean (range).

Table 2 Primer sequences for polymerase chain reaction

Gene	Sequence (5'-3')	Product (bp)	T (℃)
IL-2	F:5' CAGCTACAACTGGAGCATTTAC R:5' TCAGTTCTGTGGCCTTCTTG	130	60
IL-4	F:5' GACCGTAACAGACATCTTTGC R:5' TCGAGCCGTTTCAGGAAT	180	60
IL-10	F:5' TTGCCAAGCCTTGTCTGA R:5' ACAGGGAAGAAATCGATGAC	160	60
IL-17	F:5' CCTCAGAGATCAACAGACCAA R:5' GGTGCCTTGATCAGACAGAA	80	60
IFN-γ	F:5' GGCAAGGCTATGTGATTACA R:5' TAAAGCACTGGCTCAGATTG	180	60
TGF-β1	F:5' CACGTGGAGCTGTACCAGAA R:5' GAACCCGTTGATGTCCACTT	219	60
18srRNA	F:5' CCTGGATACCGCAGCTAGGA R:5' GCGGCGCAATACGAATGCCCC	112	60

resection margin ($\geqslant$ 10 cm away from the tumor site) that was confirmed to be tumor-free based on routine pathology. The basic data regarding the study population are shown in Table 1.

Patients were excluded if they (1) had already undergone CRC surgery or had been diagnosed with locoregional recurrence; or (2) were receiving any anticancer therapy, corticosteroids or other nonsteroidal anti-inflammatory drugs at the time of peripheral venous blood collection.

During the study period, peripheral blood was also collected from 25 healthy donors serving as a control group. Healthy controls were free of chronic pain, cardiovascular complaints, or other chronic inflammatory diseases. They were matched with patients in age and sex and showed no significant differences from patients.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from patients using Ficoll density gradient centrifugation. Fresh tumor and paratumor samples were washed three times in RPMI 1640; after which, fatty, connective and necrotic tissues were removed. Samples were cut into 1-2-mm cubes, transferred to a 50-mL beaker, and incubated for 3 h at room temperature with a triple-enzyme digestion medium containing 1 mg/mL collagenase IV, 30 μg/mL DNase I and 0.1 mg/mL hyaluronidase (Sigma, St. Louis, MO, United States). Dissociated cell suspensions were filtered through a 70-μm nylon mesh, then tumor-infiltrating lymphocytes (TILs) were isolated from cell suspensions using discontinuous density gradient centrifugation[18]. LAP-CD4+ T cells and LAP+CD4+ T cells were isolated using a Magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was analyzed by flow cytometry as described below.

Flow cytometry

TILs and PBMCs were stimulated in culture for 4 h at 37 ℃ with 50 ng/mL phorbol-12-myristate-13-acetate,

1 μg/mL ionomycin, and 0.7 μL/mL GolgiStop reagent in a 5% CO_2 incubator. T cells were identified based on surface or intracellular expression of markers labeled using antibodies (eBioscience, San Diego, CA, United States) against the following human antigens: LAP, CD4, forkhead box (Fox)p3, cytotoxic T-lymphocyte-associated protein (CTLA)-4, chemokine CC receptor (CCR)4, and CCR5. Antibodies were conjugated with one of the following fluorophores: phycoerythrin (PE), fluorescein isothiocyanate, PEcy5.5, PEcy7, peridinin chlorophyll protein (PerCP)-cy5.5, or allophycocyanin. Labeled cell suspensions were analyzed using a FACS Calibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, United States) and FlowJo software (Tree Star, Ashland, OR, United States).

Real-time quantitative polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), and first-strand cDNA was generated using oligo (dT) primers and the SuperScript III First-Strand Synthesis System (Invitrogen). Levels of mRNAs encoding cytokines secreted by LAP+CD4+ T cells and LAP-CD4+ T cells (TGF-β, INF-γ, IL-2, IL-4, IL-10 and IL-17) were determined using SYBR-based real-time polymerase chain reaction (7500 StepOnePlus system, Applied Biosystems, Carlsbad, CA, United States) and primers purchased from TaKaRa Biosystems (Table 2). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to levels of β-actin mRNA.

Statistical analysis

Data were expressed as mean ± SD. Differences between two groups were assessed for significance using the Mann-Whitney *U* test, *t*-test, or paired *t*-test, as appropriate. All statistical tests were performed using SPSS version 16.0 (SPSS, Chicago, IL, United States), and the threshold of significance was defined as *P* < 0.05.

Table 3 Proportions of LAP⁺CD4⁺ T cells in tumor tissues and in relation to clinicopathological characteristics

	n	LAP⁺CD4⁺ Treg (%)	t	P value	95%CI
Age, yr					
< 60	29	11.15 ± 2.03	0.747	0.458	-1.35-2.94
≥ 60	21	11.96 ± 4.51			
Sex					
Male	31	11.37 ± 3.24	0.444	0.659	-1.60-2.50
Female	19	11.82 ± 3.89			
Location					
Colon	22	10.35 ± 3.45	0.652	0.517	-1.11-2.18
Rectum	28	11.89 ± 3.17			
TNM stage					
I / II	23	8.45 ± 2.98	4.973	0.000	3.85-9.07
III / IV	27	14.90 ± 5.58			
Pathological pattern					
Tubular/ papillary	43	11.09 ± 3.54	1.335	0.188	-0.73-3.60
Myxoma/ ring cell	7	12.83 ± 3.26			
Differentiation					
High	42	10.50 ± 3.22	0.877	0.385	-1.45-3.70
Low	8	12.43 ± 3.87			
Metastasis					
Yes	9	12.51 ± 4.17	4.322	0.000	2.49-6.82
No	41	7.85 ± 2.61			
Ileus					
Yes	9	12.22 ± 3.49	0.904	0.470	-1.30-3.44
No	41	11.15 ± 3.14			
CEA (ng/mL)					
≤ 5	34	9.94 ± 3.15	2.692	0.010	0.81-5.58
> 5	16	13.13 ± 4.06			
CA199 (U/mL)					
≤ 37	32	11.34 ± 3.21	0.854	0.370	-1.51-3.85
> 37	18	12.42 ± 4.35			

RESULTS

LAP⁺CD4⁺ T cells are elevated in PBMCs and tumor tissue of CRC patients

PBMCs were isolated preoperatively and TILs were isolated postoperatively from patients who underwent radical resection for CRC. To understand further the roles of LAP⁺CD4⁺ T cells in the tumor microenvironment in patients with CRC, the proportion of LAP⁺CD4⁺ T cells in PBMCs and tissues was detected by flow cytometry (Figure 1). The proportion of LAP⁺CD4⁺ T cells was significantly higher in peripheral blood from patients (9.44% ± 3.18%) than healthy controls (1.49% ± 1.00%, *P* < 0.001; Figure 1B and C). Among CRC patients, the proportion of LAP⁺CD4⁺ T cells was significantly higher in tumor tissue (11.76% ± 3.74%) compared with paratumor tissue (3.87% ± 1.64%, *P* < 0.001; Figure 1B and D).

Relationship between proportion of LAP⁺CD4⁺ T cells in tumor tissues and clinicopathological characteristics of CRC

We also observed positive correlations between the proportion of LAP⁺CD4⁺ T cells and TNM stage (*P* < 0.001; Figure 2A), distant metastasis (*P* < 0.001;

Figure 2B) and serum level of carcinoembryonic antigen (CEA) (*P* < 0.05; Figure 2C) (Table 3).

LAP⁺CD4⁺ Treg cell phenotype in CRC microenvironment

Further studies of phenotypic marker expression revealed differences between LAP⁺CD4⁺ T cells and LAP⁻CD4⁺ T cells. In contrast to LAP⁻CD4⁺ T cells, LAP⁺CD4⁺ T cells showed lower Foxp3 expression but significantly higher levels of CTLA-4, CCR4 and CCR5 (*P* < 0.01; Figures 3 and 4).

Magnetic-activated cell sorting in vitro

Magnetic-activated cell sorting gave an overall enrichment of LAP⁺CD4⁺ T cells (95.02% ± 2.87%; Figure 3A) and enrichment was similar for LAP⁻CD4⁺ T cells (94.75% ± 2.76%; Figure 3B).

Cytokine expression

The expression levels of cytokine profiles were measured by real-time qPCR, LAP⁺CD4⁺ T cells expressed significantly larger amounts of IL-10 and TGF-β but lower levels of IL-2, IL-4, IL-17 and IFN-γ, compared with LAP⁻CD4⁺ T cells (Table 4).

DISCUSSION

Many carcinomas, including colorectal, gastric and nasopharyngeal cancer, are associated with elevated numbers of Treg cells[19-21], and it is suggested that Treg cells promote tumor development and metastasis by inhibiting the proliferation of effector T lymphocytes[22]. LAP⁺CD4⁺ T cells, a recently identified subset of CD4⁺ Treg cells, have 50-fold more potent immunosuppressive activity than traditional CD4⁺CD25⁺ T cells[13,23]. Here we present several lines of evidence correlating LAP⁺CD4⁺ T cells with CRC progression. These cells were more abundant in peripheral blood and tumor tissue from patients with CRC compared with healthy controls. In CRC patients, the abundance of these cells correlated positively with TNM stage, metastasis, and serum level of CEA. CEA is the most widely used serum marker and is related to the prognosis of patients with CRC. The main use of CEA in CRC is in surveillance following curative resection for primary cancer[24,25]. These results suggest that LAP⁺CD4⁺ T cells, like traditional CD4⁺CD25⁺ Treg cells, accumulate in the tumor microenvironment and postoperative monitoring of the LAP⁺CD4⁺ T cells in CRC patients may be useful for assessing prognosis and predicting distant metastasis.

In our study, expression of CCR4 and CCR5 was higher in LAP⁺CD4⁺ T cells than in LAP⁻CD4⁺ T cells. CCR4 and CCR5 are highly expressed in tumor microenvironments and appear to act as proinflammatory cytokine receptors[26,27]. Some studies have reported that CCR4 and its ligands are associated with increased tumor recurrence and impaired overall survival in patients with gastric cancer[28,29]. Wang *et al*[30] have shown that the CCL5/CCR5 axis

**Table 4 Cytokine expression by LAP+CD4+ T cells and LAP-CD4+ T cells in colorectal cancer microenvironment**

	IL-2	IL-4	IL-10	IL-17	IFN-γ	TGF-β
LAP+CD4+	0.22 ± 0.01	0.32 ± 0.12	1.13 ± 0.23	0.38 ± 0.10	0.18 ± 0.08	1.40 ± 0.15
LAP-CD4+	1.49 ± 0.37	0.86 ± 0.23	0.86 ± 0.22	0.98 ± 0.23	0.69 ± 0.21	0.89 ± 0.11
t	8.811	5.505	-2.327	6.435	5.981	-7.316
P value	0.000	0.000	0.038	0.000	0.000	0.000
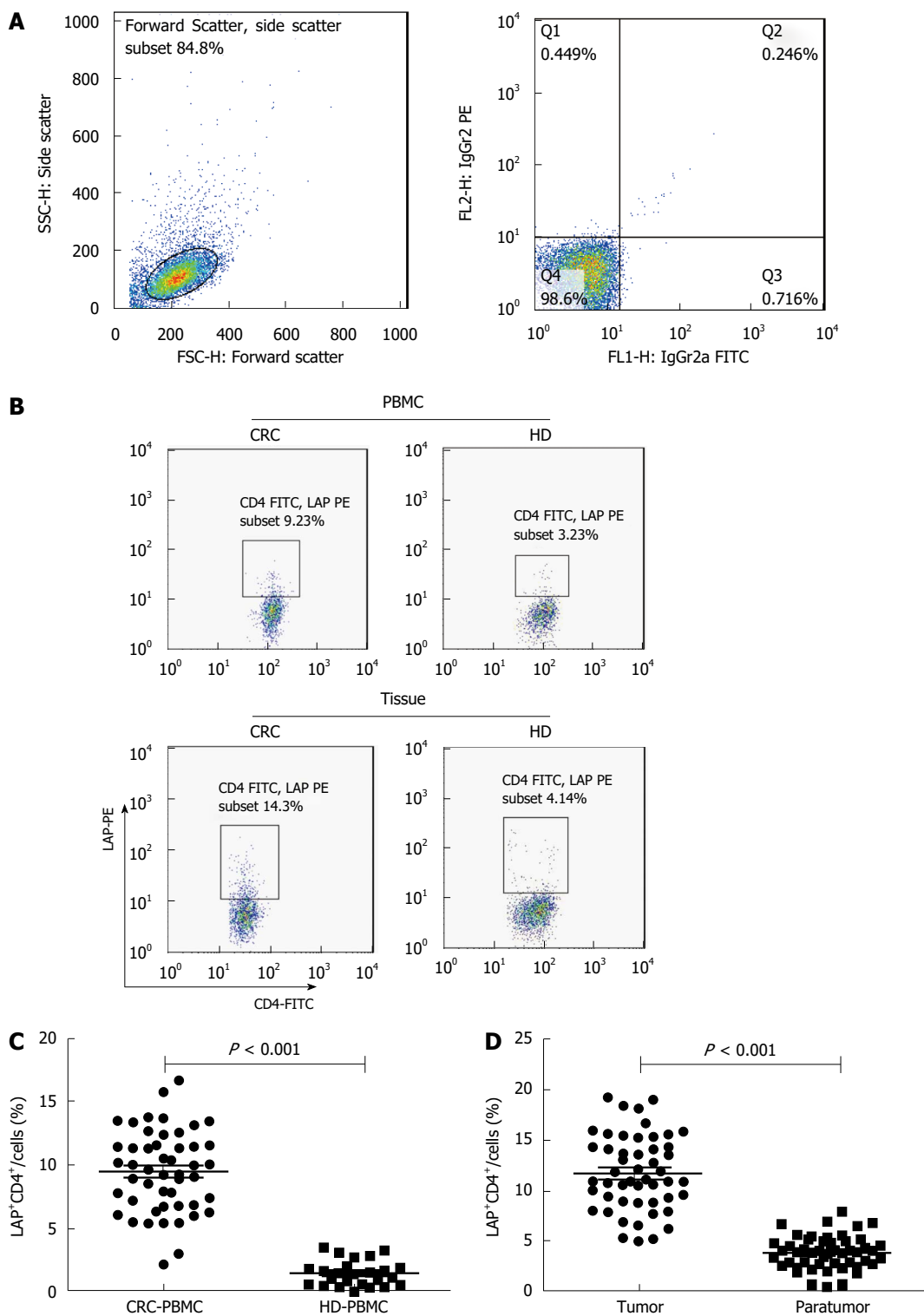
Figure 1 Abundance of LAP+CD4+ T cells in the colorectal cancer microenvironment based on flow cytometry. A: Gated on FSC/SSC, the proportion of LAP+CD4+ T cells in the CD4+ subset is presented in quadrant Q2; B: Flow cytometry to measure the proportion of LAP+CD4+ T cells in PBMCs and tissues; C: Proportion of LAP+CD4+ T cells in PBMCs; D: Proportion of LAP+CD4+ T cells in tissues.

A

B

C
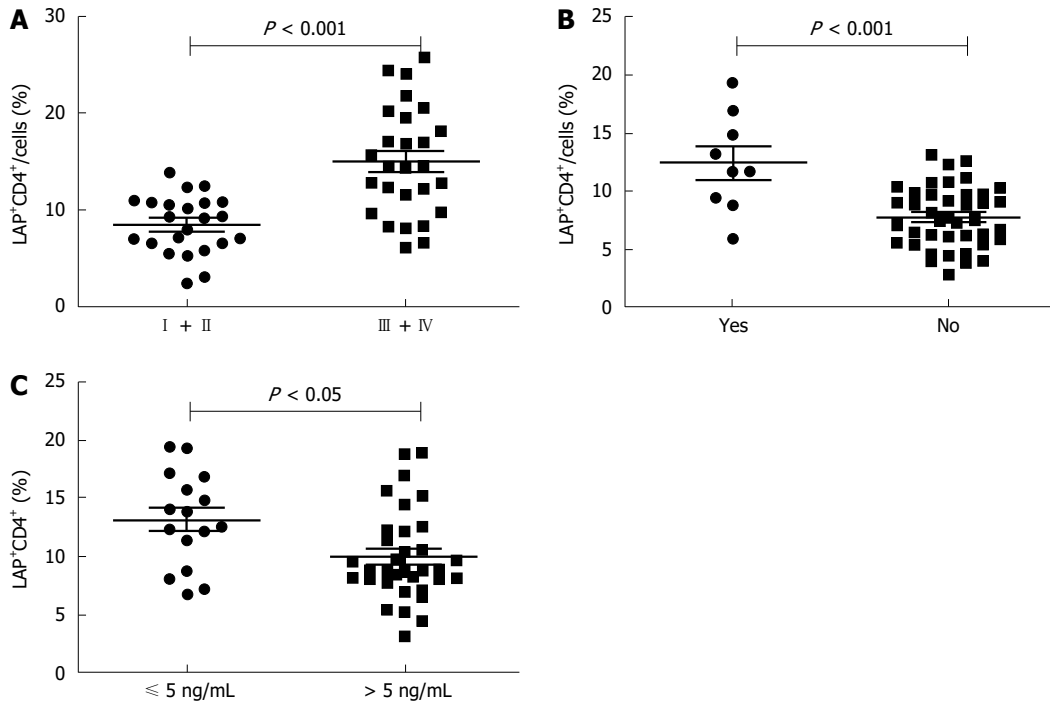
Figure 2 Correlation of LAP⁺CD4⁺ Treg cell abundance with clinicopathological characteristics of colorectal cancer, based on flow cytometry. A: TNM stage; B: Distant metastasis; C: Level of CEA.

A

B

C
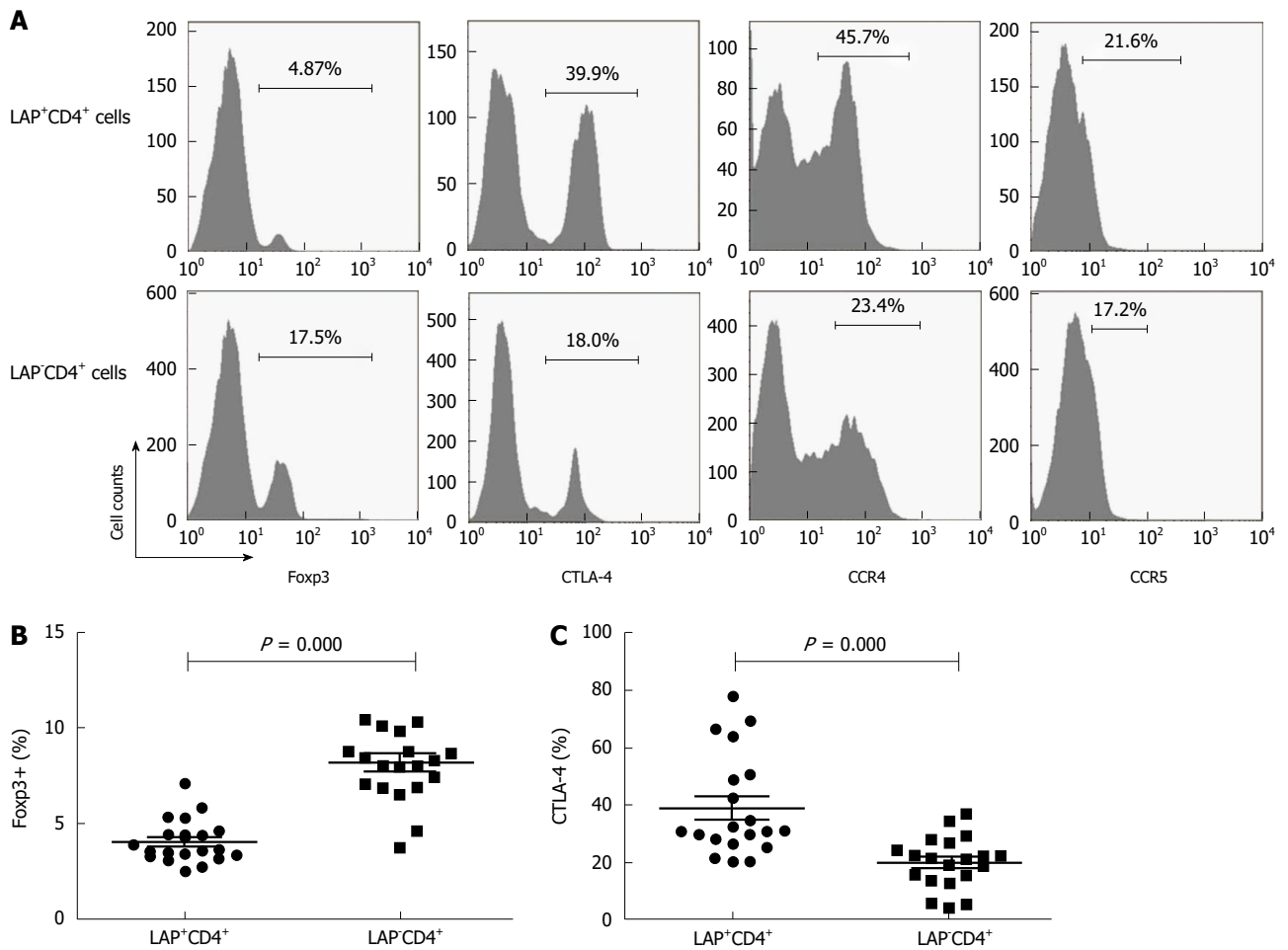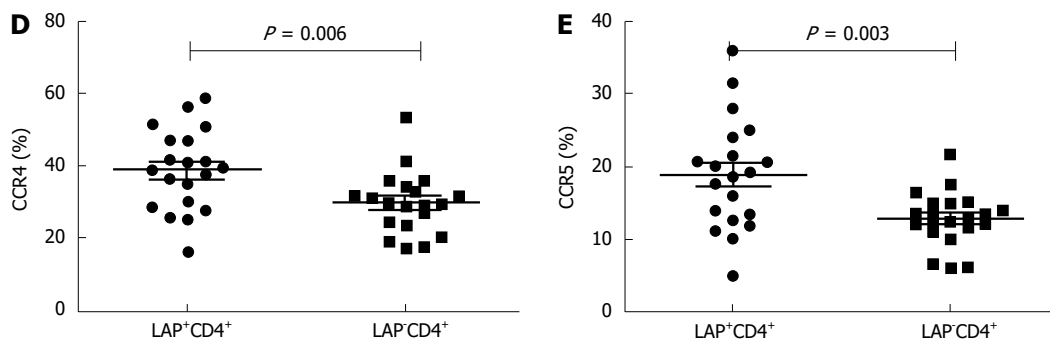
Figure 3 Expression of phenotypic markers by LAP+CD4+ T cells and LAP-CD4+ T cells. A: Typical histograms of the expression of Foxp3, CTLA, CCR4 and CCR5 in LAP+CD4+ T cells or LAP-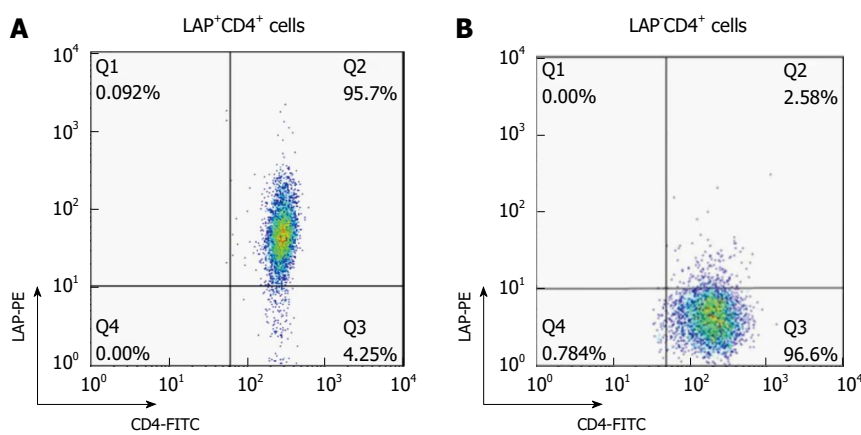CD4+ T cells are depicted. The expression levels of Foxp3, CTLA, CCR4 and CCR5 were measured and compared between LAP+CD4+ T cells and LAP-CD4+ T cells; B: Foxp3; C: CTLA-4; D: CCR4; E: CCR5.

Figure 4 Purity of cells. A: LAP+CD4+ T cells; B: LAP−CD4+ T.

modulates angiogenesis and metastasis that dictate cancer development in the tumor microenvironment. We identified similarities and differences between LAP+CD4+ T cells and traditional CD4+CD25+ Treg cells. Foxp3, previously identified as important in the differentiation and development of Treg cells[31,32], was expressed at detectable levels in only 4% of LAP+CD4+ T cells. This means that LAP+CD4+ T cells differ from traditional CD4+CD25+ Treg cells in marker expression and their immunosuppressive activity is independent of Foxp3. In contrast, LAP+CD4+ T cells expressed abundant levels of CTLA-4, which is used by CD4+CD25+ Treg cells to modulate immune responses[33]. CTLA-4 on CD4+CD25+ Treg cells has been shown to suppress immune function through several mechanisms[34,35]: increasing numbers of CD4+CD25+ CTLA-4 T cells; inhibiting production of proinflammatory factors such as IFN-γ; increasing production of IL-2, IL-4, IL-10 and TGF-β1; and blocking tryptophan synthesis by antigen-presenting cells[36]. Under normal circumstances, these mechanisms can promote self-tolerance and prevent autoimmune disease and transplant rejection. Our results suggest that the CTLA-4 on LAP+CD4+ T cells help CRC tumors evade the host immune system, and one mechanism may be by inhibiting proliferation of effector T lymphocytes.

Our results reproduce most of those of Mahalingam *et al*[37], using different procedures. We isolated LAP+CD4+ T cells and LAP-CD4+ T cells using a magnetic cell sorting system and analyzed cell purity by flow cytometry. Our results revealed that, after sorting, the purity of these two cells was > 90%. This is the first time that LAP+CD4+ T cells were isolated using a magnetic cell sorting system. In contrast to Mahalingam *et al*[37], we found that LAP+CD4+ T cells expressed high levels of IL-10 and TGF-β. These cytokines play key roles in suppressing immune responses in mouse models of cerebral meningitis and allergic inflammation[13,38,39]. The immunoregulatory activity of Treg cells has been linked to several molecules, such as CTLA-4, TGF-β, and IL-10[40,41]. TGF-β has been shown to play an important role in the differentiation, maintenance and function of natural Treg cells[42-45]. However, several studies have revealed the role of IL-10 in Treg cell suppression. It has been demonstrated that IL-10 is required for the homeostatic maintenance of the T cell number by Treg cells[46] and is involved in Treg-cell-mediated suppression in murine models of transplantation, graft-versus-host disease, chronic parasite infection, colitis, and a rat model of type 1 diabetes[47]. Like classical CD4+CD25+ Treg cells, our experiments suggest that the immunosuppressive activity of LAP+CD4+ T cells

could be mediated by IL-10 and TGF-β.

In conclusion, we provide evidence that patients with CRC have elevated proportions of LAP⁺CD4⁺ T cells in the peripheral blood and tumor microenvironment, and their accumulation at tumor sites correlates with CEA level, TNM stage and distant metastasis. LAP⁺CD4⁺ T cells express high levels of IL-10 and TGF-β, which may be involved in tumor immune evasion. Our findings suggest that investigating the functions and regulation of LAP⁺CD4⁺ T cells in CRC may improve our understanding of disease progression and treatment.

COMMENTS

Background

LAP⁺CD4⁺ T cells are a newly identified subset of regulatory T (Treg) cells that express latent-associated peptide (LAP). They function within the latent transforming growth factor (TGF)-β complex to block interaction between TGF-β and receptors on immune cells, of the various Treg cell populations, LAP⁺CD4⁺ T cells are endowed with more potent immunosuppressive function than traditional CD4⁺CD25⁺Foxp3⁺ Treg cells, and they have been associated with autoimmune disease progression. However, they are unaware of any studies examining whether LAP⁺CD4⁺ T cells contribute to colorectal cancer (CRC) progression. Thus, The authors analyzed the abundance, phenotype and cytokine secretion of LAP⁺CD4⁺T cells in the tumor microenvironment in patients with CRC.

Research frontiers

This is the first time that LAP⁺CD4⁺ T cells were isolated by using magnetic cell sorting system.

Innovations and breakthroughs

The authors found that LAP⁺CD4⁺ T cells accumulated in the tumor microenvironment of CRC patients and were involved in immune evasion mediated by interleukin-10 and TGF-β.

Applications

These findings suggest that investigating the functions and regulation of LAP⁺CD4⁺ T cells in CRC may improve our understanding of disease progression and treatment.

Terminology

LAP⁺CD4⁺ T cells are a newly identified subset of Treg cells that express LAP, and function within the latent TGF-β complex to block interaction between TGF-β and receptors on immune cells. CRC progression is a complex process involving interactions between host cellular immunity factors and the tumor, which take place in the so-called tumor microenvironment.

Peer-review

"The role of LAP⁺CD4⁺ T cells in the tumor microenvironment of colorectal cancer" from Zhong *et al* is a decent and interesting manuscript, and worthy to be published.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Multitarget stool DNA tests increases colorectal cancer screening among previously noncompliant Medicare patients

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Author contributions: Prince M, Lester L and Berger B contributed to study concept and design; Prince M, Lester L, Chiniwala R and Berger B contributed to data acquisition; Prince M and Berger B contributed to analysis, interpretation and manuscript drafting and revision; Prince M, Lester L, Chiniwala R and Berger B contributed to critical revision for intellectual content; Prince M and Berger B contributed to statistical analysis; Prince M and Lester L contributed to clinical study supervision and clinical data acquisition; Berger B contributed to laboratory data acquisition and histopathologic report analysis.

Institutional review board statement: This cohort study was performed under the peer-reviewed quality assurance guidelines involving only retrospective records review by the medical institutions involved in the care of patients and was waived for IRB review.

Conflict-of-interest statement: Mark Prince, Lynn Lester, and Rupal Chiniwala have no conflict of interest related to the manuscript. Barry Berger is an employee and owns stock in Exact Sciences Corporation.

Data sharing statement: De-identified medical records have been archived for review at USMD by Mark Prince, MD, MBA, AGAF Director of Gastroenterology USMD Health System, 801 W Interstate 20, Ste 132, Arlington, TX 76017, United States.

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Abstract

AIM

To determine the uptake of noninvasive multitarget stool DNA (mt-sDNA) in a cohort of colorectal cancer (CRC) screening non-compliant average-risk Medicare patients.

METHODS

This cross sectional primary care office-based study examined mt-sDNA uptake in routine clinical practice among 393 colorectal cancer screening non-compliant Medicare patients ages 50-85 ordered by 77 physicians in a multispecialty group practice (USMD Physician Services, Dallas, TX) from October, 2014-September, 2015. Investigators performed a Health Insurance Portability and Accountability Act compliant retros-

pective review of electronic health records to identify mt-sDNA use in patients who were either > 10 years since last colonoscopy and/or > 1 year since last fecal occult blood test. Test positive patients were advised to get diagnostic colonoscopy and thereafter patients were characterized by the most clinically significant lesion documented on histopathology of biopsies or excisional tissue. Descriptive statistics were employed. Key outcome measures included mt-sDNA compliance and diagnostic colonoscopy compliance on positive cases.

RESULTS
Over 12 mo, 77 providers ordered 393 mt-sDNA studies with 347 completed (88.3% compliance). Patient mean age was 69.8 (50-85) and patients were 64% female. Mt-sDNA was negative in 85.3% (296/347) and positive in 14.7% (51/347). Follow-up colonoscopy was performed in 49 positive patients (96.1% colonoscopy compliance) with two patients lost to follow up. Index findings included: colon cancer (4/49, 8.2%), advanced adenomas (21/49, 42.9%), non-advanced adenomas (15/49, 30.6%), and negative results (9/49, 18.4%). The positive predictive value for advanced colorectal lesions was 51.0% and for any colorectal neoplasia was 81.6%. The mean age of patients with colorectal cancer was 70.3 and all CRC's were localized Stage Ⅰ (2) and Stage Ⅱ (2), three were located in the proximal colon and one was located in the distal colon.

CONCLUSION
Mt-sDNA provided medical benefit to screening non-compliant Medicare population. High compliance with mt-sDNA and subsequent follow-up diagnostic colonoscopy identified patients with clinically critical advanced colorectal neoplasia.

Key words: Multitarget stool DNA; Advanced adenoma; Screening compliance; Colonoscopy; Colorectal cancer screening; Preventive

Core tip: The availability of multitarget stool DNA (mt-sDNA) colorectal cancer screening led to high screening compliance (88%) and diagnostic colonoscopy compliance on mt-sDNA positive cases (96%) in a cohort of previously screening non-compliant Medicare patients ages 50-85 years in a multi-specialty group practice setting.

INTRODUCTION

Colorectal cancer (CRC) is among the top three causes of cancer related death in men and women in the United States[1]. Despite the longstanding availability and recent broad third party coverage of screening tests without patient out-of-pocket expense (including fecal occult blood (FOBT/FIT), sigmoidoscopy, and colonoscopy) under the United States Affordable Care Act, a large percentage of Americans are not up to date with CRC screening[2,3]. Given the reluctance of some patients to have an invasive structural screening test like sigmoidoscopy or colonoscopy or the required annual testing using FOBT/FIT, a high sensitivity noninvasive screening test with a longer screening interval may provide an effective alternative that could increase the participation in and performance of CRC screening programs[4-7].

USMD (USMD, Dallas, TX) is an integrated health system in Dallas/Fort Worth, Texas and is focused on preventive care to improve population health. Patient compliance with colorectal cancer screening is a quality metric for USMD primary care physicians and is documented with the USMD electronic health record (EHR). Despite repeated efforts by clinicians, some patients continuously refuse CRC screening *via* colonoscopy and FOBT/FIT. We implemented mt-sDNA (mt-sDNA) screening in general clinical practice to provide a new strategy to increase colorectal cancer screening in our previously screening-non-compliant Medicare patients.

Mt-sDNA is an FDA approved, noninvasive, high-sensitivity CRC screening strategy (Cologuard®, Exact Sciences Corporation, Madison WI) for patients at average risk for colorectal cancer. Average risk includes individuals 50 years of age and older who are asymptomatic and have no personal history of colorectal cancer or colorectal adenoma; no family history of a first degree relative developing colorectal cancer at age 60 or younger; or of any two first degree relatives with colorectal cancer developed at any age; or an inherited predisposition to colorectal cancer including adenomatous polyposis coli or Lynch syndrome (Hereditary non-polyposis colorectal cancer) or no other rare inherited CRC predispositions, or inflammatory bowel disease.

Mt-sDNA is a candidate test for increasing population based screening. It has documented superior sensitivity for CRC, high grade dysplasia, advanced adenoma, and sessile serrated adenoma/polyps compared to fecal immunochemical testing (FIT) alone, albeit with somewhat lower specificity[8,9]. The CRC screening system which includes mt-sDNA was purposefully designed to address patient preference issues including the need for screening support, which is managed through an embedded nationwide patient navigation system. Mt-sDNA was recently included as a recommended routine CRC screening

test by the United States Preventive Services Task Force (2016) and is included in the American Cancer Society (2014) and National Comprehensive Cancer Network (2016) screening guidelines at three-year intervals[10-12]. It is covered by Centers for Medicare and Medicaid Services (CMS) at three year intervals and described by test specific descriptive codes (HCPCS G0464, CPT code 81528)[13]. The National Committee on Quality Assurance (NCQA) has added mt-sDNA to the 2017 Healthcare Employer Data Information Set (HEDIS 2017) which provides physicians the opportunity to receive quality credit for using mt-sDNA screening in the HEDIS effectiveness-of-care domain which covers preventive health measures[14].

Mt-sDNA is a multi-analyte test with algorithmic analysis that provides a single qualitative dichotomous positive or negative test result for each patient[15]. The test requires no preparation, change in medication, or dietary restrictions. The test result is based on a composite score derived from the quantitative values of the 11 biomarkers included in the test: 10 DNA markers [aberrantly methylated *NDRG4* and *BMP3* gene promoter regions, 7 *Kras* point mutations, *β-actin* (reference gene)] and fecal hemoglobin (immunochemical technique) analyzed as a group in a logistic regression algorithm[8]. Scores exceeding the composite score threshold are reported qualitatively as "positive." Individual biomarker results are not reportable and are not associated with biomarker specific reference ranges for clinical evaluation.

This study evaluated mt-sDNA uptake in a cohort of screening non-compliant average-risk Medicare patients aged 50-85 and the subsequent diagnostic colonoscopy usage for those patients with a positive mt-sDNA result. We correlated positive mt-sDNA results with colonoscopy findings.

MATERIALS AND METHODS

Physicians at USMD began offering mt-sDNA routinely to patients as of October 2014 in an effort to improve CRC screening among previously non-compliant Medicare patients. We performed a Health Insurance Portability and Accountability Act compliant retrospective EHR-based medical records review (October, 2014-September, 2015) to identify mt-sDNA use in average-risk Medicare patients treated by USMD Physician Services (Dallas, Texas) who were not previously compliant with colorectal cancer screening. We offered mt-sDNA to patients who were either > 10 years since last colonoscopy and/or > 1 year since last fecal occult blood test. Follow-up colonoscopy was advised for all patients with a positive mt-sDNA result.

Mt-sDNA was ordered as part of the USMD physician's daily clinical practice without any modification. Providers ordered the test, patients engaged with the mt-sDNA patient navigation system, collection kits were shipped directly to patients' homes, samples

were collected by the patients, and the completed tests were returned to the laboratory using pre-paid shipping labels. The samples were then processed and analyzed and the results reported to the USMD ordering physicians. The kit and patient process is illustrated in Figure 1.

Mt-sDNA testing is provided by a single source clinical laboratory (Exact Sciences Laboratories, LLC, Madison, WI, United States) that is accredited by the College of American Pathologists and certified by the CMS Clinical Laboratory Improvement Amendments (CLIA '88) program for high complexity testing. It is supported by a patient navigation system that is available *via* telephone at all hours, every day and supports patients, ordering providers, and health systems to assure successful screening events. A laboratory report with an mt-sDNA qualitative "Positive" or "Negative" clinical result was the measure of a completed test that was used to calculate screening compliance with a test order (intent-to screen compliance). Data was compiled and analyzed using descriptive statistics.

USMD physicians referred the patients with positive results for diagnostic colonoscopy. Patients with negative results were returned to the screening pool to be screened again in three years.

Colonoscopy and pathology findings on all mt-sDNA positive patients were tabulated and included: histologic classification, size, location, and total number of adenomas and non-adenomatous polyps. Patients were categorized by the most advanced finding (index lesion) as described on pathologic analysis of colonoscopically directed biopsies and any subsequent surgical excisional tissues[8]. Major categories of index lesions were CRC, advanced adenoma (AA), non-advanced adenoma (NAA), and negative findings. Advanced adenomas are further categorized as: tubular adenoma (TA) with high grade dysplasia or significant villous component of any size; and tubular adenoma or sessile serrated adenoma/polyp without other advanced features ≥ 10 mm in greatest dimension. Non-advanced adenomas are further characterized as; 1-2 TA's > 5 mm but < 10 mm; > 3 TA's < 10 mm; 1-2 TA's ≤ 5 mm. as these may have differing post-colonoscopy clinical surveillance intervals. Negative findings include absence of colorectal neoplasia but may include the presence of hyperplastic polyps (HP's) < 10 mm. High risk patients were excluded from this study including those patients who were symptomatic and/or had a significant personal or family history of colorectal neoplasia or inflammatory bowel disease.

RESULTS

Over 12 mo, 77 providers ordered mt-sDNA tests for 393 screening-noncompliant Medicare patients and 347 patients completed the test (88.3% intent-to-screen compliance). Successfully screened patients (347) had a mean age of 69.8 (range 50-85) and
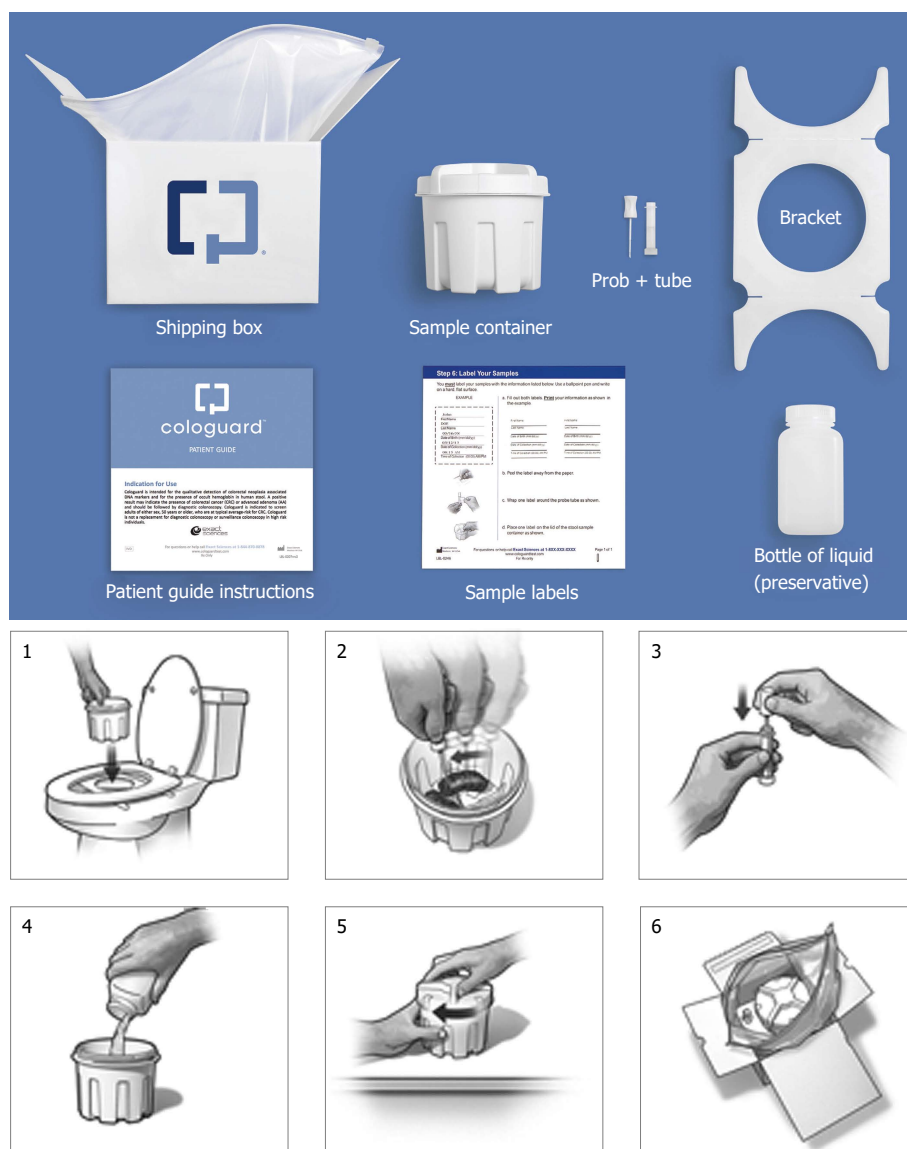
Figure 1 Multi-target stool DNA collection kit and collection process. A: Contents of Cologuard collection kit: Top row, left to right: collection kit/shipping box, stool collection container, hemoglobin sample tube, collection container support bracket for toilet; Bottom row - instructions for use, DNA preservative buffer; B: Collection process - 1, set up collection container; 2 and 3, sample for hemoglobin; 4, add DNA preservative buffer; 5, close container; 6, replace hemoglobin tube and sample container in box, seal and ship.

were 64% female. Unsuccessfully screened patients (46; 11.7%) had a mean age of 71.2 (range 61-83), and were 59% female. The mt-sDNA result was negative in 296 patients (85.3%), mean age 69.1 (range 50-85) and 61% female and positive in 51 patients (14.7%), mean age 71.8 (range 65-83), 49% female (Figure 2).

Diagnostic colonoscopy was subsequently performed on 49 mt-sDNA positive patients (96.1% diagnostic colonoscopy compliance) and two patients were lost to follow up. Index findings among 49 positive patients included: 4 patients with colorectal cancer (8.2%), 21 patients with advanced adenoma (42.9%), 15 patients with non-advanced adenoma (30.6%), and 9 patients with negative results (18.4%) (Table 1). The positive predictive value for advanced colorectal neoplasia was 51.0% (25/49) and for any colorectal

neoplasia was 81.6% (40/49).

The four CRC patients were ages 66, 68, 73, and 74 years and included 2 men and 2 women. All CRC's were localized, Stage I (2) and Stage II (2), and three were located in the proximal colon and one was located in the distal colon. The 21 advanced adenoma patients, median age 73 (65-83), 43% female (9/21), included: one with high-grade dysplasia in a 20 mm rectal tubulovillous adenoma in a 72-year-old female; 9 with tubulovillous or villous adenoma; 10 with tubular adenoma $\geq$ 10 mm without other advanced features; and one with a 10 mm sessile serrated adenoma/polyp. Index lesion location was specified in 20 advanced adenomas and 40% (8/20) were in the proximal colon.

The 15 non-advanced adenoma patients, mean age 70 (range 64-81) and 24% female (6/15),

Table 1 Distribution of most advanced findings on colonoscopy

Most advanced finding	*n* (%)
Colorectal cancer	4 (8.2)
Advanced adenoma[1]	21 (42.9)
Non advanced adenoma[2]	15 (30.6)
1-2 adenomas, > 5 and < 10 mm	7
> 3 adenomas, any size < 10 mm	5
1-2 adenomas, ⩽ 5 mm	3
No colorectal neoplasia	9 (18.4)
HP only	5
No findings	4
Total Patients	49 (100)

[1]Includes adenoma with high grade dysplasia, villous adenoma, tubulovillous adenoma and tubular adenoma and sessile serrated adenoma/polyp ⩾ 10 mm; [2]Non advanced adenoma includes tubular adenomas < 10 mm with no advanced features. HP: Hyperplastic polyp.

included: 7 patients with 1-2 tubular adenomas > 5 mm but < 10 mm; 5 patients with > 3 tubular adenomas < 10 mm; and 3 patients with 1-2 tubular adenomas ⩽ 5 mm. The specimen of one patient with a well described 8 mm polyp that was removed but not retrieved and is included in the non-advanced adenoma total. No sessile serrate adenomas/polyps were recorded that were < 10 mm. There were 9 patients without colorectal neoplasia, median age 71 (65-80) and 67% female (6/9), including 5 with hyperplastic polyps < 10 mm and 4 not requiring a biopsy. Figure 3 includes a summary of the findings.

The size distribution of CRC and advanced adenoma cases is provided in Table 2. The four CRCs were 14, 20, 25, and 40 mm in greatest dimension. Advanced adenoma index lesions include 5 at 10 mm, 9 at 11-19 mm, 5 at 20-29 mm, and 2 at ⩾ 30 mm.

DISCUSSION

Preventing colorectal cancer morbidity and mortality primarily rests on the ability of providers to successfully screen patients for premalignant and malignant colorectal neoplasia and treat accordingly. Colonoscopy is the most widely used and effective screening tool for those who will take advantage of it and ensures the screening compliance of the vast majority of screening compliant Americans[3]. However, there are millions of patients who remain unscreened or only intermittently screened using FOBT/FIT only and who will not use colonoscopic screening for a variety of reasons including risk, inconvenience, preparatory requirements and embarrassment[1,2,4,16-21].

Consequently, in the United States, colorectal cancer remains the second leading cause of cancer related death overall and third leading cause of death for each sex. The age-adjusted incidence of new CRC cases reported by the US Surveillance, Epidemiology, and End Results Program for the period 2009-2013 was 47.1 and 36.0 per 100000 for men and women respectively. Incidence increases with age with 82.4%
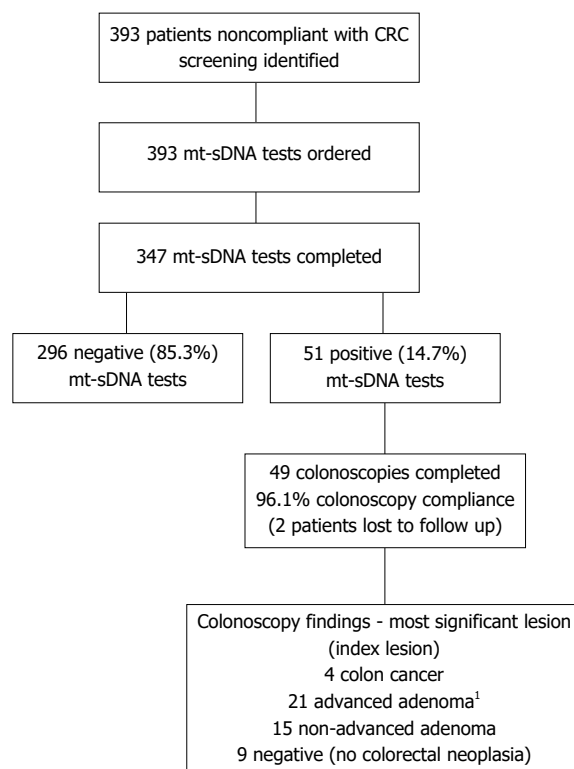
Figure 2 Enrollment outcomes. [1]Advanced adenoma: colorectal adenoma or sessile serrated adenoma/polyp ⩾ 1.0 cm in diameter; or adenoma with high-grade dysplasia or ⩾ 25% villous component, of any size. mt-sDNA: Multitarget stool DNA.

of new cases occurring patients age 45-84 years and with 21.8%, 24% and 21.8% of new cases seen in patients 55-64, 66-74, and 75-84 respectively[22,23].

Our study documented the experience of an integrated multispecialty medical practice working to increase screening effectiveness in its screening-noncompliant Medicare age patients. This population is of critical importance; CRC incidence increases with age and CRC's that present with symptoms rather than being detected through asymptomatic screening are more likely to be of late stage with increased related morbidity and cost[24].

We studied whether the availability of non-invasive CRC screening with mt-sDNA in USMD's general medical practice for routine use might encourage providers and patients to achieve successful screening. Our study did not address the discriminate features of mt-sDNA testing that led to increased patient uptake and compliance. Common patient preference issues that contribute to screening program performance include concerns around privacy, convenience, accuracy, extended screening intervals, and/or direct patient support through an embedded patient navigation system. Additionally, the long-term benefits of decreased patient and provider screening burden related to performing, tracking, administering, and navigating the mt-sDNA screening process on patient compliance were not assessed.

Our data demonstrate that mt-sDNA, with an

Group	Category	*n*	PPV			
Advanced colorectal neoplasia	CRC	4	8.2%	51%	82%	Any colorectal neoplasia
	Advanced adenoma	21	42.9%			
Nonadvanced colorectal neoplasia (adenoma < 10 mm)	NAA	15	30.6%			
HP's and no findings	Negative	9	18.4%			
Total positive		51	-			
Positives with colonoscopy		49	100%			
Colonoscopy compliance			96.1%			

Figure 3 Summary of colonoscopy findings in 49 mt-sDNA positive patients. PPV: Positive predictive value; CRC: Colorectal cancer; NAA: Non-advanced adenoma; HP's: Hyperplastic polyps.

Table 2 Features of the advanced colorectal neoplasms found in Cologuard positive patients on colonoscopy

Index lesion	Greatest dimension (mm)					Proximal colon
	10	11-19	20-29	30+	Total	
CRC[1]	0	1	2	1	4	75% (3/4)
HGD	0	0	1	0	1	0% (0/1)
TVA/VA[2]	1	3	4	1	9	38% (3/8)
TA	3	6	0	1	10	40% (4/10)
SSA/P	1	0	0	0	1	100% (1/1)
Total	4	10	7	3	24	40% (8/20)

Index lesion only. [1]Stage Ⅰ (2), Stage Ⅱ (2); [2]1 location not reported. Index lesion: Most clinically significant lesion found on colonoscopy; CRC: Colorectal cancer; HGD: High grade dysplasia; TVA/VA: Tubulovillous adenoma/villous adenoma; TA: Tubular adenoma ≥ 10 mm with no HGD or villous features; SSA/P: Sessile serrated adenoma/polyp ≥ 10 mm.

88.3% intent-to-screen compliance, provided an acceptable CRC screening strategy for previously screening-noncompliant Medicare patients. Further, there was almost universal compliance with follow-up diagnostic colonoscopy (96.1%) by mt-sDNA positive patients. Congruent with the purpose of CRC screening, mt-sDNA screening identified patients with early stage CRC (4/4) and advanced adenoma that were amenable to definitive surgical treatment and/or colonoscopic excision. Therefore, screening with mt-sDNA could reasonably be expected to contribute to CRC related mortality reduction and prevention respectively.

Because of the small size of the study, population based statistics are only somewhat informative. CRC incidence was elevated at 11.5/1000 (1.2%) which is likely consistent with population age and advanced adenoma incidence was unremarkable at 60.5/1000 (6%). The positive predictive value of mt-sDNA for CRC and advanced adenoma exceeded that seen in the much larger and more diverse DeeP-C mt-sDNA screening study, again likely more reflective of the study population of unscreened and under-screened patients than changes in test performance[24].

The study is limited by relatively small size (393) but it is strengthened by the diversity of the provider group participating (77); the use of mt-sDNA in routine daily clinical practice with a focus on shared decision making; and strong compliance data for both mt-sDNA screening and post-positive test colonoscopy. The findings may not be generalizable to non-Medicare-age patients and may reflect disease incidence particular to this geographic area.

In conclusion, the availability of mt-sDNA colorectal cancer screening provided significant medical benefit to Medicare patients cared for in a large multi-specialty group practice who were previously screening-noncompliant. Patients with clinically significant advanced colorectal neoplasia were identified as a result of high compliance with both mt-sDNA screening and subsequent diagnostic colonoscopy. Broader implementation of mt-sDNA screening into patients ages 50-65 should be evaluated to ascertain similar benefits in screening compliance in younger patients.

COMMENTS

Background

A significant percentage of Americans are not up-to-date with colorectal cancer (CRC) screening. A high sensitivity noninvasive screening test with a multi-year screening interval could provide a strategy to increase CRC screening program participation. The multitarget stool DNA (mt-sDNA) was recently included in the recommendations of the Unites States Preventive Services Task Force for average risk colorectal cancer screening. In this study, The authors determine the uptake of mt-sDNA screening in a cohort of previously screening non-compliant Medicare patients age 50-85 years.

Research frontiers

While several options are available to patients for screening for colorectal cancer and pre-malignant polyps, patient compliance has been lower than that seen on breast or cervical cancer screening. In the United States, a new screening strategy, multitarget stool DNA, provides a systems approach including high cancer and high-grade dysplasia sensitivity in a non-invasive format and an embedded national patient navigation system to support successful screening. Studies to evaluate the clinical uptake and compliance with this test will help inform decisions on applicability to national screening programs.

Innovations and breakthroughs

This is the first report of mt-sDNA use for routine average risk colorectal cancer screening in daily clinical practice by typical primary care clinicians. This medical-records-review based study demonstrates that clinicians using this system-based colorectal cancer screening strategy, combining high sensitivity mt-sDNA and a patient navigation system, achieved high levels of screening compliance (88%) in 393 patients age 50-85 years who were previously non-compliant with screening and who were participating in the United States. Medicare program. Diagnostic colonoscopy compliance in 51 test positive cases was almost universal (96%) and revealed significant disease in 25 patients (4 colorectal cancers all Stage I or II, and 21 advanced adenomas). This first small but significant study reveals that mt-sDNA screening has the potential to enhance non-invasive screening success and performance in previously non-compliant individuals in daily practice.

Applications

The results of this study support the implementation of mt-sDNA in daily clinical practice, especially in patients that have been screening non-compliant. The article also demonstrates that compliance with a diagnostic colonoscopy in mt-sDNA positive patients was almost universal (96%) and that the positive predictive value for advanced colorectal neoplasia was high (51%), justifying the expenditure of colonoscopy resources in this group.

Terminology

Advanced colorectal neoplasia: includes patients with colorectal cancer or advanced adenoma, the lesions which are the primary targets of screening.

Advanced adenoma: the group of colorectal neoplasm most likely to progress to colorectal cancer over time. The group includes adenomas with high-grade dysplasia or $\geq$ 25% villous component of any size and/or adenomas 1.0 cm in size or greater.

Affordable care act (ACA): United States regulation that includes a requirement for certain screening tests to be provided to individual participating in that program to be free of any costs to those individuals.

Average risk patient: Individuals age 50 and older who are asymptomatic and have no personal history of colorectal cancer or colorectal adenoma, no family history of a first degree relative developing colorectal cancer at age 60 or younger or any two first degree relatives with colorectal cancer developed at any age, or an inherited predisposition to colorectal cancer including adenomatous polyposis coli or Hereditary non-polyposis colorectal cancer (Lynch syndrome) or other rare inherited predispositions, or inflammatory bowel disease.

Medicare: The United States government based medical insurance system for persons 65 years of age and older and certain qualifying younger patients. Medicare pays the full expense for mt-sDNA tests for these patients ages 50-85 years once, every three years.

Mt-sDNA test: a stool based assay for colorectal cancer screening. It includes 11 biomarkers (10 DNA and 1 fecal hemoglobin) evaluated as a group in a logistic algorithm to provide a single composite result of "positive" or "negative".

Peer-review

This paper contains interesting results which merit publication. The mt-sDNA CRC screening seems to be helpful for colorectal cancer in average-risk population.

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