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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007
[3]Passed away on June 14, 2008

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EDITORIAL

New technologies in gastrointestinal research

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Abstract

This issue presents different new techniques aiming to increase our understanding of the gastrointestinal system and to improve treatment. The technologies cover selected methods to evoke and assess gut pain, new methods for imaging and physiological measurements, histochemistry, pharmacological modelling *etc*. There is no doubt that the methods will revolutionize the diagnostic approach in near future.

Key words: Pain; Gut; Brain; Sphincter; Imaging; Drug

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New technologies are emerging in gastroenterology. During the last decade, methods such as intraluminal ultrasound, other imaging modalities and high resolution manometry have become available for routine work, dramatically improving the ability to correctly diagnose and treat gastroenterological diseases. Experimental methods to assess the sphincter regions and the pain system have also become available with the possibility to get more insights into basic physiological mechanisms and understanding of how drugs affect the gastrointestinal tract. This has lead to an improvement in the treatment of patients with both organic and functional diseases. Unfortunately, many of these methods are still only available in the most advanced laboratories, but commercial systems are currently being developed with the aim of getting a more widespread use of the most promising techniques.

In the current issue of *World J Gastroenterol*, different techniques which are either commercially available or in development are presented. The technologies cover the selected methods to evoke and assess gut pain experimentally and in the clinic[1]; neuroimaging of the brain-gut axis[2]; new methods for ultrasound and imaging[3]; devices to assess sphincter functions and force measurements[4-6]; emerging experimental methods to measure blood flow and histochemical changes in the tissue[7,8]; manometry and impedance measurements as well as pharmacological and modelling techniques[9,10]. There is no doubt that these methods will revolutionize the diagnostic approach in near future. In particular, combining the different techniques with development of miniature equipment will lead to multimodal experimental procedures that can be done in one procedure with less discomfort and complications for the patients. Today, it is theoretically possible to combine probes for manometry, imaging, assessment of sphincter function and axial force in the oesophagus with endoscopy (including biopsies) and comprehensive testing of the pain system (including electrophysiological assessment of the brain-gut axis), all in one procedure. In the current issue, examples of such combined techniques are demonstrated. It has been shown that multimodal assessment may lead us to much a better understanding of diseases and symptoms in various patient groups. Individual genetic and environmental factors among others have now led to the understanding that patients with the same disease do not necessary respond to standard treatments. New pharmacological methods giving insight into drug mechanisms in individual patients are available and the methods provide the possibility for tailoring specific and individualized treatment in the near future. Naturally, there is still a long way to go before the ideal probes and equipment are available for general use; but with the necessary knowledge exchange and open-mindedness in the research community, there is no doubt that near future will revolutionize the way we look at the gastrointestinal tract and treat our patients.

The papers in the current issue show how innovative development and research can bring new ideas as well as refinement of older techniques into play in the gastrointestinal tract. The clinician should have at least some knowledge of these techniques as a close relationship and sharing of ideas between research and

the clinic are a sine qua none to find the right indications for the methods. In the research community, there are many more emerging new methods than those dealt with in this issue; but with the main focus on the upper gastrointestinal tract, the selected techniques represent broadly the evolution and forthcoming procedures in this important field.

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New technologies in the gastrointestinal clinic and research: Impedance and high-resolution manometry

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Abstract

The last five years have been an exciting time in the study of esophageal motor disorders due to the recent advances in esophageal function testing. New technologies have emerged, such as intraluminal impedance, while conventional techniques, such as manometry, have enjoyed many improvements due to advances in transducer technology, computerization and graphic data presentation. While these techniques provide more detailed information regarding esophageal function, our understanding of whether they can improve our ability to diagnose and treat patients more effectively is evolving. These techniques are also excellent research tools and they have added substantially to our understanding of esophageal motor function in dysphagia. This review describes the potential benefits that these new technologies may have over conventional techniques for the evaluation of dysphagia.

Key words: Dysphagia; Multichannel intraluminal Impedance; Bolus transit; High-resolution manometry; Esophagogastric junction; Achalasia

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INTRODUCTION

The evaluation of a patient with dysphagia should begin with a careful evaluation for structural causes for obstruction, such as webs, rings, strictures and mass lesions. Once these entities are ruled out with either endoscopy or fluoroscopy, the work-up should shift its focus to defining esophageal motor function and bolus transit abnormalities. Although fluoroscopy can provide information on bolus transit, it is burdened by the requirement of radiation, and the inability to detect the pressure profile of the peristaltic event. Currently, only manometry can define the pressure profile of both peristalsis and LES relaxation. However, this technology is limited by an inability to provide information on bolus transit and emptying. Furthermore, conventional manometry has been hindered by a lack of standardization of methodology and conflicting notions of how to analyze the data. Direct evidence of this predicament can be found in a recent publication highlighting the poor inter-observer agreement in the analysis of clinical manometry; even amongst expert practitioners[1].

Given the above observations, two technologies have been developed that have the potential to improve the management of dysphagia: high-resolution manometry and intraluminal impedance monitoring. While high-resolution manometry is an evolution of current manometric techniques, impedance monitoring is a new technology that complements manometric data by providing information on bolus transit without the need for radiation or an additional test. These two techniques have advanced our understanding of esophageal motility and it is very likely that they will also improve clinical management.

MULTICHANNEL INTRALUMINAL IMPEDANCE (MII)

MII: Technical aspects

As mentioned previously, fluoroscopic evaluation

of the esophagus is an excellent tool to assess the intraluminal anatomy of the esophagus as well as bolus transit. However, it requires radiation and also lacks the detail to define and classify esophageal motor function. Intraluminal impedance monitoring was devised to circumvent the requirement of radiation in assessing bolus transit by monitoring intraluminal resistance. Impedance monitoring works by using an alternating current generator to apply an electrical potential between pairs of metal electrode rings separated by an isolator. The electrical current loop can only be closed through the conduction of electrical charges by the surrounding material bridging the two metal electrode rings. Air, liquid (saline/refluxate), and the esophageal mucosa each have unique impedance characteristics, thereby allowing definition of which material resides between each pair of electrodes. Air is highly resistant to current flow and, thus, has a high impedance value, while saline and gastric juice have relatively low resistance to flow and, thus, have a low impedance value. Esophageal mucosa has an intermediate impedance range and, thus, serves as a baseline during monitoring.

By dispersing the impedance electrodes along a catheter, and defining impedance changes at adjacent pairs of rings, one can determine the direction of bolus transit within the esophagus and also document whether complete bolus clearance has occurred (Figure 1)[2-4]. Studies using combined fluoroscopy and impedance have validated the convention that liquid bolus entry is characterized by a 50% drop in impedance at the recording site while bolus exit is characterized by a return to at least 50% of baseline[5,6]. Studies assessing the correlation between simultaneous barium videoesophagram and impedance revealed agreement in over 97% of swallows for determining normal bolus transit or retrograde escape and stasis[7]. Thus, this technique provides qualitative evidence of esophageal emptying. However, it does not provide quantitative data regarding volume.

MII: Investigations into dysphagia pathogenesis

An important first observation using combined MII and conventional manometry was focused on describing bolus transit patterns associated with various esophageal motor patterns. Tutuian *et al*[8] described an experience in 350 patients presenting for evaluation of esophageal function. Bolus transit was assessed based on conventional manometric criteria of swallowing function. All patients with manometrically defined achalasia and scleroderma had abnormal bolus transit. In contrast, only half of the patients with ineffective esophageal motility (IEM) and spasm had abnormal bolus transit. The majority of patients with intact peristalsis and various LES abnormalities had normal bolus transit. The authors theorized that impedance monitoring could potentially categorize esophageal motor abnormalities into more clinically relevant groups based on abnormalities of both bolus transit and pressure as opposed to pressure alone.

Looking to provide more focused information regarding a clinical role for impedance, Tutuian *et al*[9] analyzed 71 subjects with DES and characterized them based on both their motor function and ability to obtain complete bolus transit[9]. Their findings suggested that DES patients with dysphagia were more likely to have abnormal bolus transit than DES patients who presented with predominant chest pain. Furthermore, they also noted that DES patients presenting with chest pain were not only more likely to have normal bolus transit, but also exhibited higher contraction amplitudes. These observations are intriguing as it appears that impedance may help define treatment strategies for various patient groups: chest pain patients with extremely high contractile amplitudes may be a sub-population amenable to treatments with nitrates and calcium channel blockers; while patients with impaired bolus transit may require alternative therapies.

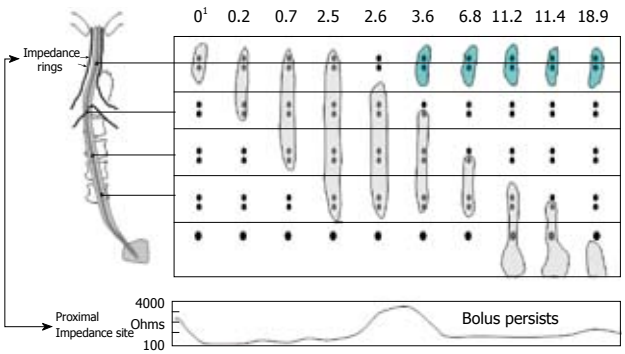

Figure 1 Example of retrograde escape and proximal stasis on simultaneous multichannel intraluminal impedance and fluoroscopy. The fluoroscopic bolus is represented by the gray bolus at each time interval during the swallow and the green bolus during retrograde escape. The bottom panel is a single impedance tracing at the proximal location. The bolus is present at the proximal impedance site until 2.6 s where the bolus tails moves distal to the two rings at that level and the impedance tracing returns to baseline. At 3.6 s, the bolus (green) reenters the recording site and once again the impedance drops consistent with bolus retention. Modified from Imam *et al*[7]. [1]Time in seconds.

MII: Clinical aspects

The clinical protocol for combined impedance/manometry is similar to the standard conventional manometric protocol with the exception that normal saline is used for the 10 liquid swallows and there is an additional portion of the study that utilizes viscous swallows. Saline is used because its high conductivity provides a contrast in impedance between the liquid bolus and the esophageal wall, while the viscous bolus is typically a gel provided by the manufacturer or another food, such as yogurt or apple sauce. Impedance parameters calculated for the evaluation of bolus transit using liquid and viscous swallows are: (1) total bolus transit time (between bolus entry at 20 cm above the LES and bolus exit at 5 cm above the LES); (2) bolus presence time (the interval between bolus entry and bolus exit at each impedance-measuring site); and (3) segmental transit times (the interval between bolus entry at a given level above the LES and bolus exit at the next most distal level)[10]. Swallows are classified as having: (1) complete bolus transit if bolus exit is recorded in all three distal impedance-measuring sites or (2) incomplete bolus transit if bolus exit is not

identified at one or more of the three distal impedance-measuring sites.

Normal values for both liquid and viscous swallows have been reported by different groups using slightly different techniques. Tutuian *et al*[10]. performed combined impedance/manometry on 43 normal healthy subjects and analyzed 10 liquid and 10 viscous swallows to determine normative ranges for total bolus transit time and the percent of swallows associated with complete bolus transit. Their results revealed that total bolus transit time for both liquid and viscous swallows were 12.5 s and greater than 90% of individuals had > 80% of the liquid swallows associated with complete bolus transit and > 70% of the viscous swallows associated with complete bolus transit[10]. Two other studies in normal controls revealed similar bolus transit parameters and thus, it appears that this technology is reproducible in normal subjects[11,12].

MII: Future directions

Although available data suggests that abnormal bolus transit occurs in many patients presenting with dysphagia, there is no clear data supporting an association of abnormal bolus transit, and the perception of dysphagia. Thus, future research should focus on the mechanisms by which abnormal bolus transit elicits symptoms. Such studies should consider the role of sensitivity, anxiety state and other potential confounders that may modulate symptoms. In addition, studies focused on whether or not abnormal bolus transit can predict clinical outcomes in dysphagia patients are also needed to support the role of impedance in clinical practice.

New technologies incorporating impedance techniques are also evolving that can provide more detail regarding anatomy and mechanical properties of the esophageal body[13,14]. The functional lumen imaging probe (FLIP) was created by McMahon *et al*[13,14] to measure cross-sectional area of the esophagus at extremely small intervals. This technique applies computer software that converts the high-resolution planimetry data into a computer animation that recreates the geometry of the anatomic zone being studied. In addition, a pressure/distention relationship can be measured to define elasticity and compliance of the lumen wall and, thus, represents the first dynamic technique to profile both anatomy and function of the esophagus with a single device. Although these devices are currently only available for research purposes, it is likely that they will be incorporated into clinical devices along with high-resolution impedance and topographic display methods.

HIGH-RESOLUTION MANOMETRY

Esophageal manometry is considered the "gold standard" for assessing esophageal motor function[15]. Accordingly, the current diagnostic classification of esophageal motor disorders is based almost entirely on manometric patterns of abnormal peristalsis and LES function[16]. Conventional manometry typically utilizes 3-8 pressure sensors positioned within the esophageal lumen to assess the contractile pattern during water swallows. A variety of sensor technologies exist, including solid-state transducers, circumferentially sensitive transducers, perfused ports, and the Dentsleeve device. The heterogeneity of the sensor types and lack of consensus regarding the optimal spacing of sensors may be partially responsible for the poor intra- and inter-observer reproducibility reported in the literature[1]. Thus, refinements in manometry are needed to improve reproducibility and accuracy of this "gold standard".

HRM: Technical aspects

High-resolution manometry is not a new technology and represents a modification of existing technology to provide greater detail by utilizing a vastly increased number of sensors spaced closely together. Thus, not only time, but also the spatial domain of the pressure profile within the esophagus can be captured as a continuum after interpolation between the sensors. The ideal system for esophageal studies should span from the pharynx to the stomach with sensor separation of no more than a centimeter apart within and around the sphincters and a temporal frequency response matched to the zone of the esophagus in which the sensors reside. High-resolution manometry can be adapted to work with any transducer technology, as long as the recording fidelity of the sensor is adequate. The frequency response required to reproduce esophageal pressure waves with 98% accuracy is 0-4 Hz, while that required for reproducing pharyngeal pressure waves is 0-56 Hz[17]. Expressed in terms of maximal recordable $\Delta P/\Delta t$, 300 mmHg/s is sufficient for studying the esophageal body while the pharynx will require a $\Delta P/\Delta t$ of 4000 mmHg/s for the pharynx.

Early studies incorporating high-resolution manometry utilized water perfused systems due to availability of appropriate multilumen extrusions, the flexibility of the sensor spacing, and the cost of solid-state pressure sensors. However, the response characteristics of the water perfused sensors were technically limited for studying the pharynx or for measuring detailed pressure gradients through both the upper and lower esophageal sphincters. Thus, the ideal system would incorporate solid-state sensors, which have become clinically available. The advantages of this type of high-resolution system are: (1) a simplified procedural set up with improved sphincter localization; (2) elimination of movement artifact; (3) simplified data interpretation; and (4) ability to perform more sophisticated analysis of esophageal function. These attributes alone make this technology more user-friendly and efficient. Thus, it has the potential to replace conventional manometry utilizing a line tracing format.

The vastly increased quantity of data and the confusing presentation of multiple overlapping tracings spaced closely together presents new challenges for interpreting high-resolution manometry. Thus, new algorithms have been devised to provide a seamless dynamic representation of pressure at every axial position from the hypopharynx through the EGJ. Advances in computerization and graphic data presentation have been adapted so that esophageal contractile activity following a swallow can be portrayed

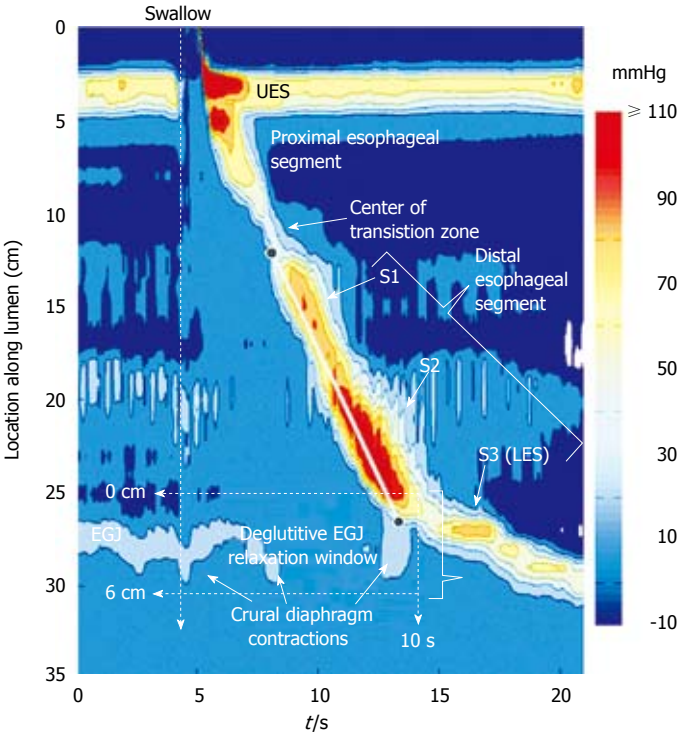

Figure 2 Typical swallow pressure topography spanning from the pharynx (locations 0-2 cm) to stomach (locations 29-35 cm) of a normal subject with normal peristalsis and normal EGJ relaxation. The transition zone, demarcating the end of the proximal esophageal segment (striated muscle) and the beginning of the distal esophageal segment (smooth muscle), is readily identified as a pressure minimum. Note that the distal segment, in fact, has three sub-segments (S1, S2, S3) within it, each with an identifiable pressure peak. Sub-segment 3, the LES, contracts at the termination of peristalsis and then descends back to the level of the CD as the period of swallow-related esophageal shortening ends. The onset of the deglutitive relaxation window is at the onset of upper sphincter relaxation while the offset is 10 s later. The spatial domain within which EGJ relaxation is assessed (the eSleeve™ range) is user defined, spanning at least 6 cm, depending on the extent of esophageal shortening after the swallow. The contractile front velocity (CFV) is the slope of the line connecting the black circle-points on the 30 mmHg isobaric contour at the proximal margin of S1 and the distal margin of S2.

in the format of color isobaric contour topographic plots (Figure 2). Clouse and Staino were the first to apply this technique in the esophagus and developed a methodology to interpolate and convert this information into topographic plots[18]. This data presentation format is more akin to an imaging technique, as opposed to conventional manometric tracings, and has the potential to greatly simplify and standardize the clinical manometric study.

HRM: Investigations into dysphagia pathogenesis

Topographic analysis of HRM data has clearly advanced our knowledge of esophageal motor function. The recent application of topographic analysis to esophageal peristalsis demonstrated that progression through the esophageal body is not seamless. Rather, it is comprised of a sequence of contractile events occurring in four discrete pressure segments (Figure 2). The first segment represents the striated muscle component of the proximal esophagus, and extends from the UES to the first pressure trough in the region of the aortic arch. This trough, representing the transition zone is usually easily identified. The distal portion of the esophagus is considered the smooth muscle dominant portion, and can be separated into two overlapping neuromuscular segments. The fourth contractile segment encompasses the LES. This segmental configuration was not appreciated by conventional manometry and underscores the strength of topographic analysis of manometric data[18-20].

Within the esophageal body one of the early achievements of HRM was the understanding of the transition zone in the mid esophagus, not just as the locus of the nadir in peristaltic pressure amplitude, but also as a physiological transition between propagated contractions of completely distinct physiology[21]. The proximal segment is that dominated by striated muscle, while the distal segment is smooth muscle. The proximal contraction is attributable to sequenced activation of motor neurons in the medulla, while the distal contraction in the smooth muscle is sequenced as a function of the balance between the excitatory and inhibitory interneurons of the myenteric plexus. This enhanced understanding of the transition zone can also account for the distinct pathology in which there is an abnormal delay between the termination of the proximal contraction and the origination of the distal contraction or a spatial gap between the two, as an explanation for dysphagia[22].

One of the first important investigations that took advantage of the detail HRM provides in defining simultaneous pressure across anatomic zones, was performed by Pal *et al.* to define pathologic constriction across the UES[23]. Their findings suggested that intrabolus pressure was an important marker of impaired bolus transit, and represented constriction at the UES. Our group built upon this work, utilizing combined HRM and fluoroscopy at the upper esophageal sphincter to determine if manometry alone could be leveraged to accurately predict bolus transit across the EGJ in patients with a spectrum of pathological conditions[24]. Using the normative data for esophago-gastric flow permissive time in 20 controls and mathematical modeling data of antegrade esophageal emptying[25], we performed a ROC analysis for the predictive value of flow permissive time that is optimal to predict complete clearance. Our findings suggest that a cut-off value of $\leqslant$ 2.5 s had the best predictive value for incomplete clearance with a sensitivity of 86% and specificity of 92%. We speculate that intrabolus pressure elevations above the EGJ may also be useful in defining pathologic constriction or impaired EGJ opening and future work should be focused on defining normative values for intrabolus pressure.

HRM: Clinical aspects

Given the fact that high-resolution manometry is a refinement of an already existing clinical tool, it can be

Table 1 Classification of individual swallows based on pressure topography criteria

Classification	Criteria
EGJ Deglutitive Relaxation (referenced to gastric pressure)	
Normal relaxation	4 s Integrated Relaxation Pressure (IRP) < 15 mmHg
Impaired relaxation	4 s IRP ⩾ 15 mmHg
Distal Segment Contraction (referenced to gastric pressure)	
Normal	⩽ 2 cm defect in the 30 mmHg isobaric contour, Contractile Front Velocity (CFV) < 8 cm/s, Intrabolus Pressure (IBP) < 15 mmHg, and Distal Contractile Integral (DCI) < 5000 mmHg × s × cm
Mild peristaltic defect	Normal appearing wavefront propagation with a 2-5 cm defect in the 30 mmHg isobaric contour
Severe peristaltic defect	Evidence of wavefront propagation with a ⩾ 5 cm defect in the 30 mmHg isobaric contour
Absent peristalsis	No propagating contractile wavefront and minimal (< 3 cm) contractile activity or pressurization greater than the 30 mmHg IBC
Nutcracker	DCI > 5000 & < 8000 mmHg × s × cm
Spastic nutcracker	DCI > 8000 mmHg × s × cm
Spasm	Simultaneous contraction (CFV > 7.5 cm/s)
Elevated intrabolus pressure	IBP > 15 mmHg compartmentalized between the EGJ and the peristaltic wavefront
Panesophageal pressurization	Esophageal pressurization UES to EGJ with > 30 mmHg IBP

easily substituted for conventional manometry in our standard evaluation of esophageal motor disorders. The clinical protocol for high-resolution manometry is almost identical to standard conventional manometry with the obvious exclusion of the initial pull-through protocol to localize the LES. This portion of the initial intubation procedure and positioning of the catheter is not required due to the increased number and close proximity of the sensors making the sphincters easily identifiable on the topographic pressure plots.

With the adoption of HRM technology and pressure topography display methodology, the classification of esophageal motility that was developed for conventional manometric systems needs to be reconsidered. Although conventional metrics can be easily measured using simple software programs to convert the topographic data back to conventional line tracings and then applying a conventional analysis to a selected set of the line tracings, this method ignores the incremental gain in information obtained from the patterns presented by the pressure topographic plots. The alternative approach is to build an analysis and classification scheme that parallels conventional manometric classification, but also enhances it based on the strengths of the technology. To that end, we recently completed a comprehensive characterization of esophageal HRM data using novel analysis paradigms devised for pressure topography interpretation. We analyzed 75 normal subjects to develop normative ranges and applied this analysis system to 400 patients[26]. The major conclusions from that work, along with relevant contributions from other research groups, has allowed the initial formulation of an analysis and classification system for clinical practice and will be summarized in the following sections and tables.

The approach to analyzing and classifying HRM studies parallels conventional manometric technique in that it is focused on defining sphincteric and esophageal body function. However, new analysis paradigms have been created to assess deglutitive EGJ relaxation and peristaltic integrity and vigor with higher accuracy and detail. Basal EGJ relaxation pressure is measured using similar methodology to that used in conventional manometry; by assessing the mean end-expiratory pressure during a sufficient baseline period at the beginning of the study. Defining EGJ relaxation, however, has been modified to quantify EGJ relaxation pressure during the entire deglutitive period. Although a single nadir pressure measurement can be easily measured, bolus emptying and flow through the EGJ is not instantaneous and may take up to 4 to 5 s based on the volume swallowed[24]. Thus, deglutitive relaxation pressure is now quantified by measuring the 4 s integrated relaxation pressure (IRP), which represents the lowest 4 s cumulative pressure values for the deglutitive time period through the anatomic zone defining the EGJ. Normative values for this parameter were derived form 75 normal controls (Table 1) and this measurement was shown to be superior to a single nadir measurement and the continuous 3 s nadir pressure[27].

Recognizing that esophageal bolus transport is effected by the interaction of resistance through the EGJ, intrabolus pressure, and esophageal closure pressure behind the bolus[28], the second step of the analysis focuses on defining peristaltic integrity. Given previous data suggesting that pressures greater than 30 mmHg are almost uniformly associated with complete bolus transit[29,30], this threshold value was applied to define an intact peristaltic wavefront. This analysis is facilitated by the generation of 30 mmHg isobaric contour plots that delineate a pressure domain such that all pressures above this threshold value are circumscribed by a dark line. Normal swallows should have a seamless intact 30 mmHg isobaric contour with a contractile front velocity (CFV) below 7.5 cm/s. Abnormalities of single swallows are defined by the defects in the 30 mmHg isobaric contour and the contractile front velocity (Table 1).

The vigor of the smooth muscle contraction is another component that can be quantified in detail with HRM using a parameter defined as the distal contractile integral (DCI). This parameter quantifies the contractile activity in a space-time box by multiplying the length of

Table 2 Distal esophageal motility disorders based on pressure topography criteria

Disorder	Criteria
With Normal EGJ Relaxation (mean IRP < 15 mmHg)	
Peristaltic Weakness	
Intermediate	More than 30% of swallows with mild or severe peristaltic defects, but numerically insufficient to constitute severe peristaltic weakness
Severe	⩾ 70% of swallows with severe peristaltic defects
Aperistalsis	100% swallows with absent peristalsis
Nutcracker Esophagus	Normal CFV, Mean DCI > 5000 and < 8000 mmHg × s × cm, can be localized to either distal subsegment or LES
Spastic Nutcracker	Normal CFV, Mean DCI > 8000 mmHg × s × cm
Distal Esophageal Spasm	Normal EGJ relaxation and spasm (CFV > 8 cm/s) with ⩾ 20% of swallows
Esophageal Obstruction	Increased IBP or panesophageal pressurization not associated with EGJ obstruction
With Impaired EGJ Relaxation (mean IRP ⩾ 15 mmHg)	
Achalasia	
Classic achalasia	Impaired EGJ relaxation and aperistalsis
Achalasia with esophageal compression	Impaired EGJ relaxation, aperistalsis, and panesophageal pressurization with ⩾ 20% of swallows
Spastic achalasia	Impaired EGJ relaxation, aperistalsis, and spasm with ⩾ 20% of swallows
EGJ Obstruction	
Mild	Elevated IBP (15-30 mmHg) that is compartmentalized between the peristaltic wavefront (normal, weak, or nutcracker) and EGJ
Severe	IBP > 30 mmHg that is compartmentalized between the peristaltic wavefront (normal or nutcracker) and EGJ

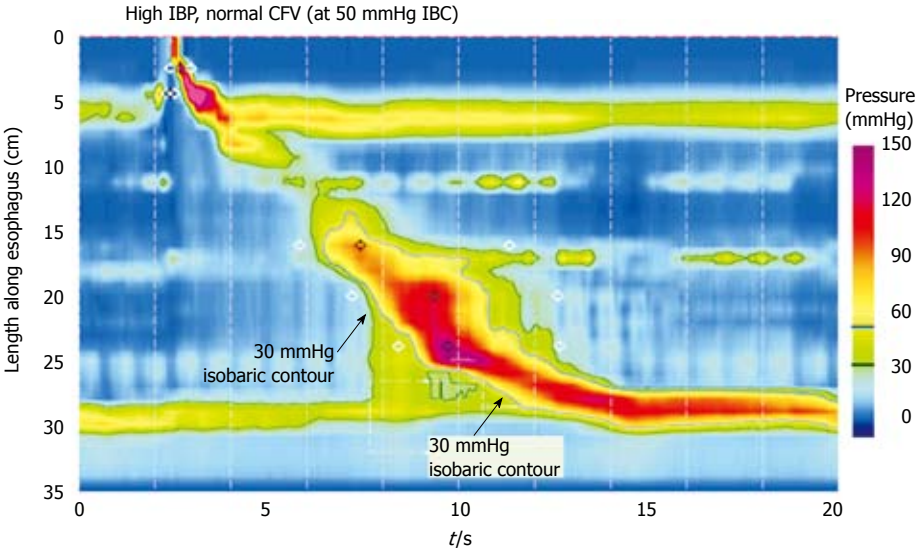

Figure 3 Defining increased intrabolus pressure using high-resolution manometry. The figure illustrates a swallow with functional obstruction at the EGJ. Note that the 30 mmHg isobaric contour line (black) deviates quickly from the 50 mmHg isobaric contour line (blue). In this case, the contractile front velocity is normal, reflecting the propagation velocity of 50 mmHg isobaric contour rather than the 30 mmHg isobaric contour. The intrabolus pressure domain is defined by the compartmentalized pressurization between the propagating contraction and the EGJ. Modified from: Pandolfino *et al*[26].

the smooth muscle esophagus by the duration of propagation of the contractile wave front, and the mean pressure value over the entire box excluding pressure signal below 20 mmHg[26]. The DCI provides much more detail than 3 isolated measurements of maximal contractile amplitude at 3-5 cm intervals, as it incorporates duration of contraction into the measurement. Although a DCI value > 5000 mmHg × s × cm exceeded the 95th percentile of normal thereby meeting the usual criterion for nutcracker esophagus, a threshold value of 8000 mmHg × s × cm distinguished a spastic nutcracker subgroup (n = 12) characterized by repetitive high amplitude contractions that was uniformly associated with dysphagia or chest pain[26]. Intrabolus pressure is another feature that can easily be defined by HRM using the isobaric contour tool to assess patterns of pressurization (Figure 3).

These measurement parameters for single swallows can be utilized to classify esophageal motor disorders[26,31]. Although HRM classification schemes have not been rigorously validated, they once again parallel conventional classification, and have the added benefit of much greater detail and an ability to characterize intrabolus pressure. Thus, it should not be difficult to incorporate these classification schemes into current clinical practice. Table 2 represents our motility laboratories version of an evolving classification scheme. Apart from changing the paradigm of categorizing peristalsis based on an intact isobaric contour, the classification of peristaltic dysfunction is very similar to conventional classification with the extra detail of distinguishing spastic nutcracker. The fundamental differences between the HRM classification system and previous conventional classification systems focuses on the sub-classification of achalasia and the category of EGJ obstruction defined by elevated IBP and impaired EGJ relaxation (Figure 3). In our analysis of 400 consecutive patients,

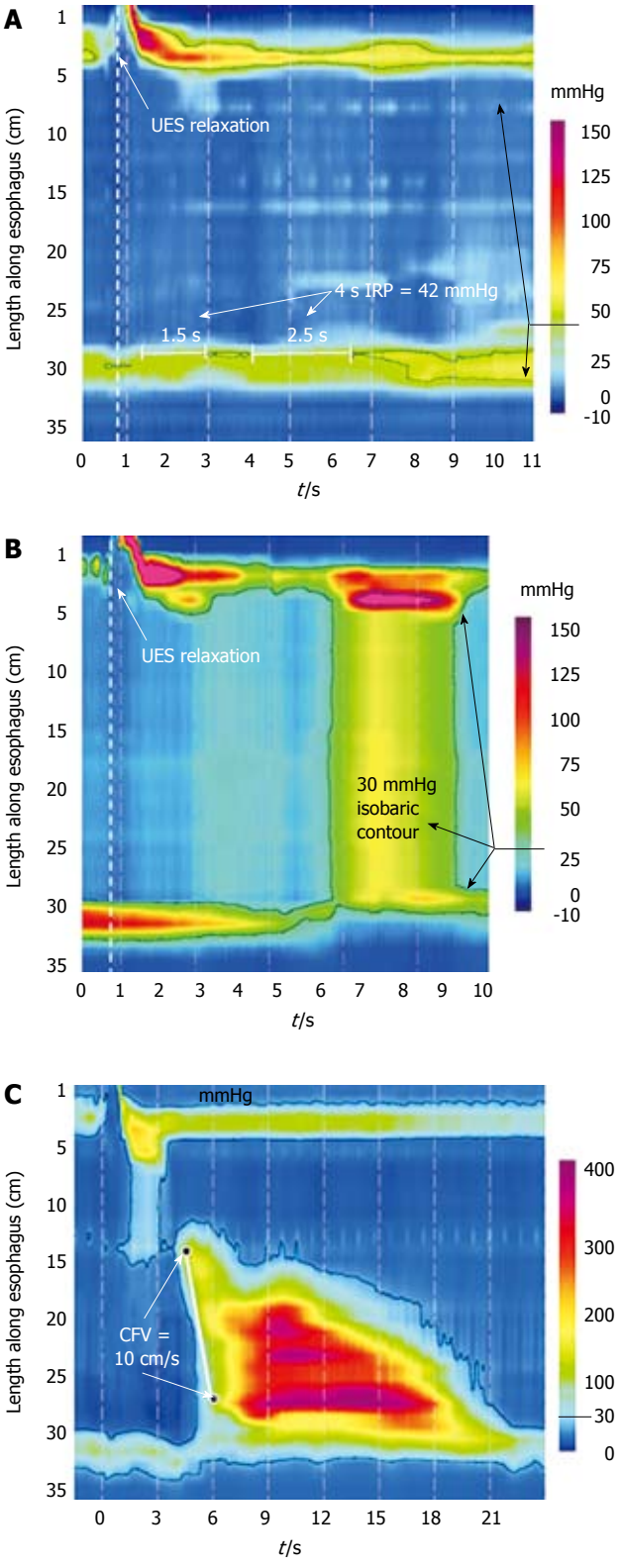

Figure 4 Achalasia subtypes based on manometric patterns of esophageal body contractility. A: Classic achalasia. There is no significant pressurization within the body of the esophagus and there is concurrent impaired EGJ relaxation (IRP of 42 mmHg in this example); B: Achalasia with compression. This subtype exhibits a rapid pan-esophageal pressurization; C: Spastic Achalasia. Although this swallow is associated with rapidly propagated pressurization, the pressurization in this case is attributable to an abnormal lumen obliterating contraction. Modified from: Pandolfino *et al*[26].

we found that 44 patients met this criteria and these patients were a heterogeneous group composed of post-fundoplication surgery, esophageal stricture, eosinophilic esophagitis and an idiopathic group that could represent an achalasia variant or pathology that could not be defined by conventional methodology[26].

A diagnosis of achalasia requires both aperistalsis and impaired deglutitive EGJ relaxation. However, there are specific subtypes that can be identified using HRM that can predict clinical outcome (Figure 4). In its most obvious form, achalasia occurs in the setting of esophageal dilatation with negligible pressurization within the esophagus (Type 1). However, despite there being no peristalsis, substantial pressurization within the esophagus can also occur. In fact, a very common pattern encountered in achalasia is of pan-esophageal pressurization (Type 2). These patients generally have a non-dilated esophagus with no obvious endoscopic or radiographic abnormalities. The other, much less common, pattern is of spastic achalasia in which there is a spastic contraction within the distal esophageal segment (Type 3). In a series of 73 consecutive achalasics, 40 (54.8%) had aperistalsis, 29 (39.7%) had pan-esophageal pressurization, and only 4 (5.5%) had spastic achalasia (Pandolfino *et al*[26]). The importance of this classification scheme was highlighted when logistic regression analysis found Type 2 to be a predictor of positive treatment response while Type 3 was predictive of negative treatment response regardless of whether treatment was medical, endoscopic or surgical.

CONCLUSION

Multichannel intraluminal impedance and HRM are new tools that can improve the accuracy and detail in describing esophageal function. These technologies should not be viewed as competing technologies, as each provides distinct information. Rather, efforts should be focused on combining these techniques, as they are largely complementary. For instance, high-resolution manometry is the best method to analyze the pressure profile of the esophagus, and certainly it should be combined with impedance monitoring if bolus transit abnormalities are shown to help define disease states and direct clinical treatment.

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Axial force measurement for esophageal function testing

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Abstract

The esophagus serves to transport food and fluid from the pharynx to the stomach. Manometry has been the "golden standard" for the diagnosis of esophageal motility diseases for many decades. Hence, esophageal function is normally evaluated by means of manometry even though it reflects the squeeze force (force in radial direction) whereas the bolus moves along the length of esophagus in a distal direction. Force measurements in the longitudinal (axial) direction provide a more direct measure of esophageal transport function. The technique used to record axial force has developed from external force transducers over in-vivo strain gauges of various sizes to electrical impedance based measurements. The amplitude and duration of the axial force has been shown to be as reliable as manometry. Normal, as well as abnormal, manometric recordings occur with normal bolus transit, which have been documented using imaging modalities such as radiography and scintigraphy. This inconsistency using manometry has also been documented by axial force recordings. This underlines the lack of information when diagnostics are based on manometry alone. Increasing the volume of a bag mounted on a probe with combined axial force and manometry recordings showed that axial force amplitude increased by 130% in contrast to an increase of 30% using manometry. Using axial force in combination with manometry provides a more complete picture of esophageal motility, and the current paper outlines the advantages of using this method.

Key words: Axial force; Traction force; Manometry; Motility; Peristalsis; Esophageal function

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INTRODUCTION

The primary function of the esophagus is to transport ingested material to the stomach. Some of the most prevalent diseases in the esophagus relate to malfunction of transport e.g. reflux of stomach contents and motility disorders. The regulation of the normal function of the esophagus is complex, and requires fine coordination of the longitudinal muscles and circular muscles[1]. Manometry is the gold standard for the diagnosis of motility diseases in the esophagus[2]. It has been used for many decades, and provides an indirect picture of the motility patterns because it only gives information about muscle contraction or radial squeeze[3]. However, any contraction - strong or weak - will only be measured by manometry if it occludes the measuring catheter. Using data from computer models, it has been argued that shortening of the longitudinal muscle plays an important role in the mechanisms of peristalsis and that pressure amplitude per se does not give any indication of the force required to drive the bolus forward[4]. Hence manometric recordings alone are insufficient to describe and quantify esophageal motility. To improve this, and gain more knowledge, modalities such as fluoroscopy[5,6] and ultrasound[7] have been used in combination with manometry. These modalities have confirmed that parameters recorded by manometry only partly describe the peristaltic wave, but these imaging modalities do not provide quantitative information on force in either radial or axial directions[8]. Furthermore, in the clinic, these extra modalities in combination with manometry are inconvenient because multiple examinations are needed.

A more physiologically related measure that gives direct information about the motility is to record the force that pushes or propels the bolus in an axial direction towards the stomach. This method of quantifying peristalsis is referred to as propulsive force[6,9-13], traction force[6,14-17] and peristaltic force[11,18]. We refer to these concepts as axial force as this is the direction of the force in contrast to manometry, which records the radial force.

AXIAL FORCE RECORDING TECHNIQUES

The number of publications in relation to axial force is limited to less than ten, with Winship *et al*[12] being the first to publish a method that recorded the axial force of the human esophagus in 1967. They used an external force transducer connected to a plastic sphere placed in the esophagus. This enabled assessments of the esophagus' ability to propel the plastic sphere against a known resistance. Pope *et al*[11] and Schoen *et al*[18] used a mercury-in-silastic strain gauge which was placed together with a plastic sphere in the esophagus. The next development by Russell *et al*[13] was similar to previous editions though the mercury in the strain gauge was replaced by saline to reduce the effect of temperature dependence. The use of a plastic sphere did not allow a change of volume *in vivo*, hence in studies with varying bag sizes, the probe had to be redrawn, the sphere replaced and swallowed again. This could introduce some errors in terms of positioning as well as irritation and secondary contractions. Williams *et al*[14] and Pouderoux *et al*[6] and co-workers used a strain gauge, but did not describe any technical details. The next series of publications were based on the use of a miniature strain gauge, and published in the period from 1992 to 1997[6,14-17]. A new technique, based on impedance planimetry, was introduced recently (2008) by our group[19]. The principle of impedance planimetry is to create an electric field between two excitation electrodes placed in a bag with conductive fluid. Two detection electrodes placed close to each other, and midway between the excitation electrodes, measure the cross-sectional area. This is possible as the impedance between the electrodes is proportional to the distance between the detection electrodes, and inversely proportional to the conductivity of the fluid and the cross-sectional area[20]. The only variable left is the cross-sectional area, as the other parameters are constants implemented in the calibration[12]. We took advantage of our experience with impedance planimetry, and redesigned the probe to have a constant cross-sectional area, but allowed the distance between the detection electrodes to vary (Figure 1). Thus, the potential between the electrodes would be linearly related to the axial force. This design enabled easier probe construction and less technical pitfalls compared to the strain gauge design.

Axial force still needs to prove its utility in relation to high resolution manometry. It has not yet been documented how high resolution manometry and axial force correlate. We believe that despite the extra information high resolution manometry can provide, it still does not record the actual function of the esophagus and any attempt to interpret the bolus transport can be flawed. An undetected transport of the bolus can still occur with manometry becauses it mainly quantifies the circular muscles' contractions

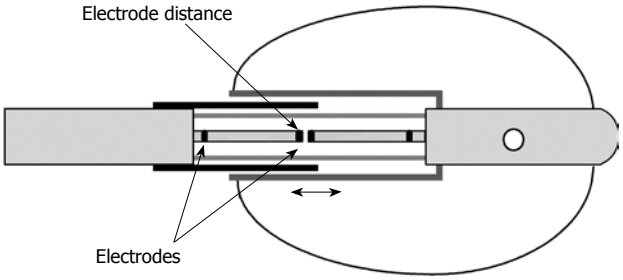

Figure 1 The redesign of impedance planimetry has enabled measurements of axial force instead of changed in cross-sectional area. The variable in the redesign is the distance between the detecting electrodes. This distance can be related to axial force by means of calibration. Any axial force applied to the bag will increase the distance between the electrodes. The thick black and dark gray lines represents rigid plastic cylinders that ensures that the construction will not bend. The design also enables the bag to be inflated *in vivo*.

Axial force, based on strain gauge or impedance, has some limitations. Despite anchoring the probe to the nose or cheek, some movement will occur. This will affect the axial force recordings but in manometry would merely relocate the recording site. This movement will primarily influence the amplitude of the axial force recordings.

Future development of axial force recordings may include multiple recordings on the same probe. Recordings at different sites would provide a more complete picture of esophageal transport function and axial forces.

AXIAL FORCE RECORDINGS IN HEALTHY SUBJECTS

The axial force can be divided into two main components; the "grip" effect, and the ability to push in the axial direction again an intra luminal object[13]. The "grip" effect is primarily determined by manometry as the squeeze effect. A better grip will decrease the chances for the peristaltic wave to slip over a given intraluminal object and, thereby, create a basis for a strong axial force. The grip effect is also determined by frictional force. Any amount and type of fluid will change the frictional forces between the probe/bag and the esophageal wall and, thereby, change the grip effect. It has been shown that a swallow of 10 mL of salad oil decreased the amplitude by more than 50% in subsequent swallows[11]. The influence of frictional forces is an issue that needs further investigation before a statement about their influence in real-life situations can be made.

The information gained from axial force recordings has differed depending on the design of the studies. As with manometry, the absolute axial force values in control subjects vary markedly[11]. The coefficient of variation has been reported to be in the same range

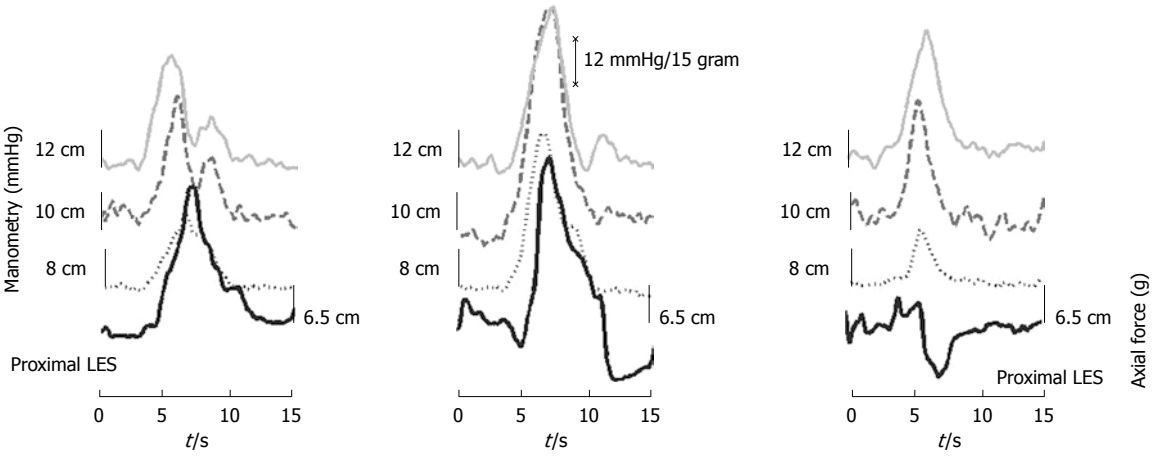

Figure 2 A voluntary dry swallow (time = 0) from three subjects with a bag volume of 2 mL. The solid black tracings are the axial force recorded 6.5 cm proximal to the lower esophageal sphincter, and the three other tracings are the pressure recorded 12 cm, 10 cm and 8 cm proximal to the lower esophageal sphincter. Using the manometric tracings, the first swallow (left) showed a normal propagating peristaltic wave and the resulting axial force response was as expected. The swallow of the second subject (middle tracings) shows a rather powerful pressure amplitude; but, it is not propagating (peak amplitudes are similar in time). In contrast, the axial force response is comparable in amplitude to that in the first subject (left tracings) although it is followed by axial force in the oral direction (negative value). In this case, the interpretation of the manometric recording would be flawed if the axial force had not been recorded simultaneously. The third person (right tracings) had lower manometric amplitudes during swallowing, without any propagation. The axial force response is different from the swallowing in the second person (middle tracings), as there is a weak reflux (oral axial force). The data shown is taken from a study in our group where the data is still being analyzed.

for manometry[22] and axial force[23]. These finding are similar to data collected in our laboratory. In assessing whether or not multiple manometric tracings along the esophagus are propagating, axial force recordings have shown to be inconsistent with the interpretation gained from manometric recordings[11]. This has also been documented using other modalities such as radiographic or scintigraphic imaging[21,24,25]. As an example from our laboratory, Figure 2 shows three dry swallows from three healthy subjects with a bag containing 2 mL saline. Three manometric tracings (12 cm, 10 cm and 8 cm proximal to the lower esophageal sphincter), and one axial force (6.5 cm proximal to the lower esophageal sphincter) tracings were recorded. These three examples show how the recordings can be very difficult to interpret when the bolus transit is deduced solely from manometry tracings.

Our probe design enables axial force recordings with various bag sizes to provide a challenge test for the esophagus (e.g. greater force with larger bag size). The effect of the bag size during force recordings was studied to some extent by Winship *et al*[12] who inflated a bag with 10 mL after which a swallow was initiated. The axial force applied to the bag from the peristaltic wave persisted until the bag was deflated. A comparable experiment was done by Pouderoux and co-workers using bag diameters of 15 mm to 20 mm[6]. During a study in our group a persisting axial force was occasionally generated at even smaller bag volumes (6 mL). However the data analysis for this study is not complete. Swallowing studies recording axial force have previously been performed with a balloon in the esophagus inflated with either air[11,12,14] or fluid[19]. Increased bag volume increased the axial force amplitude[11,14,15]. This could be due to an improved grip effect. These studies extracted parameters from axial force and manometric recordings and found little or weak correlation[6,11,13,14]. Thus, additional information is likely to be gained by recording axial force. Using clips attached to the esophageal mucosa, the shortening/elongation of the esophagus was measured in a study by Pouderoux and co-workers[6]. They combined shortening information with axial force and manometry recordings. A good correlation was found between axial force and (1) shortening of the distal esophagus, (2) the maximal contraction of the distal esophageal segment, and (3) the extent of aboral movement during the period of axial force recordings. These factors are all involved in the longitudinal muscle contraction. Hence, axial force recordings may show defects in this area where manometry provides poor information. Unpublished data from our laboratory on pressure and axial force recorded simultaneously have shown that the amplitude of manometry increases only slightly proximal to the bag (approximately 30%) when the volume is increased whereas axial force increased to a major degree (approximately 130%) (Figure 3). Somewhat similar findings were obtained in a previous study[11]. Axial force was also present with an empty bag. This could be due to the size of the probe (5 mm in diameter) as the grip effect is sufficient to generate an axial force. The amplitude for axial force, and manometry increased when dry and wet swallows were compared.

Using area-under-curve for manometry tracings as a parameter to predict the axial force response has also failed as there was very little correlation between this parameter and the area-under-curve for the axial force tracings[21].

The clinical standard procedure with manometry during swallow tests does not include a bag being inflated, as this only affects the manometric recordings to a minor degree[26,27]. On the other hand, increasing the bag volume would present a challenge test to the esophagus similar to an electrocardiogram recorded during exercise. During exercise, the electrocardiogram

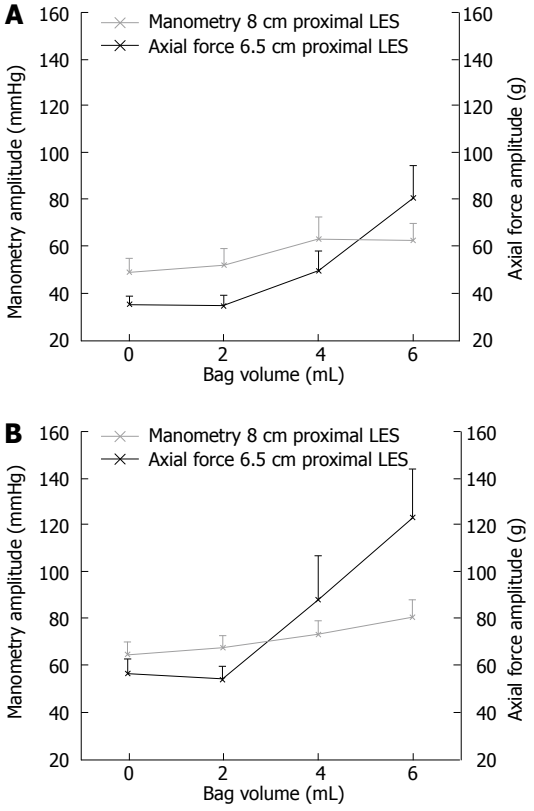

Figure 3 Swallow test with increased bag volume. A: Dry swallow; B: Wet swallow. The pressure was recorded 8 cm proximal to the lower esophageal sphincter and the axial force was recorded 6.5 cm proximal to the lower esophageal sphincter. Both graphs show that the increased amplitude for axial force was greater when compared to manometry during both dry and wet swallows. Data are presented as mean ± SE from 10 volunteers.

can reveal abnormalities not seen at rest[28]. Thus, a "challenge test" could provide a more sensitive test when recorded with axial force. This would be of great interest when applied to patients with motility-related diseases.

AXIAL FORCE RECORDINGS IN PATIENTS

Axial force is not a widely used technique and only a limited number of patients have been examined. In one study, a group of eight subjects complaining of dysphagia were examined. Radiography failed to reveal an organic narrowing of the esophagus, and no abnormal motor activity was observed by the fluoroscopist. Furthermore, the manometric examination did not reveal any dysfunction of peristalsis. On the other hand, axial force recordings clearly separated these subjects from healthy controls[11]. The patients were divided into two subgroups, one which had abnormally high amplitudes, and the other with abnormally low amplitudes. In 30 gastro-esophageal reflux patients with erosive disease and normal manometry, six patients showed impaired axial force amplitudes. This was based on a protocol with ten wet swallows (10 mL) for each patient. A similar pattern was shown in the same study where six out of twelve patients suffering from functional dysphasia had impaired axial force amplitudes but normal manometric amplitudes[17]. These results point in the direction of diagnosis being based on both manometry and axial force. If the result of multiple modalities can be provided by one examination, it will minimize the number of investigations and inconvenience for the patient without affecting the final diagnosis. Axial force has been measured simultaneously with manometry and, if not incorporated in the same catheter[11,13,19], manometry can be recorded next to the axial force probe[12,17]. The information gained from such investigations would be pressure and axial force generated by esophageal contractions. The preliminary results from studies in the last forty years have indicated that axial force recordings add further information to traditional manometry, and it remains unanswered why axial force is not more widely used.

CONCLUSION

Axial force measurement provides additional and more physiological information about the swallowing function compared to manometry. In future studies, combined axial force recordings and manometry may provide more useful information on esophageal muscle function in basic and clinical studies, especial protocols where a challenge test of the esophagus is incorporated will be of major interest in basic and clinical studies. It has been suggested that manometry could subgroup gastro-esophageal reflux disease patients into those requiring partial or total fundoplication treatment[29-31]. However, studies have not been able to prove that preoperative manometry could predict postoperative outcome in terms of dysphagia[32-34]. Measurement of axial force is more likely to identify patients who would develop postoperative dysphagia, although proof awaits clinical studies.

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GUIDELINES CLINICAL PRACTICE

Asbjørn Mohr Drewes, Professor, MD, PhD, DMSc, *Series Editor*

Do we really understand the role of the oesophagogastric junction in disease?

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Abstract

The role of the oesophago-gastric junction (OGJ) in gastro-oesophageal reflux disease is still not completely understood, and there is no clinically used method to assess the OGJ function in patients. Only indirect methods such as pH studies are carried out. The OGJ acts a valve controlling the flow of solids, liquids and gases between the oesophagus and the stomach. Manometry can determine if a sphincter is toned or relaxed; but, it cannot confirm that the sphincter region is actually open. Distension is a new technique for measuring function in the OGJ. By measuring the cross-sectional area through the narrow region in the junction during distension of a catheter mounted bag, much more information on the opening and closing patterns of the junction can be determined. This technique has already been demonstrated to show changes in the OGJ after surgical treatments for reflux disease. New measurement ideas around the concept of distending the OGJ offer new hope that a clinically useable test for compliance at the junction can be developed and could potentially help in determining appropriate therapy.

Key words: Achalasia; Distensibility; Impedance Planimetry; Intraluminal impedance manometry; Oesophago-gastric junction

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INTRODUCTION

If you ask many clinicians, even those specialising in gastroenterology, what is the mechanism between the oesophagus and stomach which prevents the backflow of stomach contents into the oesophagus? Their instinctive reply will be the lower oesophageal sphincter (LOS). While the LOS certainly contributes to the mechanism it is not the sole effecter. Many common definitions of the mechanism have not even been updated. Medlineplus© defines gastroesophageal reflux disease as "backward flow of the gastric contents into the oesophagus due to improper functioning of a sphincter at the lower end of the oesophagus and resulting especially in heartburn[1]". The more clinical definition agreed at the Montreal Consensus does not make any mention of the relevance of the LES; "GORD is a condition which develops when reflux of stomach contents causes troublesome symptoms and/or complications". A definition very much based on effect rather than cause[2].

Of course with a little reading, most of us will know that the barrier at the oesophago-gastric junction (OGJ) is rather complex with a number of mechanisms impinging on it. Mittal and Balaban's New England Journal of Medicine article from 1997 highlights the fact that the lower end of the oesophagus is

guarded by an intrinsic smooth muscle called the lower oesophageal sphincter, an extrinsic skeletal muscle called the crural diaphragm, the intra-abdominal location of the LOS, integrity of the phrenoesophageal ligament, and maintenance of the acute angle of His promoting a "flap valve" function[3-5]. It has also been concluded that this structure is very sophisticated in the neurophysiologic control of opening, and its anatomical configuration, and with respect to dynamic changes in forces from active and passive muscle structures in this region of the body.

DISEASES RELATED TO OGJ FUNCTION

Gastro-oesophageal reflux disease

Gastro-oesophageal reflux disease (GORD) is one of the most common diseases in Western Civilisation. GORD can progress to severe complications if untreated. It is a highly prevalent disorder and affects 10%-20% of Western populations[6]. GORD originates from a disturbance in the structure and function of the LOS barrier. Anatomical or structural abnormalities occur often in the presence of a hiatal hernia and physiological predispositions from abnormal motor function of the LOS and oesophageal body. Dysfunctional oesophageal motility coupled with a weak LOS can cause uncoordinated propulsion of food, regurgitation of food and acid into the oesophagus, particularly after meals, and in the horizontal position, an inadequate acid/bile clearance from the oesophagus.

Achalasia

Achalasia is a motor disorder of the oesophagus characterized by loss of oesophageal peristalsis and failure of the LOS to relax completely upon deglutition. There is a poor correlation between higher sphincter pressures, oesophageal emptying and achalasia symptoms in general[7]. The origin of achalasia is still poorly understood. There is evidence to implicate familial, autoimmune, infectious or environmental causes. Currently, it is not possible to determine how the OGJ or the sphincter function changes as the disease develops.

DIAGNOSIS OF OGJ RELATED DISEASES

By far the most common OGJ disorder is GORD. For most people, GORD is diagnosed by symptoms usually at a visit to their general physician or community doctor. However, for many people, pharmaceutical treatment of GORD does not completely eradicate their symptoms, and they must be referred to a gastroenterologist for further evaluation and treatment. Usually, the first test a gastroenterologist will carry out is an upper gastrointestinal endoscopy. This visual test will quickly identify the presence of any erosive disease in the region of the OGJ, and if there are visible signs of this erosion or Barrett's metaplasia.

These referrals may also undergo other tests if endoscopy is negative. Traditionally, this involved oesophageal manometry to evaluate peristalsis and to confirm sphincter position and activity. This is followed by a 24 h pH study using a catheter based system that can incorporate impedance monitoring or a catheter-free telemetry-type system. Intraluminal impedance is increasingly being used to determine whether non-acid reflux may play a role in patients with PPI-resistant reflux symptoms, chronic unexplained cough, excessive belching, and rumination[8].

Despite the important role the OGJ plays in reflux disease, apart from standard manometry[9], techniques have not focused on objectively measuring its function.

ROLE PLAYED BY OGJ

Even with a number of new tests for reflux disease, it is still difficult to determine the actual role of the OGJ in this disease. Of course, the only test which directly measures some aspects of the junction is manometry, and this has been shown, for some time now, not to be a reliable predictor of GERD on its own[10]. There has been little emphasis on assessing the mechanical properties of OGJ opening, and little is known about the dynamics of retrograde flow across the OGJ. Thus, it is time to revisit this and look at new evidence.

DO WE HAVE THE COMPLETE PICTURE?

If the OGJ has a role in digestive disease, and if none of the parameters we use to determine the severity of a patient's reflux disease relate to any direct objective and quantitative parameter or parameters, do we really have the complete picture? Should more research not be carried out into methods to determine the role of the OGJ in disease? If pH studies determine that there is too much acid refluxing into the oesophagus, can we always be confident that this is due to dysfunction of the LOS? Could it be some other mechanism in the process of digestion, such as peristaltic dysfunction and poor oesophageal acid clearance which is malfunctioning and causing the disease?

THE OGJ AS A VALVE

If we accept that the main action or combined actions at the OGJ is to narrow and close the junction between the stomach and the oesophagus, then we must accept that it works in a similar way to a valve. The medical dictionary defines a valve as "a bodily structure that closes temporarily a passage or orifice or permits movement of fluid in one direction only" (Merriam-Webster Online Dictionary copyright© 2005 by Merriam-Webster, Incorporated). In the context of the OGJ, this definition is quite limiting, and I prefer the more general web definition of "A valve is a device that regulates the flow of substances (either gases, fluidized solids, slurries, or liquids) by opening, closing, or partially obstructing various passageways[11]". The OGJ function is probably better described in this way. It is as much a control valve as an "on" and "off" valve. While food boluses and liquids travel antegrade, air and on occasion small

amounts of stomach contents must travel retrograde back up the oesophagus for venting purposes.

HOW CAN WE MEASURE VALVE FUNCTION?

This, in effect, can be a difficult question. In engineering, we can easily measure the performance of an on/off valve by checking it visually. If the open valve condition is created, it is usually possible to observe flow through the valve. In the closed valve condition, it can normally be inspected for leaks or other forms of dysfunction. If the valve controls flow rather than shutting off completely, then normally there will be a device included to determine the flow.

Obviously the situation is much different in the OGJ. Much of the anatomical structure in the region is soft tissue. This does not image well radiologically, although historically barium studies have given a good, but subjective indication of flow during swallowing. The OGJ tends to be very dynamic, and so does not image well using magnetic resonance. In addition, the image resolution of current MR technology and CT is of insufficient quality to be satisfactory.

Historically, the LOS has been evaluated using manometry which uses pressures measured at precise points on a luminal catheter to determine the forces applied by the squeezing or pushing of the sphincteric regions in luminal organs. Manometry has evolved into a very sophisticated procedure for determining pressure changes in luminal organs during motility, but it is no longer recommended for the diagnosis of reflux disease[10].

FUNCTIONAL TESTING FOR DIAGNOSIS

The flow diagram in Figure 1 outlines the pathway for a patient with gastro-oesophageal reflux disease. In up to 20% of cases, there can be a negative or non-optimal response to drug therapies such as PPIs or H_2 blockers. These patients are usually referred to a gastroenterologist or in some cases directly to a GI surgeon where the severity of their condition is evaluated.

Referred patients will most often undergo an upper GI endoscopy so that erosive signs of the disease or Barrett's metaplasia can be excluded. However, up to 80% of patients will have a normal endoscopy, and will be referred for oesophageal function testing[12]. Most of these patients will undergo pH and manometry studies. Manometry can be utilized to document landmarks for placement of the pH electrode. The older manometry method is to perform a catheter pull-through study which will identify the respiratory inversion point and establish the high pressure zone at the LOS. This would also then indicate the position of the z-line and determine the position of the 24 h pH catheter, usually 6 cm above the z-line or 5 cm above the proximal margin of the LOS. The 24 h pH study is then used to determine the total time the pH < 4 or a composite

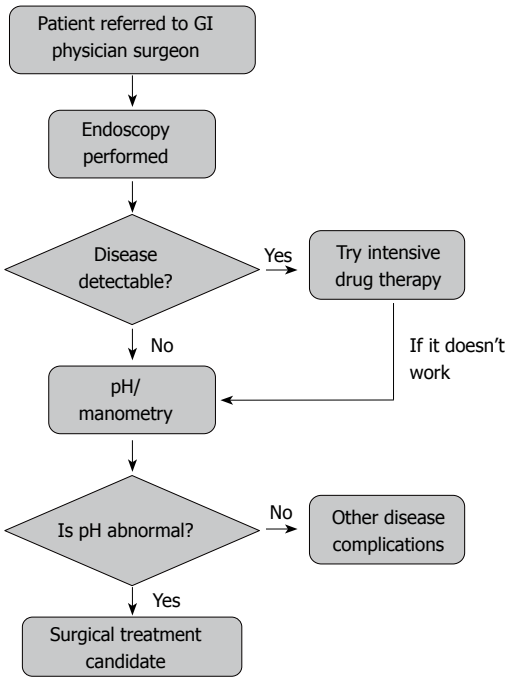

Figure 1 The pathway for a referred patient with gastro-oesophageal reflux disease.

score of oesophageal acid exposure. The DeMeester score uses 6 parameters (supine reflux, upright reflux, total reflux, number of episodes, number of episodes longer than 5 min, and the longest episode) to calculate a score which indicates the severity of reflux.

Newer methods for determining oesophageal function include high-resolution manometry (HRM), intraluminal impedance and wireless pH measurement. HRM is a method which uses sensors at 12 radial orientations and 36 longitudinal positions (1 cm intervals). In effect, this sophisticated probe can monitor the dynamic activity throughout the oesophagus from above the upper oesophageal sphincter to below the LES. Recent clinical evaluation has shown two major strengths of pressure topography plots compared with conventional manometric recordings. These strengths are the ability to (1) delineate the spatial limits, vigor, and integrity of individual contractile segments along the oesophagus and (2) to distinguish between loci of compartmentalized intra-oesophageal pressurization and rapidly propagated contractions[13].

In terms of reflux monitoring, the main advantage of the wireless pH system is the ability to attach a capsule to the inner mucosal surface of the oesophagus, thus negating the need to run an uncomfortable catheter through the patient's nasal passage[14].

The recent development of intra-luminal impedance as a measurement technique has added another option to the clinician's diagnostic armoury. This technique has been shown by numerous studies to be a sensitive and reliable means of detecting fluid or gas movement within the oesophagus. Hence it is a useful tool for determining the presence and extent of acid and non-acid gastro-oesophageal reflux, but not the quantity[15-17]. Despite these sophisticated tests none can measure the objective

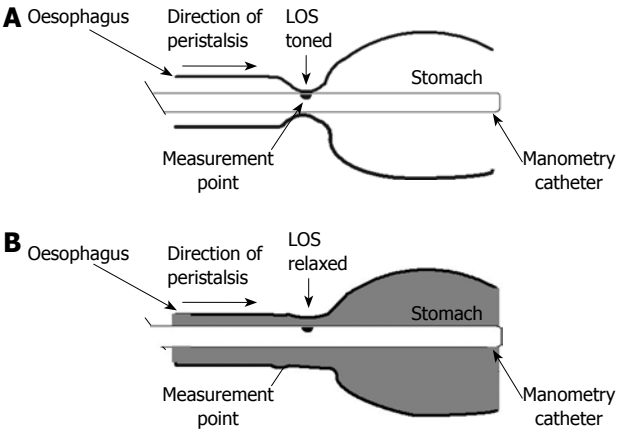

Figure 2 Simple manometry catheter shown in position in the LOS when it is toned. A: Manometry catheter with the black dot on the catheter indicating the measurement point in the oesophago-gastric junction when a normal LOS is toned or competent causing the sensor to be engulfed or occluded by the sphincter; B: The pressure is indicative of the state of the chamber created by the lumen (area in grey) opening up between the oesophagus and stomach.

role of the valvular effect at the OGJ in oesophageal disease.

CAN MANOMETRY MEASURE OGJ FUNCTION?

Since the work of Ingelfinger, Code and Fyke in the 1950s, most of the evidence which identifies the OGJ as the prime suspect in reflux disease has been based on manometric studies[18,19].

From the basic pull-through techniques to the development of the sleeve sensor, manometry has been used to describe sphincteric action at the junction. However, all the evidence in recent years suggests that it is not the ideal measurement technique. Recently, the AGA has advised that manometry should not be used to verify the presence of reflux disease. Yet, it is often still used as a technique in research studies[10,20-22].

A manometer is actually an instrument designed for measuring gas pressure. We should also remember from physics that pressure is a measure of force per unit area. Oesophageal manometry is often incorrectly defined as the degree of pressure exerted by the muscles of the oesophageal wall. The less specific definition of manometry as a method of recording pressures within the oesophagus is probably more appropriate. Strictly speaking muscles do not exert a pressure, they exert a force or rather forces in several directions. It could be argued that a manometric system cannot measure these forces; however, what it can do is use pressure as a rough proxy.

Consider the case of a manometry sensor on a catheter type probe suitable for inserting into luminal organs such as the oesophagus. Whether it is a water perfusion 4 port catheter or a state-of-the-art 36 position, 12 radial sensor measuring high resolution manometry catheter, the sensing principle is the same. Figure 2 indicates how this concept of measuring pressure may

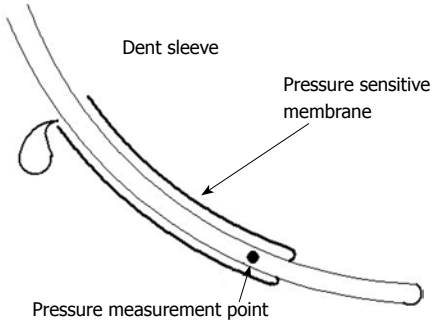

Figure 3 The dent sleeve.

be limiting. In Figure 2A, we can see the manometry catheter with the black dot on the catheter indicating the measurement point. This point represents a sensor on any manometry catheter. When a normal LOS is toned or competent, it exerts a squeeze or force on the sensor causing it to be engulfed or occluded by the sphincter. Hence, the pressure measured provides a reasonable representation of the force in the tightened sphincter. However, if the sphincter is relaxed, as demonstrated in Figure 2B, then rather than the lack of squeeze or force present being represented by no or low pressure at the sensor, the pressure here is more indicative of the state of the chamber created by the lumen opening up between the oesophagus and swallowing. Hence, the pressure observed at the measurement point is the force over a large area indicated by the shading in the diagram. We can conclude from this, when making manometric measurements in the LOS, that when the sphincter is in the toned state, we are actually measuring under a different set of parameters than when it is relaxed or not exerting a force on the pressure measuring sensor point. The measurement being made during swallowing is also altered by the peristaltic wavefront creating a bolus pressure that is pushing through the relaxed OGJ-highlighted bolus flow pressure and elastic properties of the OGJ.

One of the biggest developments in manometry was the introduction of the sleeve sensor in 1976[23]. Difficulties with reliable monitoring of maximal sphincter pressure with perfused side holes led to development of the Dent sleeve (Figure 3). The Dent sleeve recognized that, because of continuous movement within the lumen of the recording catheter and the narrowness of the region in the sphincter at peak pressure, it is impossible to guarantee that you are measuring the peak pressure continuously with a normal point measurement catheter. The sensing function of the sleeve ensures that it records maximal sphincter pressure, regardless of where the sphincter is positioned along the sleeve length. These unique recording properties make the sleeve the only sensor that can monitor sphincter pressure reliably using a static probe. However, new high-resolution manometry catheters are not limited by this movement artifact. The pressure drop measured by the Dent sleeve or high-resolution manometry is sometimes mistakenly assumed to be correlated with sphincter opening.

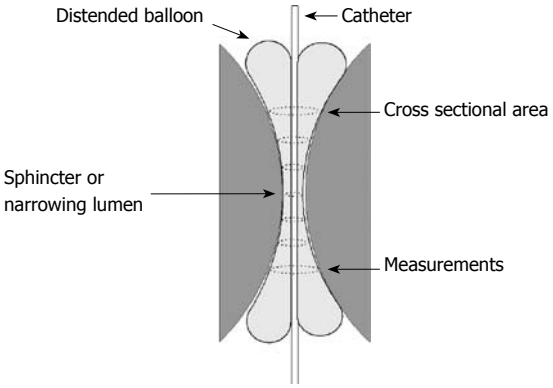

Figure 4 The functional luminal imaging probe (FLIP) showing how a cylindrical shaped bag mounted on the distal end of a catheter can use impedance planimetry to measure multiple cross-sectional areas (CSAs) at fixed intervals along a catheter.

DISTENSIBILITY AS A NEW MEASURE

Recently, a number of groups have been looking at the concept of distending the junction as a better measure of its performance. This suggestion is not a new idea. In fact as early as the 1960's, Harris and Pope identified that sphincters do not necessarily need to squeeze or contract tightly to be competent and, therefore, resistance to distension by measurement of radial force should be the prime determinant of sphincteric strength[24]. Studies on yield pressure in the stomach were probably an early distension method[25,26]. This involved filling air into the stomach and monitoring the pressure reading when the OGJ was forced to open to allow air to travel up into the oesophagus.

In 2002, Pandolfino and coworkers demonstrated that the OGJ of patients with hiatal hernias was more distensible and shorter than normal subjects[27]. This was done using manometry, fluoroscopy and stepwise, controlled barostatic distension of the OGJ. Then by controlling the pressure, the OGJ diameter and length were measured during deglutitive relaxation. In reality, it is difficult to control pressure in a bag located at the distal end of a narrow diameter catheter.

Shaker *et al*[28], demonstrated that under different conditions, the effects of muscle action and passive elastic tissue on the distensibility of the junction could be determined. The investigators used a barostat bag, cylindrical in shape, placed across the OGJ and recorded pressure, volume and distensibility as changes in pressure related to changes in volume. These changes were only a rough proxy for distensibility changes in the digestive lumen at the oesophago-gastric junction, since changes in the bag volume could be attributed to all forces on the bag, and not just those relating to valvular or sphincter action. Nevertheless Shaker *et al*[28] went on to show how this technique could indicate an increase in pressure at stepped bag volumes, and implied that this was related to a reduction in the distensibility of the OGJ. This work clearly demonstrates that a distensibility test may be useful in evaluating the effects of the different components on OGJ function.

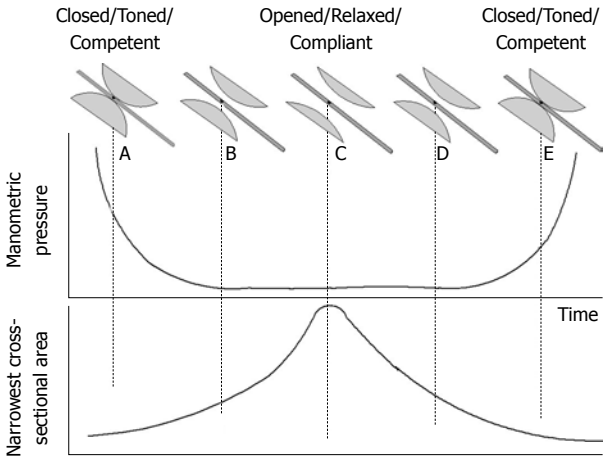

Figure 5 Diagram showing the difference between manometry and cross-sectional area (CSA) for measuring luminal changes in the oesophago-gastric junction during opening and closing. At point A where the junction is closed, there is a high pressure and a low CSA representing the toned sphincteric region. As the sphincter relaxes, very quickly the manometry catheter is no longer squeezed, and at point B the pressure is zero or very low. However, this opening is detected by an increase in CSA at B. At C the sphincter is fully relaxed; but, there is still no useful information from manometry despite the CSA increasing even further. The pressure does not start to rise again until the sphincter is fully closed and toned at E.

The functional lumen imaging probe (FLIP) takes distensibility testing to a higher level. FLIP allows a cylindrical shaped bag mounted on the distal end of a catheter to measure multiple cross-sectional areas (CSAs) at fixed intervals along the catheter. These CSAs can be used to build a dynamic three dimensional representation of the luminal geometric changes in the OGJ as the bag is distended on the catheter (Figure 4). In this way, luminal changes in the OGJ can be visually represented instantaneously, and with high spatial and temporal resolution[29,30]. This technique has been shown to demonstrate differences in patients undergoing endoluminal therapy for reflux[31].

MANOMETRY VERSUS DISTENSIBILITY

So what is the difference between measuring contraction or squeezing with manometry, and measuring opening or relaxation patterns using a distensibility technique? At the top of Figure 5 a simple series of drawings demonstrates the pattern of the sphincter part of the OGJ, and how it changes from being toned, compliant or competent to being relaxed or incompetent and then as it moves through the cycle back to being toned again. It can be seen how this cycle is measured using a manometry catheter or a distensibility probe such as FLIP, which can measure the narrowest CSA in the OGJ region. At the time point, represented by the dashed line A, the sphincter is contracted or squeezed, manometry will indicate by pressure that the force on the catheter is relatively high. Moving in time to the point at line B, it is evident that the sphincter has started to relax; the manometric pressure has dropped significantly since the sphincter no longer obscures or squeezes the pressure measuring point. Then, at time point C the sphincter

is fully relaxed or open; there is no significant change in the manometric pressure reading from time point B. However, the CSA value continues to increase to a maximum value. In practice, pressure can tell us the difference between a toned and a relaxed sphincter. CSA can tell us the difference between an open and closed sphincter. It can, in effect, tell us the amount of opening. As the sphincter starts to close and is no longer relaxed, it can be observed that manometry is very insensitive to this action at time point D, whereas CSA has detected the narrowing pattern. Then, eventually, at time point E, manometry has detected the squeezing effect of the toned sphincter, and the CSA has reduced to the smallest measurable value.

The most significant difference between manometry and distensibility is in the type of measurement. Manometry measures pressure which is a proxy of the combined active and passive forces during squeezing. Ideally, it does not interfere with the measurement. In reality, you cannot measure a toned sphincteric region using a pressure point on a catheter which is running through the sphincter without interfering with its function. Distensibility, on the other hand is more of a challenge test. By inflating the liquid filled bag as it straddles the OGJ, we are able to measure its response to ramped or stepped distension.

CONCLUSION

There are no available objective tests for testing OGJ function in routine clinical practice. Current gold standard tests for GORD or achalasia tell us very little about the role of the OGJ in disease. Much of the evidence for the role of the OGJ is related to manometric studies carried out more than 20 years ago. The experts advise us that manometric measurements should not be used to confirm GORD. Yet, most practitioners accept that there is a strong relationship between OGJ dysfunction and reflux disease. It is clear that manometry, while being a great tool for assessing motility, does not provide optimal information for OGJ evaluation.

A distensibility test using a bag catheter probe such as FLIP may provide better information on the opening and closing dynamics of the OGJ, rather than just relying on the sphincter tonic state as measured by manometry. New measurement ideas around the concept of distending the OGJ offer new hope that a clinically useable test for compliance at the junction can be developed and could potentially help in determining appropriate therapy.

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Sensory testing of the human gastrointestinal tract

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Abstract

The objective of this appraisal is to shed light on the various approaches to screen sensory information in the human gut. Understanding and characterization of sensory symptoms in gastrointestinal disorders is poor. Experimental methods allowing the investigator to control stimulus intensity and modality, as well as using validated methods for assessing sensory response have contributed to the understanding of pain mechanisms. Mechanical stimulation based on impedance planimetry allows direct recordings of luminal cross-sectional areas, and combined with ultrasound and magnetic resonance imaging, the contribution of different gut layers can be estimated. Electrical stimulation depolarizes free nerve endings non-selectively. Consequently, the stimulation paradigm (single, train, tetanic) influences the involved sensory nerves. Visual controlled electrical stimulation combines the probes with an endoscopic approach, which allows the investigator to inspect and obtain small biopsies from the stimulation site. Thermal stimulation (cold or warm) activates selectively mucosal receptors, and chemical substances such as acid and capsaicin (either alone or in combination) are used to evoke pain and sensitization. The possibility of multimodal (e.g. mechanical, electrical, thermal and chemical) stimulation in different gut segments has developed visceral pain research. The major advantage is involvement of distinctive receptors, various sensory nerves and different pain pathways mimicking clinical pain that favors investigation of central pain mechanisms involved in allodynia, hyperalgesia and referred pain. As impairment of descending control mechanisms partly underlies the pathogenesis in chronic pain, a cold pressor test that indirectly stimulates such control mechanisms can be added. Hence, the methods undoubtedly represent a major step forward in the future characterization and treatment of patients with various diseases of the gut, which provides knowledge to clinicians about the underlying symptoms and treatment of these patients.

Key words: Endoscopy; Intestine; Experimental; Neurophysiology; Pain

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INTRODUCTION

Abdominal pain is very common in the general population[1], and pain is the most prevalent symptom in the gastrointestinal (GI) clinic[2]. Gastroenterologists face a challenge in treating these symptoms. Consequently, characterization of visceral pain is one of the most important issues in the diagnosis and assessment of organ dysfunction, as diseases giving rise to deep pain often are difficult to diagnose. This is partly due to the sparse and diffuse termination of visceral afferents in the spinal dorsal horn that overlap several segments, which is further complicated by convergence with somatic afferents (spinal convergence), autonomic (involvement of vagal nerve and spinal afferents) and enteric nervous (homeostatic and secretory) systems. Hence, activation of pe-

ripheral sensory afferents may lead to symptoms related to GI motor function (sweating, vasodilation, nausea and vomiting), which blurs the clinical picture. Consequently, complaints related to the autonomic nervous system or related to referred somatic pain are a clinical challenge. To understand the sensory system and how it can be tested, it is important to understand the basic neurophysiological mechanisms behind GI pain.

In healthy subjects, most visceral afferent activity does not reach higher brain centers, except information regarding filling of the esophagus, stomach, rectum and bladder. However, when the internal organs are potentially in danger - e.g. *via* inflammation and diseases - symptoms such as discomfort and pain are typically reported. The feeling is mostly vague and difficult to characterize, in contrast to distinct localization and characterization in somatic diseases. The different neuroanatomical structures of the two systems explain to some degree why visceral pain is more challenging to diagnose than its somatic counterpart (Figure 1). Visceral afferents that mediate conscious sensations run predominantly together with sympathetic nerves that reach the central nervous system (CNS), although some afferents join parasympathetic and parallel pathways. However, the upper esophagus and rectum also possess somatic innervation. The importance of this dual innervation is not clear, although the rectum has more complex functions than most other visceral organs and may need a more differentiated innervation. The peritoneum and parietal serous membranes of the lungs and heart possess their own parietal nerve supply, which is organized like the skin[3]. Hence, pain from these structures gives a distinct, intense and localized pain, which is comparable to the pain evoked by skin lesions. Most of the visceral afferents converge with lamina I , II and V spino-thalamic tract (STT) neurons, which receive input from both superficial and deep somatic tissue as well as other visceral organs[4]. Although the neuronal mechanisms are more complex, this convergence leads to referred somatic pain as well as viscero-visceral hyperalgesia. The latter phenomenon may explain several comorbid conditions such as increased number of anginal attacks in patients with gallbladder calcinosis, and increased number painful sensations to normal air and feces in the gut in patients primarily suffering from dysmenorrhea[5-9]. Most visceral organs exhibit spinal representation overlapping multiple segmental levels[10]. This widespread and low-density nature of visceral sensory innervation explains why large areas of the gut appear to be relatively insensitive to pain stimuli. The extensive resulting CNS activation may also explain the diffuse and unpleasant nature of visceral pain. Finally, unlike the somatic system, where prolonged or summated stimuli such as during inflammation are necessary to activate the N-methyl-D-aspartate (NMDA) receptor, it seems as though - in the visceral context - the NMDA receptor can be more easily activated by short-lasting and low intensity stimuli[11,12]. The resulting amplification of nociceptive processing explains why the manifestation of visceral pain is so often unpleasant and intense in its clinical presentation.

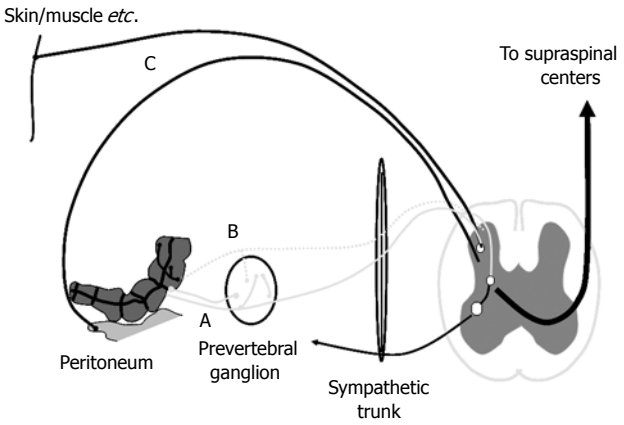

Figure 1 Afferent nerve supply of the gut. True visceral afferents innervate the gut, and most run temporarily together with either the sympathetic or parasympathetic nerves to enter the spinal cord. During inflammation, silent afferents (dashed line) may become activated and contribute to the sensory response. The peritoneum and parietal serous membranes of the lungs and heart have their own parietal nerve supply, which is organized like that of the somatic structures.

THE RATIONALE FOR EXPERIMENTAL STIMULATION OF THE HUMAN GUT

In clinical practice, several symptoms of underlying diseases confound the characterization of pain. These may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions such as fever and general malaise[13]. Furthermore, analgesic treatment and other medications often cause sedation and/or other side effects, which invariably bias the clinical evaluation of pain-related symptoms. Consequently, most studies evaluating drug efficacy in sensory functions of the gut have included a large number of patients. As a result of the above factors, together with the heterogeneity of the material, complicated statistical models have frequently been used - albeit often with equivocal effects. In the clinical situation, this is not a major problem. But, in assessment of analgesics in clinical trials, these confounders can easily invalidate the outcome.

However, in experimental pain models, the confounding factors can often be turned to advantages in the assessment of basic GI functions, mechanisms of disease and treatment efficacy. Under these circumstances, the investigator controls the experimentally induced pain (including the nature, location, intensity, frequency and duration of the stimulus), and provides quantitative measures of the psychophysical, behavioral or neurophysiological responses[13-15]. Different experimental animal models have been used in this context. The advantages of these models are obvious: neuronal activity can be studied directly in anesthetized or spinalized animals with invasive recording techniques or *via* assessment of behavior[16]. However, as neurobiology of the pain system differs substantially even between animal species, translation from animal studies to human pain studies has some major shortcomings.

Human experimental pain studies have for those rea-

sons gained much interest during recent years. In man, pain is closely related to culture, linguistic terms and expressions, and should be regarded as the net effect of complex multidimensional mechanisms that involve most parts of the CNS including intensity coding, affective, behavioral and cognitive components. This explains some of the difficulties and challenges in quantifying human sensory experiences with simple neurophysiological and/or behavioral methods, and why interest in more advanced human experimental pain studies has increased rapidly during the last decade[13,17]. The ultimate goal of advanced human experimental pain research is to obtain a better understanding of pain mechanisms involved in pain transduction, transmission and perception under normal and pathophysiological conditions, such as clinical pain. Obviously, the risk of perforation and other complications during invasive procedures limits the testing possibilities when stimulating the gut. As a result of these difficulties in accessing the GI tract, visceral experimental pain testing is far more resource-intensive and challenging than the more traditional somatic pain stimulations. As a result, most previous visceral studies have relied on relatively simple mechanical or electrical stimuli. These methods are easy to apply. But they have numerous limitations[13]. One should bear in mind that as pain is a multidimensional perception, the response to a single stimulus of a given modality only represents a limited fraction of the entire pain experience. Hence, the possibility of combining different methods to gut stimulation and induction of hyperalgesia will provide the possibility to more closely imitate the clinical situation, and provide extensive and differentiated information on the visceral nociceptive system[13,18]. Ideally, experimental stimuli to elicit gut pain in humans should be physiological, minimally invasive, reliable in test-retest experiments, and quantifiable. Preferably, the pain should mimic observations in diseased organs by inducing phenomena such as allodynia and hyperalgesia. Most experimental studies have been completed in functional diseases such as functional chest pain, non-ulcer dyspepsia and irritable bowel syndrome; but, to some degree organic diseases (e.g. ulcers, inflammatory bowel disease and chronic pancreatitis) have also been investigated[18-23]. The different types of stimulation (electrical, mechanical, thermal, chemical and ischemic) that evoke visceral pain in humans, as well as their limitations, have been described in detail previously[13,24]. In this review, we focus on novel developments regarding test systems that allow standardized stimulation of the GI tract and their applications.

MECHANICAL STIMULATION OF THE GUT

In the last decade, several studies have addressed the mechanical and sensory function of the GI tract by means of mechanical distension. Simple and physiological gut distension, such as ingestion of well-defined meals, may be useful in clinical studies[25]. Balloon or bag distension is, however, the favored method as the mechanical stimulation intensity is easier to control. Most recent studies have used the Barostat based on volume changes in an air-filled balloon kept at constant pressure levels, and several protocols and stimulation paradigms have been recommended for the Barostat, such as phasic and tonic distension. These stimulation paradigms have been thoroughly discussed, and will not be described here - for review see Whitehead *et al*[26] and van der Schaar *et al*[27]. The major advantages of the Barostat system and similar pressure-volume-based methods are the relatively low cost and the documented reproducibility between laboratories[28]. Furthermore, they are reliable and easy to use for routine purposes. Such systems have also been used for assessing sensory and pain thresholds, and under different conditions, attempts have been made to calculate the compliance and tension of the organs[26,27,29,30].

A major pitfall in early balloon distension studies was the use of latex balloons causing large errors because of latex deformability and lack of control of the stimulation field. Consequently, polyurethane or polyethylene bags are now recommended generally. There are, however, still several limitations and sources of error with systems based exclusively on volume and pressure. These include the fact that the data obtained must be corrected for compressibility of air as well as other major concerns, for further details see Drewes *et al*[31]. Basically, a common mistake in GI distension studies is to consider the mechanoreceptors as pressure, volume or tension receptors. In fact, the sensory rating may not be strictly related to pressure (or tension) during gut distension.

Circumferential strain or stress are more likely parameters correlating with receptor responses to stimulus intensity[32]. This is partly due to the fact that strain is a non-dimensional parameter independent of the geometry of the organ and directly associated with tissue deformation. In fact, recent studies have clearly demonstrated that circumferential strain is an important determinant of mechanoreceptor-mediated responses[33-36]. Correspondingly, studies providing tension calculations based on Barostat methods have shown conflicting results, e.g. in a recent study of the stomach, the estimated tension seemed to correlate with the sensation[29,37], whereas another study showed a high inter-individual variability in the sensation score to the applied tension, which suggests that factors other than wall tension influence the sensation[38]. However, uncertainties in the assumptions given above, and lack of adequate geometric and biomechanical considerations can also explain these findings[39].

Methods based on impedance planimetry allows recordings of luminal cross-sectional area directly and calculation of the radius in the distended GI segment[33,35,36,40-42]. As an example, a schematic drawing of the multimodal rectal probe is shown in Figure 2. Estimates of circumferential wall tension, stretch and strain based on measured radius are more accurate than estimates based on volume exclusively[32]. Findings during rectal distension support this theory, as stretch ratio at pain detection threshold produces an excellent intra-class coefficient of 0.98, both

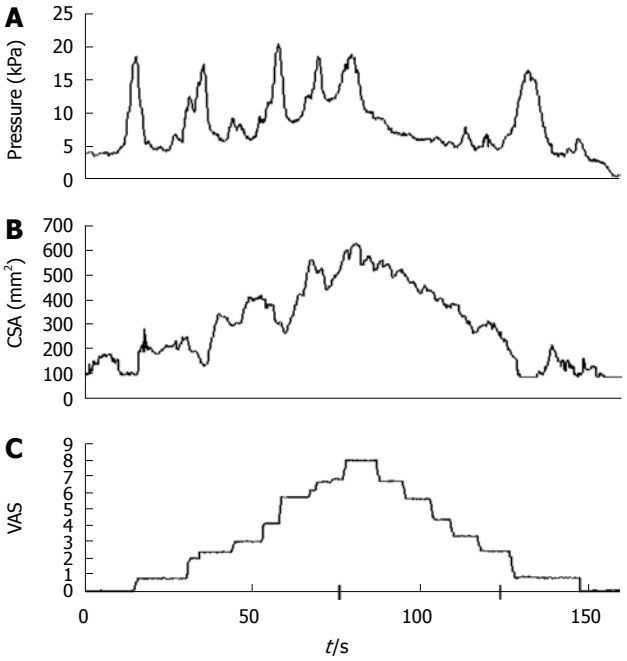

Figure 2 Illustration of mechanical stimulation in the esophagus. The bag was filled at an infusion speed of 25 mL/min. During distension, (A) pressure, (B) cross-sectional area (CSA) and (C) pain intensity was recorded on line. The increase in CSA corresponded with increasing stimulus intensity after the bag was filled with water, whereas there was little relation between the pressure waves and the sensation. The pain intensity was rated on a visual analogue scale (VAS), with 5 as the pain threshold. An intensity of 8 on the VAS resulted in reversal of the pump. For details see Drewes *et al*[35].

with and without administration of the antimuscarinic drug butylscopolamine[43]. An example of a rectum probe is shown in Figure 3. To reliably compute, e.g. rectal stress and strain, more complex modeling is necessary. Thus, in future studies mechanical distension combined with, for example, ultrasound methods or magnetic resonance imaging may offer the possibility of a better anatomical characterization of the GI tract[44,45].

ELECTRICAL STIMULATION OF THE GUT

Electrically induced depolarization of sensory afferents has been widely used as an experimental stimulus in humans. Electrical stimuli have proved to be safe in all parts of the GI system; however, it is recommended to monitor the heart during esophageal stimulation, as previous experiments have documented that atrial capture may occur[46].

Electrical stimulation of the GI tract has been used to study, for example, basic pain mechanisms[7,42,47-49] *via* evoked brain potentials to gut stimuli[50-52], and the effect of analgesics in both healthy volunteers and patients[53]. The main advantage of electrical stimulation is its reproducibility[43,54]. Also, its dynamic range (i.e. the range from sensation to pain threshold) is relatively high, which allows more robust assessment of sensory thresholds. A further advantage is that electrodes are easily implemented on different GI probes[13,43,54]. The well defined on- and offset of the stimulus makes it suitable to study pain mechanisms related to time, such as temporal summation[48,55] and cerebral evoked potentials.

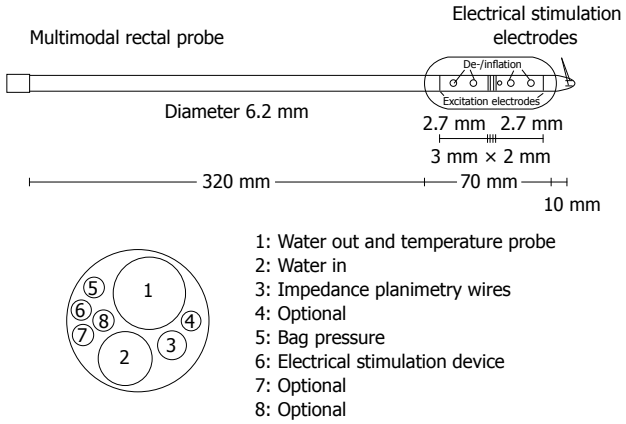

Figure 3 Probe used for measuring rectal CSA during distension.

There are, however, also limitations and drawbacks. Depending on the probe design and the electrodes, it may be difficult to obtain optimal mucosal contact between the electrodes and the GI tract because of, for example, longitudinal esophageal mucosal folds. Hence, it is necessary to measure and control the impedance between electrodes during stimulation, preferably at different frequencies. Further, electrical stimuli are neither natural nor specific for any sensory modality, and the electrical stimulation bypasses receptors, which stimulates all afferent nerves directly, including silent fibers. Consequently, electrical stimulation reflects the central nervous response rather than peripheral afferents. However, as most gut afferents are polymodal[56] and respond to a wide range of stimuli, specificity may be of minor importance. The electrical stimulation creates an electrical field, and the action potential is partly determined by the extracellular electrical potential, and partly by the nerve properties, including myelin and ion-channel configuration. Thus, there may be some selectivity relating to fiber type as the non-myelinated afferents (C-fibers) possess a higher activation threshold than myelinated fibers[17,57,58]. Hence, increasing stimulation intensity may depolarize myelinated fibers first, followed by C-fiber recruitment at higher stimulation intensities.

Electrodes can be either unipolar with a reference placed on the skin or bipolar with a set of electrodes. As a result of safety considerations, bipolar stimulation is recommended because the electrical field is more localized. Cardiac arrhythmia may theoretically be evoked by stimulation of nearby organs. Normally, atrial captures can be seen; but, this has no clinical significance. Arrhythmia can be avoided by either turning patch-electrodes away from the dorsal side of the heart, or by using bipolar ring electrodes that exhibit good mucosal contact[42]. Impedance should preferably be less than 3 kΩ before stimulation is initiated. Numerous stimulation paradigms have been recommended and no general consensus exists with respect to the configuration of the optimal electrical pulse. In fact, the stimulus should reflect the purpose, e.g. it is crucial to use single pulses in electrophysiological studies, where early peaks of evoked brain potentials are wanted. On the other hand, a single stimulus in the gut demands

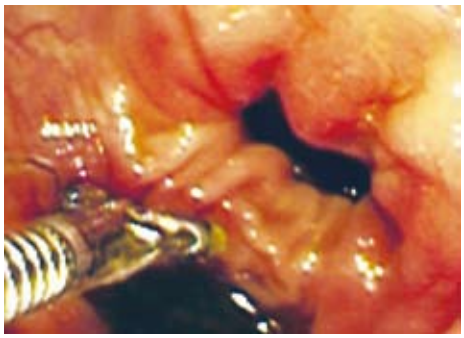

Figure 4 An example of targeted colonic stimulation is shown, which demonstrates the electrode position. The controlled position, which can be altered in case of stimulation in vicinity of somatic structures and nerves, is a major advantage.

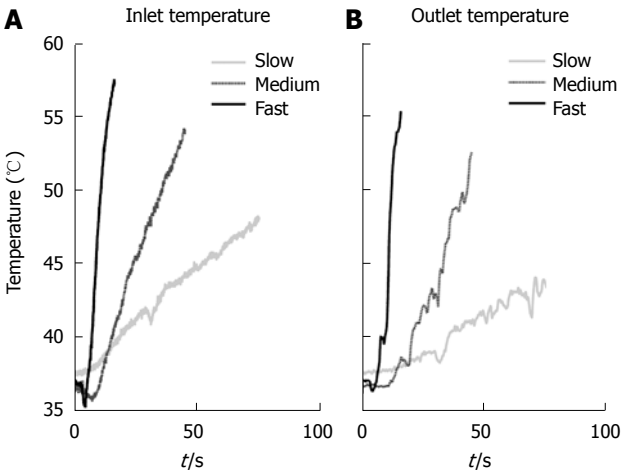

Figure 5 Temperature stimuli with three different incline rates. A: Left graph shows the temperature measured at the inlet of the bag; B: Right graph shows the temperature measured at the outlet of the bag.

rather high intensity to evoke pain, and trains or continuous series of pulses can be used in order to investigate temporal summation to a repeated series of stimuli (termed "wind-up" in animal experiments). Based on this experience, we use either: (1) single square pulses (duration 0.2-2 ms) in electrophysiological studies assessing evoked brain responses; (2) trains of five constant current pulses (rectangular with a duration of 1 ms applied at 200 Hz termed "single burst stimuli"), as such stimuli demand less current to evoke sensory responses; (3) "repeated burst stimuli" given as a series of single burst stimuli to investigate, for example, the central amplification of the repeated stimuli[48,55,59]; or (4) tetanic stimulation to be used for sensory thresholds of temporal summation[60]. This is applied as a train of pulses (0.2 ms, 100 Hz) that is linearly increased from zero. The advantage of this stimulation is that it is precise and less time-consuming.

Blind, untargeted stimulation is avoided by integrating electrodes onto endoscopic biopsy forceps, which makes stimulation of well-defined areas in the esophagus, stomach, duodenum, terminal ileum and colon possible[48,55]. An example of targeted colonic stimulation is shown in Figure 4. The major advantage of this modification is that electrode position is controllable and can be altered in case of stimulation in the vicinity of somatic structures and nerves. Further, mucosal contact is secured and evoked motor phenomena such as secondary contractions can be studied directly. Hence, the method allows characterization of local and referred pain to stimuli in most areas of the intestine relevant to localized pathology. However, the subjects have to cope with the rather thick endoscopes during experiments, which may be unpleasant especially in the upper GI tract, and which may cause bias in the pain assessment.

As gut segments exhibit differences in anatomy and innervation, a general consensus regarding stimulation location is warranted. Consequently, as described above, stimulation paradigms have a major influence on pain assessment. Thus, future studies should include standardized and validated optimal parameters such as stimulus duration, shape, polarity, frequency and intensity, which allows comparison between different laboratories.

THERMAL STIMULATION

In contrast to mechanical and electrical stimuli, thermal stimuli activate selectively, for example, being either mucosal heat-responsive TRPV1 receptors with temperatures above 43℃, or mucosal cold-responsive TRPA1 with temperatures less than 17℃[61]. Thermal stimulation has been used to study basic pain mechanisms[42,43,48,49,54,61,62], functional[63] and organic gut disorders[22] and analgesic efficacy in healthy volunteers and patients[53].

Rectal heat pain stimulation has been performed using a Peltier device[61]. To improve thermal stimulation in the gut, we have developed the multimodal esophageal probe - for details see Drewes and Gregersen[24]. Thermal stimulation is based on recirculation of cooled and/or heated water in the bag with a temperature sensor placed inside the bag. The method has also been integrated onto a multimodal rectal probe. In both cases, the most reliable proxy of the thermal energy applied is the area under the temperature curve[43,64]. In the esophagus, the method aims at having a constant high or low temperature in the bag until the pain threshold is reached. This method has been shown to be reliable and robust in drug experiments[54]. In studies of healthy subjects, it has shown some limitations, as not all subjects reach a pain threshold during the 2 min stimulation that were empirically found to be safe. To improve control over the stimulation intensity and duration, the method has recently been changed in order to obtain a linear increase in temperature, with an adjustable temperature ramp. Such stimulation is shown in Figure 5. In these experiments, the stimulation intensity can continue until the subject reaches the pain threshold, an improvement that is expected to result in better validity and reliability of the method.

CHEMICAL STIMULATION

Chemical stimulation of the GI tract resembles clinical inflammation and approaches the ideal experimental

visceral pain stimulus[7]. Such stimuli have successfully been applied to the skin[14,17,65] and muscles[66], but are also widely used in the gut. As an example, esophageal acidification is commonly used as a method to sensitize the gut evoking allodynia/hyperalgesia[67,68], but the model may also be used for direct stimulation[69]. The major relevance of the model may be induction of sensitization of visceral afferents to subsequent experimental stimulation. Chemical stimulation has been used to study, for example, basic pain mechanisms[42,43,48-50,62,70,71] and functional gut disorders[22,50]. However, drawbacks of chemical stimuli include a relative long latency time to onset of effects, and that the effects are often not reproducible[7]. Other stimuli such as glycerol, alcohol, bradykinin and other chemicals[72-75] have been used in uncontrolled studies, but their applicability has yet to be established. Recently, capsaicin has been used to evoke pain in the small and large intestine[76-78]. Chemical stimulation has also been used to explore basic functions such as autonomic changes in referred pain[79]. Hammer *et al*[77] have shown that activation of chemosensitive pathways induces symptoms that differ from those induced by mechanical activation, although animal data do not allow such a strict separation[80,81]. As a result of the relative inconsistency of the effects of acid perfusion in the esophagus[82], we recently used perfusion of a combination of acid and capsaicin in the human esophagus[83]. It is believed that capsaicin has an additional effect on acid because of synergistic mechanisms on the transient receptor potential vanilloid type 1 (TRPV1) channels[83]. The perfusion induced locally reproducible hyperalgesia to subsequent heat and electrical stimulation, and an expansion of referred pain in all subjects. The increased referred pain reflects convergence mechanisms on second-order neurons in the spinal cord, and can be used to elucidate the central component of hyperalgesia. A further step is achieved by the demonstration of viscero-visceral hyperalgesia in the rectum following esophageal perfusion with acid and capsaicin[84]. Hence, the model may be more robust than acid perfusion alone, but further studies are needed.

SPATIAL AND TEMPORAL SUMMATION

Lewis[85] has found that distension of the gut is most painful when long, continuous segments of the gut are distended simultaneously. Even greater pressures within smaller segments of the gut are not as efficacious in producing painful sensations. Hence, spatial summation is clearly an important contributor to visceral pain mechanisms. An experimental design to achieve spatial summation is mounting either multiple inflatable bags or one long bag on a probe, and then assessing the distension volume at pain detection threshold (PDT) derived from each bag, compared to the distension volume at PDT during simultaneous distension of multiple bags or the long bag.

Also, integration over time-temporal summation-is important. If electrical stimuli are repeated over time, both pain and the area of referred pain increase progressively[47]. The same phenomenon is seen following repeated distensions[7].

ACTIVATION OF INHIBITORY MECHANISMS

Pain inhibits pain, and impairment of descending control mechanisms is believed to be an important part of the pathogenesis of chronic pain[86]. Descending inhibition involves a spinal-supraspinal-spinal feedback mechanism, which results in direct or indirect inhibition of spinal neuronal responses[87,88]. Supraspinal sites can, however, also facilitate nociception, and the measured net output is either predominantly facilitation or inhibition[89]. Hence, experimental studies assessing human inhibitory processes measure the balance between these phenomena.

The most common method to provoke the noxious inhibitory system is the cold pressor test that is performed by immersing a hand or foot in 2.0 ± 0.3℃ ice-cooled water for at least 2 min. Efficacy of the descending control can then be investigated by comparing two stimuli separated by the cold pressor test. The cold pressor test has been used to study basic pain mechanisms and functional gut disorders[90].

MULTIMODAL APPROACH

Five to ten years ago, the available probes did not possess the ability to produce more than a few of the above-mentioned stimuli. Some authors have combined mechanical and electrical stimuli[91,92], and others have used electrical stimuli combined with sensitization to acid[50]. The esophageal multimodal probe and its use in basic, pharmacological and clinical studies has been reviewed recently, and the reader is referred to Drewes *et al*[18]. Recently, the model has been used in basic science, including other gut segments such as the duodenum[62] and rectum[43]. We are currently using the multimodal rectal approach in pharmacological and clinical studies of functional and organic disorders.

One limitation of multimodal pain stimulation in the gut is that it is done without visualization of the inside of the intestine. Hence, diseases such as esophageal erosions cannot be excluded. Recently, we have made an attempt to combine the multimodal probe with endoscopy, as illustrated in Figure 6, by passing a small (2.8 mm) video-endoscope into a multimodal probe, which allows mechanical, thermal and chemical stimulation. A schematic drawing of the probe is shown in Figure 6. As electrical stimulation can be done with electrodes attached at the biopsy forceps for the endoscope[48], the probe allows full multimodal stimulation including mucosal inspection and biopsies (albeit small) for histology and specific immunohistochemical staining. In diseases such as esophagitis, the TRPV1 receptor has been shown to be important[71] and the receptor also seems to play a role in sensation to heat and acid[93]. Hence, combined information about the sensory profile and

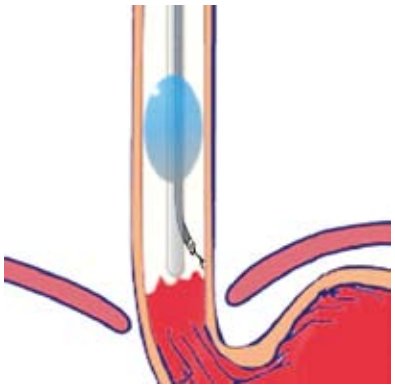

Figure 6 Newly developed multimodal endoscopic probe that allows comprehensive sensory information combined with visual mucosal inspection and biopsy specimens.

histological findings may be important in evaluation of the pathogenesis in diseases. Theoretically, the endoscope can be replaced with an ultrasound probe that allows assessment of the gut wall and, therefore, can be used for advanced mechanical modeling[94].

CONCLUSION

Over the last few years, the technical limitations of sensory testing in the GI tract have been increasingly surmounted. Multimodal esophageal, duodenal and rectal probes have been developed, which allow the investigator to use different stimulus modalities in the gut. The probes have proved to be robust across sessions, and have shown high reproducibility in all modalities. Future experiments using experimental testing will undoubtedly shed light on the pathogenesis of GI disorders, as well as assisting in finding new treatment modalities.

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GUIDELINES CLINICAL PRACTICE

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Imaging of the gastrointestinal tract-novel technologies

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Abstract

Imaging of the gastrointestinal tract is very useful for research and clinical studies of patients with symptoms arising from the gastrointestinal tract and in visualising anatomy and pathology. Traditional radiological techniques played a leading role in such studies for a long time. However, advances in non-invasive modalities including ultrasound (US), computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI), *etc*, have in the last decades revolutionised the way in which the gastrointestinal tract is studied. The resolution of imaging data is constantly being improved and 3D acquisition, tools for filtering, enhancement, segmentation and tissue classification are continually being developed. Additional co-registration techniques allow multimodal data acquisition with improved classification of tissue pathology. Furthermore, new functional imaging techniques have become available. Altogether, the future of gastrointestinal imaging looks very promising which will be of great benefit in clinical and research studies of gastrointestinal diseases. The purpose of this review is to highlight the capabilities of the newest techniques to explore the detailed morphology, biomechanical properties, function and pathology of the gastrointestinal tract.

Key words: Computed tomography; Gastrointestinal tract; Imaging; Magnetic resonance; Radiology; Ultrasound

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INTRODUCTION

Examinations with visualisation of the anatomy and pathology of the gastrointestinal (GI) tract are often mandatory in the diagnosis of GI diseases. For this purpose, traditional radiological techniques played a leading role for a long time. However, improvements in endoscopic examinations, the latest including wireless capsule endoscopy, have radically changed the possibilities for direct visualisation and intervention in the GI tract. The introduction and advances in non-invasive imaging modalities including ultrasound (US), computed tomography (CT), positron emission tomography (PET) and magnetic resonance imaging (MRI) have in the last decades revolutionised the way in which the GI tract is studied[1]. The resolution of imaging data is constantly being improved and 3D acquisition, tools for filtering, enhancement, segmentation and tissue classification are continually being developed. Additional co-registration techniques allow multimodal data acquisition (PET-CT, MR-PET, CT-US, *etc*) with improved classification of tissue pathology. Each modality is characterised by a distinct profile of favourable and unfavourable features, and the technique used depends upon availability, accuracy, usefulness, safety and costs. The diagnostic performance in terms of sensitivity, specificity and accuracy depends on several factors: the specific method and equipment used, the part of the GI tract investigated, patient constitution and preparation, most importantly the sort

of pathology being studied, and not least which "gold standard" the method is being compared to.

The purpose of this review is to highlight the capabilities of the newest imaging techniques to explore the detailed morphology, biomechanical properties, function and pathology of the GI tract. Table 1 provides an overview of the advantages and shortcomings of the most frequently used modalities in the study of the GI tract.

CONVENTIONAL RADIOLOGICAL EXAMINATIONS

Using non-contrast radiography, normal GI segments with no or little gas content cannot be separately visualised; but normal and abnormal gas accumulations, air-fluid levels, calcifications and motility of air contained in the intestines can be visualised[2].

In mono-contrast examinations, the intestinal lumen is filled with a positive contrast material in order to visualise peristalsis, emptying and pathological changes such as stenosis, dilatation, luminal filling defects and external compression. In double contrast examinations, the inner surface is coated with contrast material and the lumen is distended with air. This allows detailed visualisation of the mucosa which is especially useful in the detection of inflammatory and neoplastic changes of the small and large intestine[2]. However, the methods do not allow direct description of the deeper wall layers and extraintestinal lesions.

ANGIOGRAPHY

Conventional angiography of the GI tract has a clear role in the visualisation and treatment of GI bleeding. However, oesophago-gastroduodenoscopy and colonoscopy are the primary methods for identifying GI bleeding; but, sometimes these approaches cannot identify the source of bleeding[3]. In these cases, the leakage may be visualised using scintigraphy with tagged red blood cells, capsule endoscopy, double balloon endoscopy and increasingly multi-detector CT (MDCT)[3,4].

ULTRASONOGRAPHY

Transabdominal US is a safe procedure without any radiation exposure and allows visualisation of the intestinal wall, fluid-filled intestinal segments and the surrounding environment. US has excellent soft tissue imaging capabilities which make it ideal for both clinical and research studies of the GI tract. This is especially valuable in the detection of GI tract inflammation, where wall thickening, disturbed wall morphology, surrounding oedema and lymphadenopathy can be visualised[5]. In cases of extraintestinal fluid collections and abscess formation, mini-invasive drainage of these collections can be performed guided by US. Endosonography using intraluminal probes allow high-resolution imaging of the wall layers[6-8]. By applying special techniques (see below), additional information can be obtained.

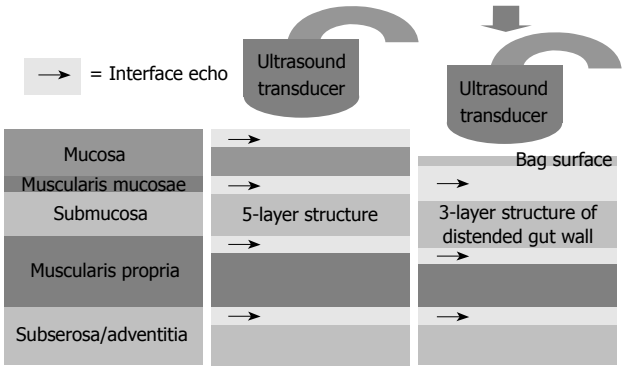

Figure 1 The principles of endosonography. The histological gastrointestinal wall layers (left) are correlated to the typical layered ultrasound appearance of the gastrointestinal wall (middle). The 5-layered appearance is due to the addition of several interface echoes at the tissue interfaces. During compression or distension the wall is further stretched (including mucosal unfolding) which together usually obscures the second echo-rich mucosal layer. Hence, the wall appears 3-layered (see Figure 2).

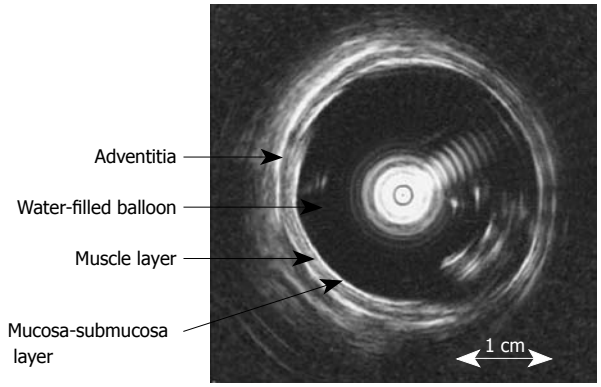

Figure 2 Cross-sectional endosonographic image of the distended distal oesophagus allows identification of three oesophageal sub-layers, i.e. mucosa-submucosa, muscle and adventitia. The white shadows inside the water-filled bag (4-5 o'clock) represent artefacts due to convulsions of the water-filled balloon, which results in reduced image quality at low degrees of distension. Modified from [17].

Perfusion of the intestinal wall and surrounding tissues can be assessed using Doppler imaging. Recently, the application of intravenous US contrast agents has improved the detection of hypervascularisation and hyperemia, especially in inflammatory bowel diseases[9,10].

Qualitative and quantitative information of intestinal motility and gastric filling/emptying can be obtained using transabdominal US[11,12]. 3D position and orientation systems allow real-time 3D visualisation with reconstructions and volumetry of the GI tract[13,14]. Intraluminal flow can be assessed using Doppler flow imaging[15]. This is especially useful in studying flow through the pylorus.

GI wall layers can be visualised endoscopically using high-frequency endosonography. This is normally used in tumour diagnosis, but has also been used experimentally to study e.g. the biomechanical properties of the GI tract[16,17]. Usually 3-7 layers of the wall can be visualised (Figures 1 and 2)[7]. The separate layers are bound together and possess dissimilar active and passive biomechanical (i.e. anisotropic) properties

Table 1 Most frequently used imaging modalities in the study of the gastrointestinal tract: Overview of main advantages and shortcomings

Modality	Advantages	Shortcomings
Multidetector computed tomography (MDCT)	High temporal and spatial resolution Fast image acquisition without motion artefacts Total evaluation of entire intestine and its surroundings 3D reconstructions and virtual endoscopy Possibility for image guided intervention	High radiation exposure Less suitable for research in healthy subjects No direct functional information Low risk of nephropathy due to intravenous iodised contrast media
Ultrasound (US)	High soft tissue resolution No radiation exposure Ideal for repeated examination and research Evaluation of intestinal wall and surroundings Information on motility, function and flow directly available using special techniques Possibility for intraluminal imaging Ideal for image guided intervention	Relatively high interobserver variability Intestinal gas lowers image quality Artifacts may be difficult to interpret Total visualisation of the entire intestine is difficult
Magnetic resonance imaging (MRI)	Good soft tissue imaging capabilities No radiation exposure Ideal for repeated examinations and research Total evaluation of entire intestine and its surroundings Functional and motility information directly available using special techniques	Motion artifacts due to intestinal motility Long image acquisition Image resolution less than CT making 3D reconstructions and virtual endoscopy cumbersome Potential long term effects of gadolinium-based contrast media (nephrogenic systemic fibrosis)
Conventional radiography	High temporal and spatial resolution Fast image acquisition Motility and function easily studied using intraluminal contrast	Only direct visualisation of luminal/mucosal properties Radiation exposure No 3D image data
Endoscopy	Direct visualisation of the mucosa Possibility for intervention (biopsies, polypectomy and endoscopic surgery)	Invasive procedure Discomfort and potential intestinal perforation No visualisation of deeper wall layers and surroundings

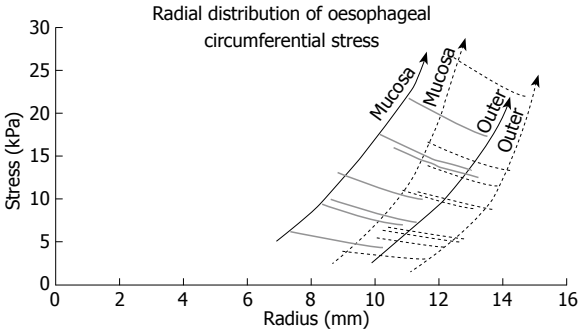

Figure 3 The oesophageal stress is calculated based on endosonography images and manometry. The alignment of solid curves represents the oesophageal stress profiles during oesophageal distension in a healthy volunteer. The alignment of dashed curves represents distension during butylscopolamine smooth muscle relaxation. As the oesophagus distends the inner radius and stress increases, i.e. the left end of the curves shift to the right and upwards. At high degrees of distension the steepness of the stress profile increases. Oesophageal relaxation shifts the alignment of the stress profiles to the right. Modified from [17].

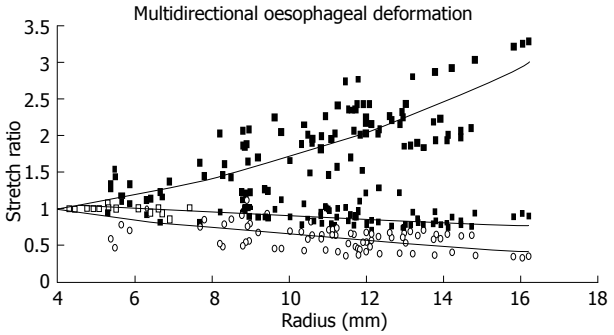

Figure 4 The circumferential, radial and longitudinal deformation of the oesophageal muscle layer is here described as the stretch ratio and as a function of the radius. The stretch ratio and radius are calculated based on endosonography. Data are from 12 healthy volunteers and shows a tensile circumferential stretch, radial compression and longitudinal shortening. Modified from [17].

which make a detailed analysis complex[16]. To assess the biomechanical properties, the intestine can be distended with fluid-filled balloons containing pressure measurement and US mini-probes providing cross-sectional images of the intestine[17,18]. The applied load on the wall can be controlled and accurately quantified. This allows calculation of passive and active biomechanical properties of the distended segment with parameters such as strain (relative deformation), tension, stress (force per cross sectional area) and stiffness of the wall layers[16,17,19]. The radial distribution of the circumferential wall stress has been assessed in the oesophagus[17]. Both the circumferential strain and stress were highest at the mucosal surface and decreased throughout the wall (Figure 3). The stiffness increased throughout the wall and was highest at the outer surface. The high stiffness of the muscle layers (compared to the mucosa) may limit the total oesophageal deformation (i.e. further distension) and protect the vulnerable and less stiff mucosa from damage when overstretched. This method has also been used to assess the multi-directional deformation and wall layer thicknesses of the oesophagus. Distension induces tensile circumferential stretch, radial compression and longitudinal shortening (Figure 4), which is not taken into account when using conventional barostat methods. This has shown to be valuable in the description of structural remodelling in organic GI disorders. Patients with longstanding diabetes

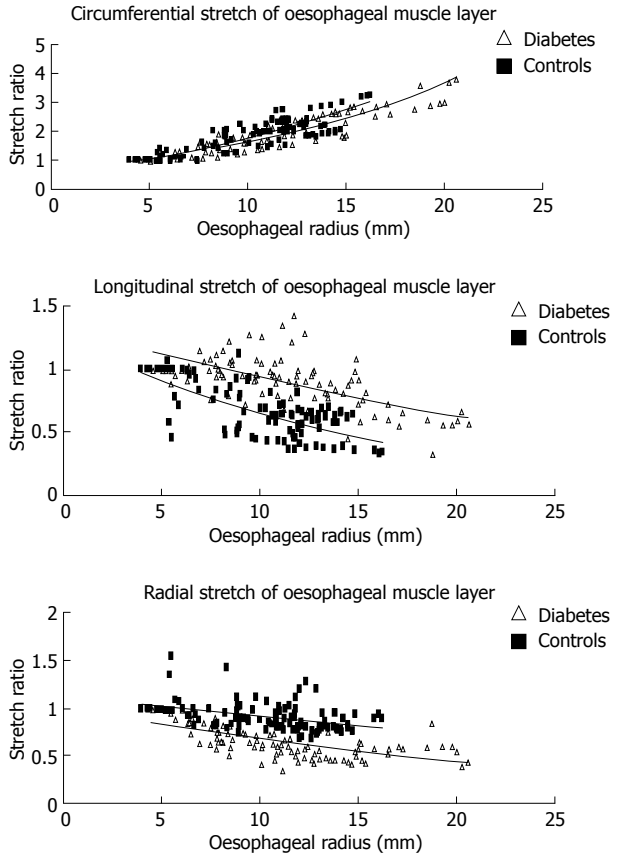

Figure 5 The graphs show the effect of diabetes on deformation of the oesophagus. The distension-induced change in oesophageal circumferential, longitudinal and radial deformation (stretch ratio) are calculated based on endosonography and illustrated as a function of the oesophageal radius. The curves were obtained during smooth muscle relaxation with butylscopolamine. The data points represent multiple measuring points during distension in diabetic patients and controls. Exponential trend lines (solid lines) of the diabetic patients and controls are shown. Oesophageal shortening during distension was clearly reduced in the diabetic patients while the radial stretch was decreased. Modified from [20].

mellitus have increased thickness of the oesophageal wall layers[20]. Also, longitudinal shortening was decreased in the diabetic oesophagus combined with a decreased radial stretch (Figure 5)[20]. Together with these structural changes indicating remodelling of the GI tract, diabetic patients also have an increased reactivity to oesophageal distensions and impaired coordination of the contractions which may reflect neuronal abnormalities due to autonomic neuropathy[20].

Further developments in the Doppler imaging technique can, when applied on the wall tissue itself, give information about movements inside the wall structure. This technique is known as strain rate imaging (SRI) and allows detailed mapping of the deformation of the wall layers with description of local tissue velocities[21]. Hence, the different contractile activity of the circular and longitudinal muscle layers can be visualised[12]. Elastography is a new US method which allows assessment of tissue stiffness. The tissue is compressed and the deformation pattern is visualised as colours (from soft to stiff) on the US image. The stiffness depends on the biomechanical properties, allowing differentiation between normal and abnormal tissues[12,22].

Cross-sectional endosonography of oesophageal contractions has shown that the cross-sectional area of the outer longitudinal muscle layer increases during contractions[23]. This indicates a contraction of the longitudinal muscle and shortening of the oesophagus which is thought to support the peristaltic force generated by the inner circular muscle[23]. Endosonographic studies have revealed that episodes of oesophageal chest pain and heartburn are associated with sustained contraction of the longitudinal muscle layer[24].

CT

The introduction of multidetector CT (MDCT) scanners with typically 64 detectors or more allows fast acquisition of thin slices and allows multi-planar reconstructions in any direction. This is a valuable tool in the study of intestinal loops[25]. Non-contrast enhanced CT scanning is increasingly replacing plain radiography in the evaluation of free intraabdominal air and intestinal obstruction. Intravenous contrast enhancement and filling of the intestinal lumen with water or positive contrast agents are performed in order to optimise imaging of the bowel wall. This is particularly valuable in the detection of inflammatory and neoplastic intestinal lesions, and allows accurate detection of extra-intestinal findings[26].

MDCT colonography is a relative new way of studying the large intestine. After proper colonic preparation, the large intestine is distended with air and the patient is scanned in the prone and supine positions[27]. The examination is reviewed in multiplanar views and as virtual endoscopy allowing flight-through of the intestine in both directions. This allows detection of smaller (> 6 mm) colonic polyps with a similar high accuracy to that of conventional colonography, while the accuracy for even smaller polyps is poor[27-29]. New generations of software with virtual dissection and unfolding of the colon will, together with computer-aided detection (CAD), probably improve the diagnostic accuracy and reduce the imaging time[30,31]. In addition, the detection of any incidental extra-colonic pathology is possible[32,33]. This technique may replace the traditional double contrast examinations in the case of incomplete colonoscopy and may also play a central role as a non-invasive screening examination.

MAGNETIC RESONANCE IMAGING (MRI)

MRI has no known short- or long-term hazards, and, therefore, provides excellent soft tissue imaging capabilities for studying the GI tract. This makes MRI favourable compared with CT which has considerable radiation exposure. However, intestinal MRI is limited by long acquisition times and a high risk of motion artefacts. Since the early days of MRI, the technology has advanced significantly and recent developments in MRI techniques such as parallel imaging, allow much faster and higher quality image acquisition.

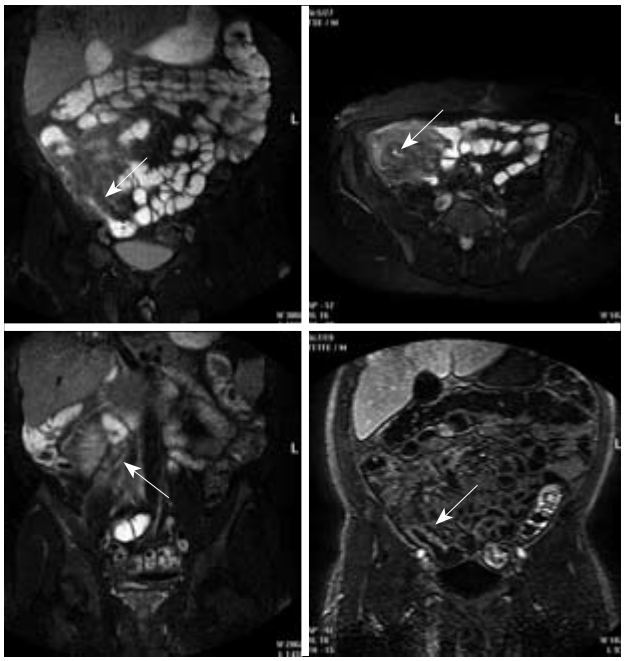

Figure 6 MRI of Crohn's disease. The coronal and axial (upper panel) fat-saturated T2-weighted MRI display marked wall thickening, mucosal irregularity and stenosis of the terminal ileum (arrows). Advanced mesenteric inflammation with hypervascularity and enlarged lymph nodes (arrow) are visualised on coronal fat-saturated T2-weighted MRI (lower left). Coronal T1-weighted MRI (lower right) shows clear wall enhancement (arrow). Modified from [36].

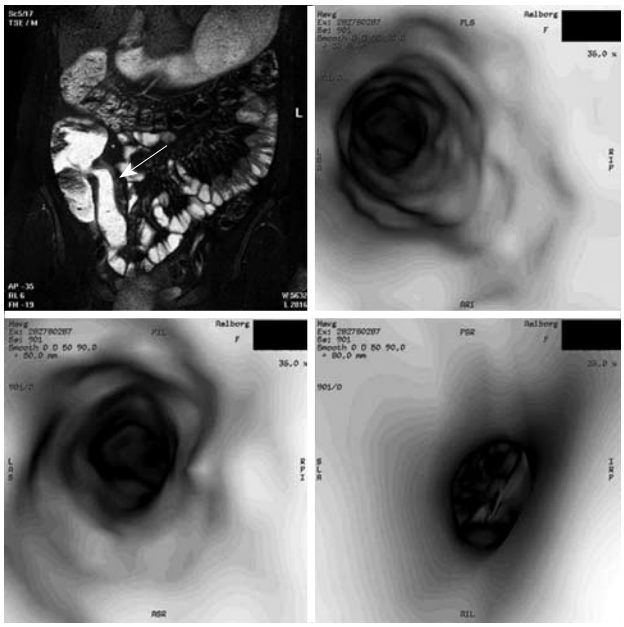

Figure 7 MRI of Crohn's disease. Virtual endoscopy views of the displayed diseased small bowel segment (arrow) shows mucosal nodularity, ileo-caecal narrowing and minor prestenotic dilatation. Modified from [36].

MRI is generally accepted as the gold standard examination in the staging of rectal cancers and inflammatory bowel diseases. Pelvic MRI, especially with endorectal coils, gives exact visualisation of infiltration of the rectal wall and perirectal fat allowing reliable TNM staging[34]. MRI of the small intestine has several advantages compared with conventional enteroclysis. It provides cross-sectional images without radiation hazards, and the entire small bowel can be visualised including other relevant abdominal pathology not directly related to the small bowel[26,35]. Luminal (stenosis, cobble stoning, and fissures), mural (wall thickening, and wall enhancement upon iv gadolinium) and exoenteric (mesenteric inflammation, fibrofatty proliferation, lymphadenopathy, hypervascularity, abscesses and fistulas) pathologies are visualised with high sensitivity and specificity (Figure 6)[35-38]. In particular, MRI is superior in the evaluation of fistulas in the anorectal region[39]. The optimal protocol for small intestinal MRI is not yet developed, since many different methods of preparation and imaging sequences exist. The intestine can be filled both orally or by intubation of the small intestine. Various positive and negative intestinal contrast materials exist. The use of water-based positive contrast agents are generally accepted with the addition of hyperosmotic substances (polyethylene glycol, methyl cellulose, bulk fibre laxative, mannitol, locust bean gum, *etc*) securing optimal distension of the entire small intestine[36,40-42]. Spasmolytics such as butylscopolamine and glucagon are usually administered to avoid motion-induced artefacts. The administration of intravenous contrast permits enhancement of hypervascular and hyperperfused areas allowing distinction between active and inactive inflammatory lesions. Acquisition of 3D images allows the possibility of virtual endoscopy which contributes to the detailed evaluation of a diseased bowel segment and the intraluminal display is easily recognized by gastroenterologists[36,43,44].

Our research group performed MRI and conventional enteroclysis in 36 patients with suspected Crohn's disease who underwent oral administration of plum juice and bulk fibre laxative[36]. Virtual endoscopy was performed with excellent demonstration of the mucosal surface (Figure 7). The main limitation of virtual endoscopy is the long and cumbersome computer post-processing and a high image quality is needed for this technique. However, this technique ensured sufficient distension of the small bowel for detecting small bowel changes. Pathological abdominal changes were found in 70% more patients using MRI compared with conventional enteroclysis[36]. MRI using this technique is preferable to conventional enteroclysis due to a superior demonstration of the entire small bowel pathology, low patient discomfort and absence of radiation exposure. In a study by Gourtsoyiannis *et al*[45], MR enteroclysis (MRE) was compared with conventional enteroclysis (CE) as the gold standard in 52 patients with small intestinal Crohn's disease. The sensitivity of MRE in the detection of superficial ulcers, fold distortion and fold thickening was 40%, 30% and 62.5%, respectively. The sensitivity of MRE in the detection of deep ulcers, cobble-stoning pattern, stenosis and prestenotic dilatation was 89.5%, 92.3% and 100%, respectively. Additional findings demonstrated on MRE images included fibrofatty proliferation in 15 cases and mesenteric lymphadenopathy in 19 cases. Hence, MRE strongly correlates with CE in the detection of individual lesions expressing small intestinal Crohn's disease, and provides

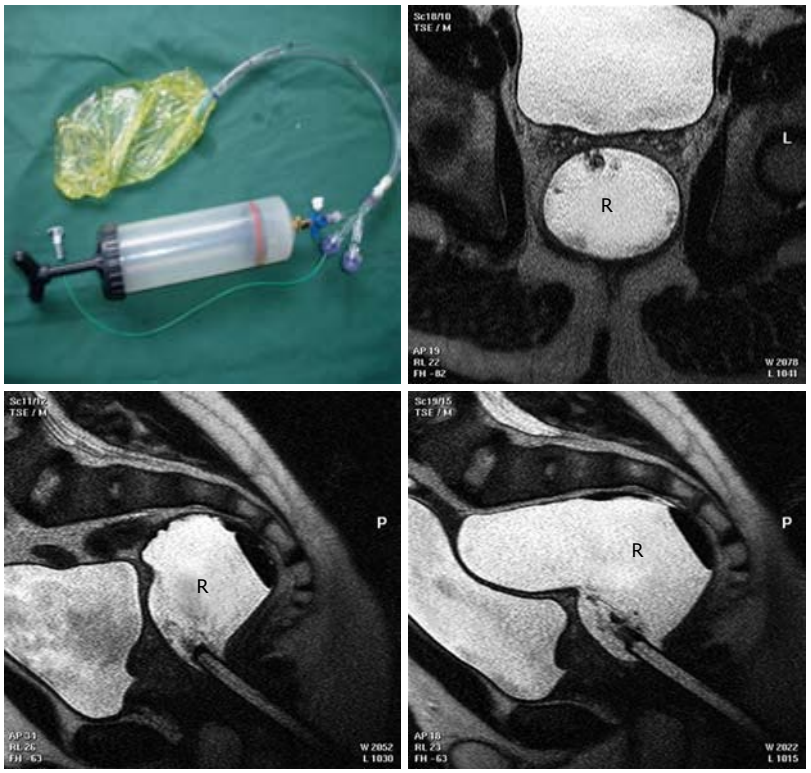

Figure 8 Stepwise distension of water filled balloon with simultaneous MRI and pressure recording. The rectal probe (upper left) allows rectal water distension and pressure measurement. MRI shows the distended water-filled bag in the rectum (R). The sagittal MRI (lower panel) shows the distension, elongation and relation to neighbouring structures at 100 mL and 300 mL inside the bag. Modified from [50].

additional information on the mesenteries. However, its capability in detecting subtle lesions is still inferior to CE. Additional functional cine-MRI will allow studies of intestinal motility with detection of intraabdominal adhesions due to surgery or inflammation[46]. The technique is also relevant for research studies on GI function and motility.

The technique of MRI colonography is also still developing[29,47,48]. Intestinal preparation is crucial in order to distinguish between polyps and intestinal residuals. Basically, the large intestine has to be cleaned and filled with water. New ways of faecal tracking with the oral application of negative contrast agents before the examination may allow less intestinal cleansing[49]. However, the method of MR colonography is not yet as sensitive as CT colonography in detecting smaller polyps.

Since MRI provides excellent soft tissue imaging capabilities without the use of radiation, it is ideally suited for research studies of the GI tract. Using advanced image processing, the three-dimensional geometry and mechano-sensory properties can be studied. Stepwise distension of water filled rectal and sigmoid balloons with simultaneous MRI and bag pressure recording was performed by our group (Figure 8)[50,51]. Based on the cross-sectional images, 3D models of curvatures, radii of curvature, tension and stress were generated and the circumferential and longitudinal strains were calculated (Figure 9). The distributions of the biomechanical parameters throughout the rectal and sigmoid surfaces were distinctly different between individuals and non-homogeneous throughout the colorectal wall due to its complex geometry. This complex geometry suggests that simple estimates of tension based on pressure and volume do not reflect the true 3D biomechanical properties of the intestine. This tool may in the future be useful in the research and clinical setting for assessing the geometry and mechano-sensory properties of visceral wall structures in health and disease.

PET

18F-fluorodeoxyglucose positron emission tomography (FDG-PET) has high accuracy in the detection and follow-up of oesophageal, colorectal and stomal cancers[52]. The advantage of PET is that metabolic changes often precede clear structural changes and, therefore, can be detected early in disease development. A combination of PET-CT is a powerful tool in the primary staging and assessment of any recurrent disease.

OTHER NOVEL TECHNIQUES

Other techniques such as impedance measurements and manometry which were initially not direct imaging modalities have developed more and more into techniques with imaging data display.

Impedance planimetry with assessment of multiple closely arranged cross-sectional areas can be displayed in 3D. This concept is known as the Functional Lumen Imaging Probe (FLIP) allowing direct on-line imaging of the luminal geometry of the GI tract[53,54]. This is particularly suitable for visualisation of the complex physiology of the GI sphincters, especially in the evaluation of gastro-oesophageal reflux and sphincter incompetence.

Oesophageal high-resolution manometry (HRM) with up to 36 pressure sensors allows on-line visual display with a spatio-temporal colour plot of oesophageal peristalsis[55]. The technique is explained

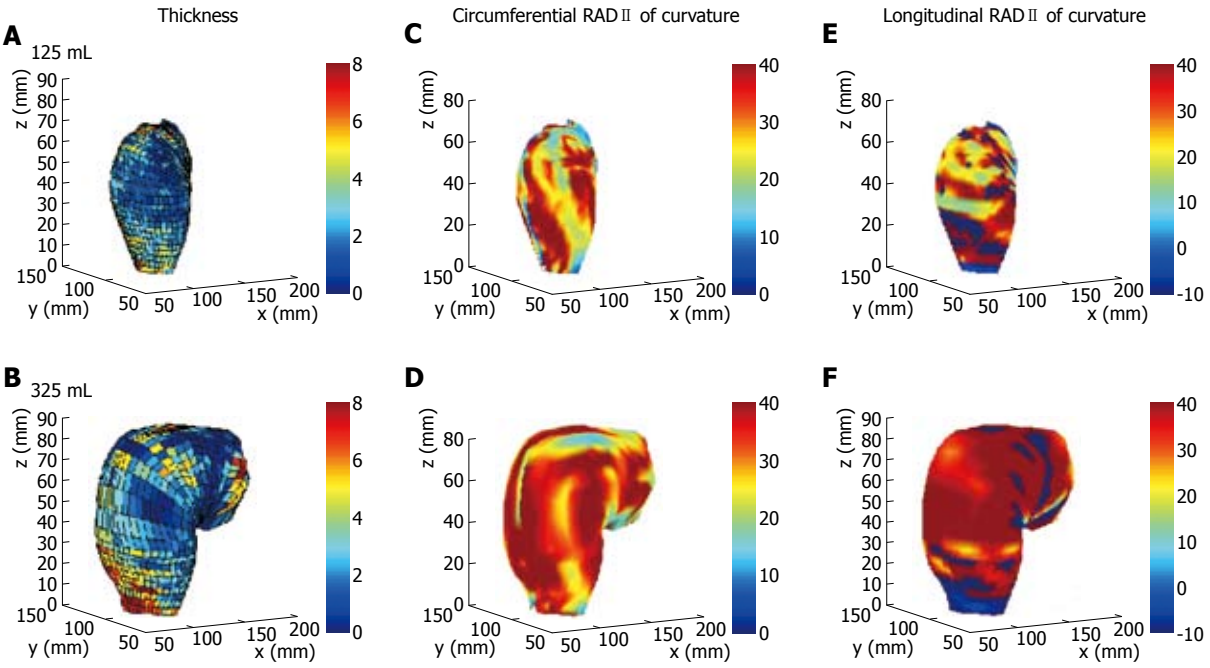

Figure 9 3D models of the rectum based on MRI and pressure recordings. The 3D distribution of the rectal wall thickness (A-B), circumferential (C-D) and longitudinal (E-F) principal radii of curvatures in one healthy volunteer at infused volumes of 125 mL (A, C, E) and 325 mL (B, D, F). The change in colour from blue to red during bag distension indicates an increase in rectal wall thickness or radius of curvature, i.e. increase in diameter. Modified from [50].

in detail in another paper in this issue. The recording reveals the complex motility of oesophageal bolus transport including sphincter function. Pathology related to oesophageal motor dysfunction is visualised with high accuracy[56]. Manometry can be combined with intraluminal impedance and pH measurements allowing further characterisation of reflux episodes (fluid *vs* air, acid *vs* non-acid).

Scintigraphy and single photon emission computed tomography (SPECT) are applied for emptying and motility studies of the GI tract[57,58]. Radionuclide transit/emptying scintigraphy is easy to perform, closely reflects physiology and provides quantitative data in the evaluation of several functional or motility disorders of the upper GI tract. However, scintigraphy has a low radiation burden. Like conventional radiography, dynamic scintigraphy with a radioactive liquid or semisolid bolus provides information on oesophageal motility useful in disorders such as nutcracker oesophagus, oesophageal spasm, non-cardiac chest pain, achalasia, oesophageal involvement in scleroderma, gastro-oesophageal reflux and monitoring response to therapy. Scintigraphy with a radiolabeled test meal represents the gold standard for evaluating gastric emptying in patients with dyspepsia, and evaluation of gastric function in various systemic diseases affecting gastric emptying. Similar scintigraphic methods are applied in the study of small intestinal and colonic transit. Recent radionuclide methods include dynamic antral scintigraphy and gastric SPECT for assessing gastric accommodation. However, US and MRI methods (see above) are still developing and the evaluation of functional GI diseases may in the future be subject to new and innovative techniques.

CONCLUSION

Imaging of the GI tract is essential in the diagnosis of GI diseases. This review highlights the capabilities of the newest techniques to explore the detailed morphology, biomechanical properties, function and pathology of the GI tract. The technological development is fast and the innovative potential enormous. Refinement of present modalities with faster image acquisition, higher resolution, increased computer power and improved software for post-processing are the main developing trends. Another trend is the development and refinement of "new sub-modalities" based on the traditional methods, and not least the fusion of different modalities into new multimodal concepts. Altogether, the future of GI imaging looks very promising, which will be of great benefit in clinical and research studies of GI diseases.

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Gastrointestinal tract modelling in health and disease

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Abstract

The gastrointestinal (GI) tract is the system of organs within multi-cellular animals that takes in food, digests it to extract energy and nutrients, and expels the remaining waste. The various patterns of GI tract function are generated by the integrated behaviour of multiple tissues and cell types. A thorough study of the GI tract requires understanding of the interactions between cells, tissues and gastrointestinal organs in health and disease. This depends on knowledge, not only of numerous cellular ionic current mechanisms and signal transduction pathways, but also of large scale GI tissue structures and the special distribution of the nervous network. A unique way of coping with this explosion in complexity is mathematical and computational modelling; providing a computational framework for the multilevel modelling and simulation of the human gastrointestinal anatomy and physiology. The aim of this review is to describe the current status of biomechanical modelling work of the GI tract in humans and animals, which can be further used to integrate the physiological, anatomical and medical knowledge of the GI system. Such modelling will aid research and ensure that medical professionals benefit, through the provision of relevant and precise information about the patient's condition and GI remodelling in animal disease models. It will also improve the accuracy and efficiency of medical procedures, which could result in reduced cost for diagnosis and treatment.

Key words: Gastrointestinal tract; Computational modelling; Biomechanics; Remodelling; Disease

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INTRODUCTION

The gastrointestinal (GI) tract, also called the digestive tract or the alimentary canal, is the system of organs within multicellular animals that takes in food, digests it to extract energy and nutrients, and expels the remaining waste. The major functions of the GI tract are digestion facilitated by motility, secretion and absorption. The various patterns of GI tract function are generated by the integrated behaviour of multiple tissues and cell types. Medical imaging methods such as ultrasonography[1,2], magnetic resonance imaging (MRI)[3,4], and endoscopic ultrasound (EUS)[5,6] are well known stand-alone clinical methods that can disclose structural and functional abnormalities of the GI tract. However, a thorough study of the GI tract requires understanding of the interactions between cells, tissues and gastrointestinal organs in health and disease. This depends on knowledge, not only of numerous cellular ionic current mechanisms and signal transduction pathways, but also of large-scale GI tissue structures and the special distribution of the nervous network. A unique way of coping with this explosion in complexity is mathematical and computational modelling; providing a computational framework and Information Communication Technology (ICT) based tools for multilevel modelling and simulation of the human gastrointestinal anatomy and physiology[7-9]. Computer-based analysis, visualisation, modelling and simulation are used routinely in fields such as engineering, meteorology or traffic control to understand the behaviour and outcomes of new designs and impact of

external phenomena well in advance of their realisation, thereby avoiding costly failures. In GI tract studies, this approach is not common, mainly because we still lack those models that can emulate the behaviour of the human body. Nevertheless, exploration of the GI tract has dramatically improved by the introduction of cross sectional imaging modalities such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI), which have revolutionised the way in which many conditions are diagnosed and treated. The ability to examine, in detail, structures inside the GI tract without resorting to surgery, has allowed clinicians to diagnose problems and plan corrective procedures with a minimum of risk to the patient[10-11]. In order to continue this exploration, it will be necessary to complement the traditional approach with an integrative approach that combines observation, theory and prediction across the temporal and dimensional scales, across scientific disciplines, and across the anatomical subsystems, all of which reflect the rather artificial divisions described.

The aim of this review is to describe the currently status of biomechanical modelling work on the GI tract in humans and animals that can be further used to integrate the physiological, anatomical and medical knowledge of the GI system.

ANATOMY AND FUNCTION OF THE GI TRACT

The GI tract is a continuous channel through the body with the biliary and pancreatic ducts as major side-branches. The GI tract consists of a series of organs, which resemble one another in constitution, being variously arranged as cylinders, spheroids, or intermediate forms. The main functions of the GI tract are transport and digestion of food. The different segments show a large variation in morphology and muscle mechanical properties, i.e. the oesophagus mainly serves to quickly transport the food bolus from the mouth to the stomach where the food in the stomach is stored for some time whilst simultaneously being broken down into smaller components. The GI sphincters serve to separate the GI tract into compartments. However, the gut is also important for immune functions[12]. The wall of the GI tract is typically composed of four layers, i.e., the mucosa, submucosa, muscle and serosa (some parts are called the adventitia where there is no epithelium) (Figure 1). The muscle layer consists of an outer longitudinal and an inner circular muscle layer. The collagen-rich submucosa and mucosa layers are inside the muscle layer. Another thin layer of muscle, the muscularis mucosae, exists almost throughout the entire tract. The motions of the GI tract accomplish a net antegrade flow in order to mix the contents and move them across the surface where absorption occurs. The contractile patterns and transit vary greatly from one part of the tract to another. The GI wall movements during digestion and absorption are the consequence of contractions of the two layers of smooth musculature. Contractions of the longitudinal muscle layer shorten the gut wall, whereas the peristaltic contractions of the circular muscle layer, in contrast, mainly produce forward transit with relatively little mixing. The contractions of circumferential and longitudinal muscles occur together, most of the time. The enteric nervous system (ENS), composed of both the myenteric (inter-muscular) plexus and the submucosal plexus, is distributed in the GI tract from the oesophagus to the internal anal sphincter[13]. A network of nerves of the myenteric plexus is embedded in the loose collagen matrix between the longitudinal and circular muscle layers (Figure 1). This set of nerves is essential for the regulation of the contractions of the adjacent musculature. Between the nerve endings and smooth muscle are the interstitial cells of Cajal (ICCs), which have been shown to be critical for the generation and propagation of the electrical slow waves that regulate the phasic contractile activity of GI smooth muscle, and for mediating neurotransmission from enteric motor neurons to smooth muscle cells[14,15]. The ENS ensures that the GI tract can fulfil essential tasks even when isolated from the rest of the body. The GI tract is - on the other hand - unable to work normally without the integrative functions of the ENS. Malfunctions of the ENS are increasingly recognized as underlying factors in many GI diseases. The exogenous nerves running together with the sympathetic and parasympathetic nervous systems are also important in regulating blood flow and secretion *etc*[16]. They also encode the conscious sensations from the gut such as fullness, urge to defecate and pain[17]. Medical imaging methods such as ultrasonography, MRI, and endoscopic ultrasound (EUS) are well known stand-alone clinical methods that can disclose structural and functional abnormalities of the GI tract[6,18]. Therefore, modelling analysis based on the anatomy and structure of the GI tract and different imaging methods can be applied to the problems related to function and pathophysiology.

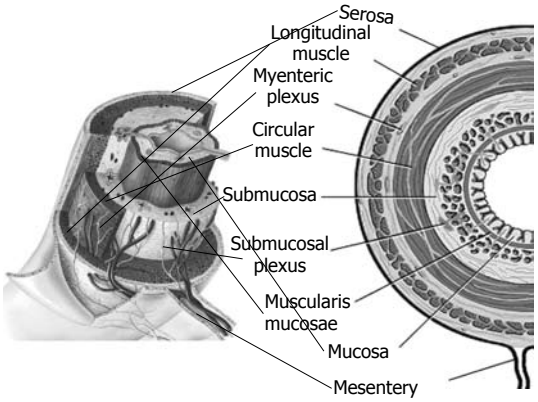

Figure 1 Schematic diagram of the GI tract.

DISEASE CAUSING TISSUE AND STRUCTURE REMODELLING OF THE GI TRACT

The GI tract, like other hollow organs such as the

Table 1 Diseases causing histomorphological and biomechanical remodeling of GI tract[19-45]

Diseases	Species	Test organs	Histomorphometric remodeling			Biomechanical remodeling		
			WT	WA	LT	OA	RES	Stiffness
Type Ⅰ diabetes	Human	Esophagus	↑	ND	ND	ND	ND	Circ NC Long↑
		Duodenum		ND	ND	ND	ND	Circ NC Long↑
	Rat	Esophagus	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long ND
		Duodenum	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long↑
		Jejunum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long↑
		Ileum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long↑
		Colon	↑	↑	Mu↑Su↑Ms↑			Circ↑Long↑
Type Ⅱ diabetes	Rat	Esophagus	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long ND
		Stomach	ND	ND	ND	ND	ND	Circ↑
Systemic sclerosis	Human	Duodenum	ND	ND	ND	ND	ND	Circ↑
Ulcerative colitis	Mice	Colon	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long↑↑
Fasting	Rat	Duodenum	↓	↓	Mu↓ Su↓	↑	↑	Circ↓ Long↓
		Jejunum	↓	↓	Mu↓ Su↓	↑	↑	Circ↓ Long↓
		Ileum	↓	↓	Mu↓ Su↓	↑	↑	Circ↓ Long↓
Low protein diet	Mink	Duodenum	↓	↓	NC	NC	NC	Circ↓ Long NC
		Jejunum	↓	↓	Mu↓ Su↓	↓	↓	Circ↓ Long NC
		Ileum	↓	↓	Mu↓ Su↓ Ms↓		↓	Circ↓ Long NC
Partial obstruction	Opossum	esophagus	ND	ND	ND	ND	ND	Circ↑Long ND
	Guinea pig	Jejunum	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long ND
Osteogenesis imperfecta	Mice	Esophagus	↓	↓	Mu↓ Su↓ Ms↓	↑	↑	ND
Irradiation	Mice	Rectum	↑	↑	ND	↑	↑	ND
Small bowel resection	Rat	Jejunum	↑	↑	Mu↑Su↑Ms↑	↑	↑	NC
		Ileum	↑	↑	Mu↑Su↑Ms↑	↑	↑	NC
EGF treatment	Rat	Esophagus	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long ND
		Duodenum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long NC
		Jejunum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↓ Long↑
		Ileum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↓ Long↑
		Colon	↑	↑	Mu↑Su↑Ms↑	↑	↑	ND

WT: Wall thickness; WA: Wall cross-sectional area; OA: Opening angle; RES: Residual strain; LT: Layered wall thickness; Mu: Mucosa; Su: Submucosa; Ms: Muscle; Circ: Circumferential direction; Long: Longitudinal direction; ND: Not done; NC: No change.

heart, blood vessels, urinary bladder and the urethra, is functionally subjected to dimensional changes. Hence, biomechanical properties are of particular functional importance. Data on the biomechanical properties are crucial for the understanding of the normal function of the GI tract and dysfunction due to disease because, (1) peristaltic motion that propels the food through the GI tract is a result of interaction of the passive and active tissue forces and the hydrodynamic forces in the food bolus and (2) remodelling of the mechanical properties reflects the changes in the tissue structure that determine a specific sensory-motor dysfunction.

Human studies have documented that diabetes melitus[19] and systemic sclerosis[20] induce biomechanical GI remodelling. Using different animal models (Figure 2), we have demonstrated that biomechanical and histomorphological remodelling occur in the GI tract due to normal physiological growth[21,22], malnutrition[23,24], inflammation, obstruction[25-27], bowel resection[28], diabetes[29-33], radiation injury[34], collagen changes[35,36] and EGF treatment. The morphometric properties are best described at the zero-stress state where no internal or external forces deform the tissue. Furthermore, knowing the zero-stress configuration is essential in any mechanical analysis since it serves as the reference state for computing stress and strain under physiological or pathophysiological conditions. With reference to the zero-stress state, combining the morphometry data and pressure data, we can compute the stress-strain relationship of the GI wall. The stress-strain distribution mainly reflects the elastic properties of the GI tract. Changes in the elastic properties reflect structural remodelling of the GI wall in different diseases. Therefore, we consider the opening angle of the zero-stress state, residual strain and stress-strain relationship as the most relevant biomechanical parameters to describe diseases causing GI remodelling. Generally, diseases and factors inducing tissue overgrowth, such as diabetes[37-40], obstruction and EGF[41-45] treatment, increase GI wall stiffness; whereas factors reducing tissue growth, such as fasting and low protein diet decrease GI wall stiffness. The more collagen in the GI wall, the stiffer the wall is[20], and vice versa. However, the effect of different factors on the opening angle and residual strain of GI tract depend on the changes of layered structure. Fung's hypothesis of non-uniform remodelling states that if the inner wall grows more than the outer wall, the opening angle will increase[46] whereas if the outer wall grows more than the inner wall, the opening angle will decrease. Table 1 summarizes the histomorphological and biomechanical remodelling of the GI tract caused by different diseases.

It is well known that mechanosensation is important for GI function. Mechanosensitive nerve endings exist extensively in the GI tract where they serve a critical role in homeostasis. The mechanosensitive afferents in

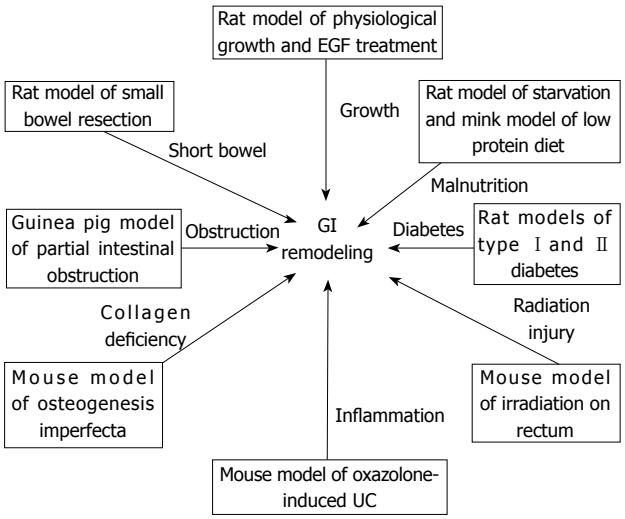

Figure 2 Disease-induced GI remodeling in animal models.

the intrinsic and extrinsic pathways have been described as low-, wide-dynamic- or high-threshold tension-receptors[12]. Therefore, the GI tract structure, as well as the stress and strain distribution in the wall, is important for the GI sensory and motor function. The GI wall structure or deformation changes caused by a disease will alter the relative positions of the mechanosensitive afferents (zero setting of the mechanosensitive afferents). The biomechanical remodelling by the disease, such as alterations of residual strain, stress distribution and wall stiffness, will alter the tension and stress distribution of the mechanosensitive afferents. As a result, the perception and motility of the GI tract will also change. Therefore, the morphological changes and biomechanical remodelling of the GI tract are likely to affect the function of mechanosensitive afferents in the GI wall and further affect the motor and sensory functions.

BIOMECHANICAL MODELLING OF THE GI TRACT

The use of numerical models and, in particular, of finite element models has been extensively studied in the field of soft tissue mechanics because of the potential they offer in the analysis of the mechanical behaviour of morphologically complex structures, with high structural hierarchy and constituents with non-linear behaviour[47,48]. The effectiveness of numerical models depends on reliable reconstructions of the morphometry of the anatomical site under investigation, the specific loading and boundary conditions, as well as the definition of constitutive models capable of describing the mechanical response of the single tissues. Gastroenterology research has traditionally been based on experimental approaches rather than on mathematical modelling. Most of the previous modelling efforts in the biological area were in the cardiac and lung field; but, other areas are in now being developed. However, in the past five to ten years several groups have independently started to model the GI tract.

The large morphological complexity of the GI tract and the variability in the different parts of the tract are well known. The complexity increases in the characteristic folds of the connection regions[49-51]. With regard to the structural conformation of GI tract tissues, the inner mucosa and submucosa layers are surrounded by the outer muscular and serosa layers. Collagen fibres of submucosa form a complex network and are oriented in different directions. The muscular layers have muscle fibres oriented in the circumferential direction (circumferential muscle layer) or the longitudinal direction (longitudinal muscle layer). As a consequence, the GI tract tissue must be considered as a multi-layered composite material, made up of tissues with different mechanical characteristics[12,52-54]. Some of the components show a specific spatial disposition of the sub-structures, such as collagen and muscle fibres, and are studied by means of a constitutive formulation already adopted in other fields for the mechanics of biological tissues, based on the theory of fibre-reinforced materials[55-57]. The most current investigations of the GI tissue properties are mainly focused on seeking the constitutive equation and the associated constitutive parameters of the physiological or pathological status. To date, most GI structure and tissue property studies have been based on animal experiments. Medical device development has made it possible to study the mechanical behaviour of the GI tract *in vivo*[58-62].

The methods and current developments in studies of the biomechanical properties of normal and disease remodelled GI tissues have been described in the above sections. Hereafter, the establishment of morphometric-related modelling of the GI tract will be briefly introduced. According to the reconstruction methods on GI modelling, the establishment of the GI models can be divided into *in vivo* medical images-based models, the anatomical-based models and the theoretical analysis-based models.

In vivo medical images-based GI models

Advances in imaging are introduced initially as research tools and subsequently as clinical diagnostic tests. Medical image-based 3-D models of *in vivo* GI organs have characterized the oesophagus, stomach, small intestine, sigmoid colon, oesophageal gastric junction and the rectum, based on cross-sectional imaging using ultrasonography, computed tomography (CT), Functional Luminal Imaging Probe (FLIP) or magnetic resonance imaging (MRI)[3,4,63-68]. With the development of the medical devices such as impedance planimetry, it is now possible to record the mechanical parameters such as the luminal pressure simultaneously with the cross-sectional medical images. Therefore, the *in vivo* mechanical behaviour of the organs can be computed on the basis of the reconstructed GI morphometric models and the recorded mechanical parameter. A reconstructed sigmoid-colon model and the corresponding tension and stress distribution on the model are illustrated as an example in Figure 3. Detailed descriptions of *in vivo* GI

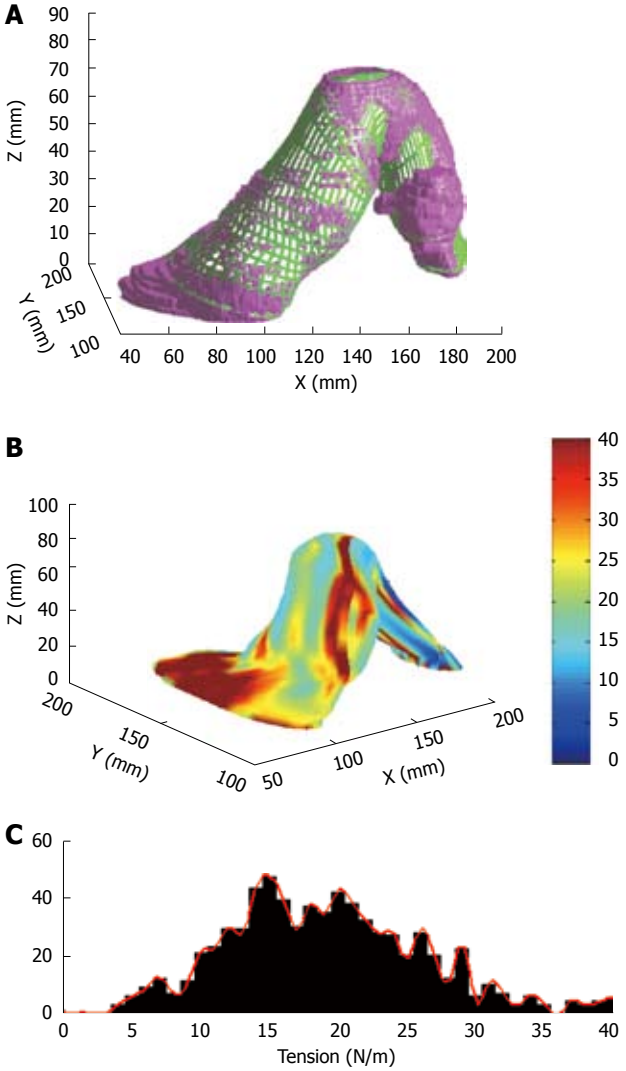

Figure 3 A reconstructed sigmoid-colon model and the corresponding tension distribution. A: A representative sigmoid colon model with the distension volume of 200 mL. The model with purple colour is the solid model generated directly from the MR images, and the green mesh is the smoothened surface, the comparison between the solid model and smoothened surface indicates that the smoothened model fits well with the original solid model; B: The circumferential curvature distribution on the surface models; C: Tension distribution of the sigmoid colon surface model.

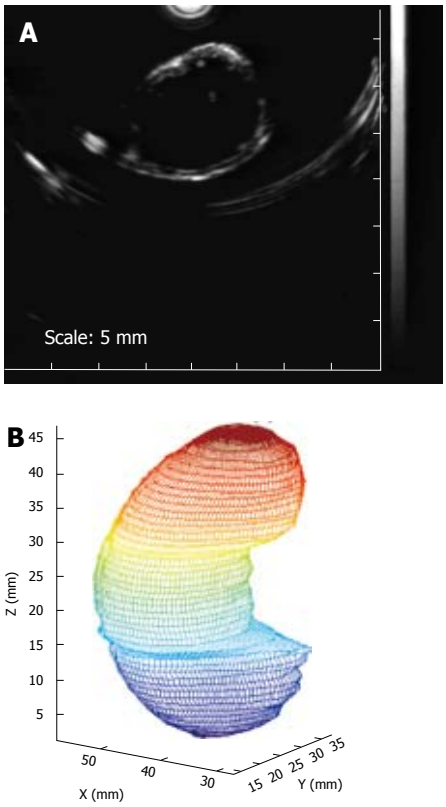

Figure 4 An example of the anatomically based *in vitro* rat stomach model generated from ultrasonic scanning. A: A representative CT scanning of a cross sectional slice of an *in vitro* rat stomach; B: The reconstructed gastric model on the basis of the CT scanning on the *in vitro* rat stomach. The distance between cross sectional slices was 1 mm, the colour change from blue to red means the increase of the stomach length in z direction.

modelling analysis can be found in studies of Liao *et al*[66] and Frokjaer *et al*[3,4].

Anatomy-based GI modelling

For modelling analysis using the *in vivo* image based models, only the tension or stress was computed on the basis of three-dimensional surface geometry using the Laplace's equation, and the wall thickness. The tissue structure was, therefore, not taken into account. To aid in understanding of the relationship between the structure and function of the GI tract in healthy and diseased states, an anatomically-based finite element model is needed. The anatomically based visualization GI model is now commercially available, however the GI anatomy based numerical modelling analyses are mainly been done by Andrew Pullan's group so far, and all models were built from the Visible Human Project[69-71]. The Visible Human project provided a set of cross sectional images of a human cadaver. On each image, data points around the organ boundary of interest were created and then the geometry models were constructed on the basis of the distinguished data clouds[71-75]. The anatomically-based models have now been used to investigate normal and pathological electrical activity of the stomach and small intestine[71-74], the muscle functions on the gastro-oesophageal junction during swallowing[75] and the blood flow in the mesenteric arterial system of the human intestine[69,70]. An example of the anatomically-based *in vitro* rat stomach model generated from ultrasonic scanning is illustrated in Figure 4.

Theoretical analysis-based GI modelling

The morphological complexity of the GI organs makes it difficult to build the anatomically-based finite element models. Hence, some numerical models of the GI organs were built on the basis of theoretical analysis by simplifying the complex GI morphometry as a regular geometry such as a circular cylinder for the oesophagus[52,57,64,68,76] and a sphere for the stomach pouch[77] and some regular tubes for describing the antrodudenal junction[78] and the oesophago-gastric junction[79]. In the morphometrically-simplified model, most of the biomechanical features such as the tissue structure, tissue properties and bolus flow can be

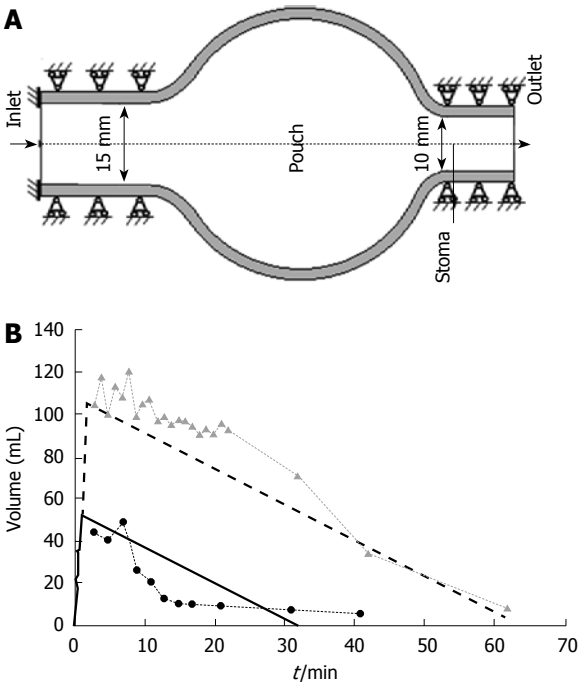

Figure 5 A simplified pouch model for describing the gastric emptying of a patient treated for obesity. A: A representative pouch model of midsized pouch with stoma diameter of 10 mm, B: Volume history in the filling and emptying phases in the mid-sized and large pouch models with stoma diameter of 10 mm. The solid line represents the mid-sized pouch, and the dotted line the large pouch. Circles and triangles represent volume data of the recorded clinical emptying curve for the mid-sized and large pouch. Pouch and stoma are a small fundic cavity and a corresponding narrow outlet between pouch and the rest of the stomach in gastroplasty and gastric bypass procedures for obesity.

expressed mathematically and, thus, the mechanical function of the GI tract can be simulated. The simplified GI tract models have existed for describing the muscle function[53,68,76], food transportation[77,78,80,81] and blood flow[65] in the GI tract in healthy and diseased situations. A simplified pouch model for describing the gastric emptying of a patient treated for obesity is illustrated in Figure 5 as an example. As can be seen, the emptying curves for the pouch based on the simplified model agree well with the clinical results.

PERSPECTIVE

GI modelling studies are focused on patient-specific computational modelling and simulation for prediction of disease or early diagnosis by integrating patient specific knowledge and predispositions obtained in biomedical imaging. Modelling studies of the GI tract will advance our understanding of the mechanisms of GI function and diseases, such as dyspepsia and visceral pain. Furthermore, an integrated GI tract simulation model will be beneficial for medical education, and for evaluation of the efficacy and safety of new drugs. The challenge of GI modelling is to develop mathematical and computational models of structure-function relations appropriate to each (limited) spatial and temporal domain, and then to link the parameters of a model at one scale to a more detailed description of structure and function on the adjacent levels. The present analytical tools can thus be integrated in order to analyze complex structures for understanding biomechanical behaviour in other visceral organs and be further integrated as a global GI model. The mechanical behaviour-related aspects of diseases of the sigmoid colon (diverticular disease, irritable bowel syndrome, *etc*), small bowel (motility disorders), stomach (motility disorders, non-ulcer dyspepsia, *etc*) and oesophagus (oesophagitis, gastro-oesophageal reflux disease, non-cardiac chest pain, *etc*) can be further developed by applying modified versions of the present models.

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Translational pain research: Evaluating analgesic effect in experimental visceral pain models

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Abstract

Deep visceral pain is frequent and presents major challenges in pain management, since its pathophysiology is still poorly understood. One way to optimize treatment of visceral pain is to improve knowledge of the mechanisms behind the pain and the mode of action of analgesic substances. This can be achieved through standardized experimental human pain models. Experimental pain models in healthy volunteers are advantageous for evaluation of analgesic action, as this is often difficult to assess in the clinic because of confounding factors such as sedation, nausea and general malaise. These pain models facilitate minimizing the gap between knowledge gained in animal and human clinical studies. Combining experimental pain studies and pharmacokinetic studies can improve understanding of the pharmacokinetic-pharmacodynamic relationship of analgesics and, thus, provide valuable insight into optimal clinical treatment of visceral pain. To improve treatment of visceral pain, it is important to study the underlying mechanisms of pain and the action of analgesics used for its treatment. An experimental pain model activates different modalities and can be used to investigate the mechanism of action of different analgesics in detail. In combination with pharmacokinetic studies and objective assessment such as electroencephalography, new information regarding a given drug substance and its effects can be obtained. Results from experimental human visceral pain research can bridge the gap in knowledge between animal studies and clinical condition in patients suffering from visceral pain, and thus constitute the missing link in translational pain research.

Key words: Visceral pain; Analgesics; Pharmacokinetics; Pharmacodynamics

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INTRODUCTION

Deep muscular and visceral pain is very frequent and causes major challenges in pain management[1]. Visceral pain is different from somatic pain because it is more diffuse, hard to localize, accompanied by autonomic reflexes, and very often described with an associated somatic referred pain. Pathophysiology and the mechanisms behind visceral pain conditions remain poorly understood. This lack of knowledge makes treatment of visceral pain challenging and often suboptimal.

Non-opioids are often insufficient in relieving pain to an acceptable level in patients suffering from severe pain originating from the gastrointestinal tract[2]. On the other hand, treatment with traditional μ-opioid agonists may not be optimal. It often fails to relieve pain sufficiently, and at the same time causes side effects such as constipation, euphoria, sedation and nausea. These adverse effects are mainly mediated through μ-receptors in the central nervous system (CNS). To encompass these problems, new therapeutic approaches have addressed opioids interacting with the peripheral κ-receptor, NMDA-antagonists and adjuvant analgesics (antidepres-

sants and anticonvulsants)[3-7].

In experimental pain models, it is important to have a robust pain measure to obtain a reliable model and to detect the analgesic effect[8]. In standardized experimental human pain models, the investigator can control the induced pain (including modality, localization, intensity, frequency and duration), and provide quantitative measures of the responses. Hence, confounding factors such as sedation, nausea and general malaise that underlie clinical pain can, to a large extent, be controlled or avoided[9]. Different experimental stimulations can be used to induce visceral pain. Thermal, mechanical, electrical and chemical stimulations can be performed with a multi-modal approach, for which, different receptor types, pathways and mechanisms can be activated. This may act as a proxy for some of the mechanisms involved in clinical visceral pain conditions[10]. Such a multi-modal stimulus regimen can be used in conjunction with a multi-tissue approach in which skin, muscles and viscera are stimulated[9].

Pharmacokinetic and pharmacodynamic (PK-PD) modeling of analgesics can be used to identify and explain potential differences between the analgesic substances in their mechanism of alleviating pain. The effect depends on the concentration at the site of action. The site of action (biophase) for many analgesic substances is within the CNS, while most pharmacokinetic studies measure analgesic concentrations in plasma. When PK-PD modeling is performed, it is, therefore, important to account for a possible delay between plasma concentration and effect[11].

PK-PD MODELING

There are various approaches to the study of opioid pharmacokinetics and pharmacodynamics (Figure 1). These can be classified according to their relative advantages and disadvantages.

Animal studies

These studies allow the investigation of fundamental mechanisms (such as cerebral equilibration rates) and the collection of arterial and venous blood concentration data. However, the dynamic information [e.g. tail flick times, changes in electroencephalography (EEG) or magnetic resonance imaging (MRI) signals] cannot be readily related to analgesia in humans. Representative studies include sheep studies performed at the University of Adelaide, Australia where a model to study the relationship between plasma and CNS concentration has been developed by Upton *et al*[12,13]. They have previously used a sheep preparation to examine the cerebral kinetics and dynamics of analgesic drugs used in the perioperative period. Physiological PK-PD models developed in sheep have been adapted to assess the clinical profile of these drugs in humans[14]. One sheep study has shown that the faster analgesic onset with oxycodone compared to morphine might be explained by a faster equilibration between blood and brain for oxycodone[15].

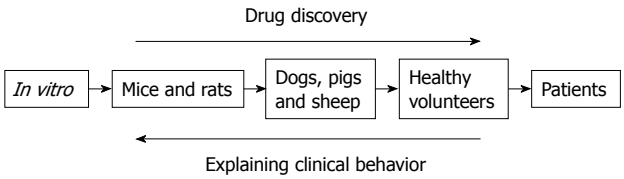

Figure 1 Understanding the effect of analgesics. An overview of the different levels of investigation of analgesic effects. A good deal of attention has been focused on progressing from left to right in drug discovery. Less attention has been focused on progressing from right to left. Reproduced with permission from Upton *et al*[33].

Surgical patient studies

These studies are typically conducted in patients just before or during surgery when patients have an arterial cannula (often for patient management). As the patients are generally sedated (and cannot report pain) and the dose is high, the pharmacodynamic information is generally derived from changes in EEG. A representative study is that of Poyhia *et al*[16] in which EEG was used to quantify the CNS effects of oxycodone during anesthesia for primary coronary artery bypass grafting.

Volunteer and awake patient studies

For ethical reasons, these subjects usually only have a venous cannula placed in an arm vein. However, they can report highly relevant dynamic information such as pain and sedation scores, and can be studied using doses and routes that are directly relevant to clinical practice. Representative studies are the opioid study by Staahl *et al*[17], or the intranasal fentanyl studies of Christrup *et al*[18] and Foster *et al*[19]. Differences in the site of action for opioids may be reflected in the delay between opioid blood and CNS concentration and the analgesic effect. These differences might be more pronounced in diseases in which liver and kidney function are reduced or affected. Understanding these differences has implications for interpretation of PK-PD opioid studies, and provides insight into optimal clinical analgesic management of visceral pain[17]. A robust pain assessment is needed to obtain a reliable model of the PK-PD relationships for opioids. Experimental pain models in healthy volunteers provide less variable and less confounded pain measures, which are suitable for PK-PD modeling. A neurophysiological objective assessment of pain response and analgesic effect is EEG, which also can support the subjective findings in experimental pain studies. Previous investigations have shown that quantitative (spectral) analysis of the increase in delta frequency band of the resting EEG is a suitable biomarker for the PK-PD correlation of opioids[20,21]. In a study of biophase kinetics within the PK-PD analysis of a wide range of opioids, morphine showed profound hysteresis between the blood pharmacokinetics and EEG effect[22]. Groenendaal *et al*[22] have concluded that within the wide range of opioids used in their study, only morphine displayed complex biophase distribution kinetics, which can be explained by its relatively low permeability of the blood-brain barrier and its interaction with active transporters present at the barrier.

TESTING OF ANALGESICS IN EXPERIMENTAL VISCERAL PAIN MODELS

Until now, only a few studies investigating the effect of analgesics in visceral experimental pain in healthy volunteers have been performed. Only two of these studies assessed the PK-PD relationship[17,23].

Opioids

Morphine: Morphine is a highly potent opiate analgesic drug and is the principal active agent in opium, and is the prototypical opioid. Morphine is one of the few opioids to have been evaluated in human experimental visceral pain models[24]. Comparing somatic and visceral tissue, differences in opioid analgesia have been observed. Morphine does not affect somatic pain. Morphine analgesia is significantly better than placebo in attenuating mechanical and electrical esophageal pain, but not thermal esophageal pain.

Oxycodone: Oxycodone is a semi-synthetic opioid with analgesic effect and, as with morphine, has been evaluated in human experimental visceral pain models[24]. As for morphine, tissue differences in opioid analgesia have been observed. One study has shown that oxycodone is significantly better than placebo in attenuating mechanical, electrical and thermal esophageal pain. Furthermore, oxycodone has a superior effect on visceral pain compared to morphine[24]. This indicates that oxycodone may interact with other visceral opioid receptors more than morphine does. This reflects the clinical situation in which visceral pain, in contrast to somatic pain, can be difficult to treat with traditional μ-opioid agonists[25].

Lalovic *et al*[23] have studied the pharmacokinetics and pharmacodynamics of oxycodone in healthy human volunteers; measurements included the time course of plasma concentrations and urinary excretion of metabolites, along with the time course of miosis, and subjective opioid side effects. The contribution of circulating metabolites to oxycodone pharmacodynamics has been analyzed by PK-PD modeling. The human study was complemented by *in vitro* measurements of opioid receptor binding and activation studies, as well as *in vivo* studies of the brain distribution of oxycodone and its metabolites in rats. Noroxycodone and noroxymorphone are the major metabolites in the circulation with elimination half-lives longer than that of oxycodone; but, their uptake into the rat brain is significantly lower compared with that of the parent drug. PK-PD modeling has indicated that the time course of pupil constriction in healthy volunteers is fully explained by the plasma concentration of the parent drug oxycodone. The metabolites do not contribute to the central effects, either because of their low potency or low abundance in circulation, or as a result of their poor uptake into the brain[23].

A pronounced delay between the plasma concentrations and analgesia will produce hysteresis when the analgesic effect is plotted against plasma concentration. This is characteristic for opioids and has been shown previously to be caused partially by the rate of transport of the opioid into the CNS (across the blood-brain barrier)[26], but also by receptor-mediated cascades[13,27]. Traditionally, hysteresis is collapsed by the implementation of a theoretical effect compartment between the plasma compartment and effect (i.e. the effect is not delayed compared to the drug concentration in this compartment)[11].

In visceral pain assessments in healthy volunteers, obvious differences are seen between oxycodone and morphine PK-PD profiles. The effect of morphine is generally described through an effect compartment, whereas oxycodone tends to be more directly linked to the plasma concentration, because no hysteresis is produced[17]. This supports the results of Lalovic *et al*[23] and the theory that oxycodone acts partially at a peripherally located receptor. One hypothesis is that this peripherally located receptor is the κ receptor, since there is some evidence that oxycodone has a partial effect at the κ opioid receptor[24,28]. In contrast to other opioid receptor types, for which central effects dominate, the peripheral κ receptor may also be important for visceral analgesia[3,29,30]. Staahl *et al*[17] have confirmed that morphine and oxycodone have somewhat different PK-PD relationships in attenuation of visceral pain and, therefore, most likely act at receptors situated in different physiological compartments. These results partly address the question: to what extent do the cerebral pharmacokinetics of a drug contribute to its clinical behavior? Furthermore, they provide insight into optimal clinical analgesic management of visceral pain.

N-methyl-D-aspartic acid (NMDA)-antagonists

Ketamine is classified as an NMDA receptor antagonist. Ketamine has also been found to bind to opioid receptors. It has the added benefit of counteracting spinal sensitization or wind-up phenomena experienced with chronic pain. It is primarily used for the induction and maintenance of general anesthesia, usually in combination with some sedative drug, because otherwise unwanted psychological side effects can occur.

The analgesic effect of ketamine has been investigated in several experimental pain models. During visceral distension, pain and unpleasantness are decreased by ketamine[4]. Apparently, deep muscular or visceral pain is treated more successfully than superficial pain[4,31]. This supports the findings in other human studies in which deep pain activates central mechanisms (involving the NMDA receptor) such as summation more quickly than superficial pain does[32].

Hyperalgesia to electrical pain has been induced in the esophagus by acid infusion. It has been shown that ketamine prevents development of hyperalgesia and reverses induced hyperalgesia[5].

Antidepressants

Imipramine is a tricyclic antidepressant of the dibenzazepine group. Imipramine is similar in structure to some muscle relaxants, and has a significant analgesic effect, and, therefore, is very useful in some pain conditions. As an example of experimental pain testing of the effect of imipramine, non-nociceptive sensation and pain to

distension of the esophagus have been investigated by Peghini *et al*[6]. In this study, only stimulation within the painful range was affected, which shows a pain-specific action of imipramine.

Amitriptyline is a tricyclic antidepressant. In terms of its mechanism of action, amitriptyline inhibits serotonin and noradrenaline re-uptake almost equally. A pain model that involved esophageal and rectal distension was not sensitive to amitriptyline[7]. This study applied a rather uncontrolled stimulation paradigm in which there was a risk for bias in stimulus intensity[9]. Hence, more studies are necessary to determine if the stimulation paradigm caused the lack of sensitivity.

Regarding the studies of ketamine and the antidepressants, more knowledge could be obtained on their effects by combining these experimental studies with studies on pharmacokinetics. However, experimental human pain studies often lead to new information, and as such studies often consist of a very complicated protocol and setup, it is not always possible to study the pharmacokinetic profile in parallel.

CONCLUSION

To improve visceral pain treatment, it is important to study the underlying physiological mechanisms of the pain and the pharmacological mechanism of action of the different analgesics. Experimental human visceral pain research bridges the knowledge gap between animal studies and clinical studies in patients suffering from pain, making it an important tool in translational pain research, as illustrated in Figure 1. An experimental pain model activates different modalities and, therefore, explores the effect of analgesics. With further understanding of the cerebral pharmacokinetics and pharmacodynamics of analgesics, opportunities may emerge to improve the efficacy and safety of these drugs in clinical practice. In combination with PK-PD studies and objective assessments such as EEG, new information regarding a given drug, its dose regimen and its effects can be obtained. Thus, evaluation of pharmacokinetics and pharmacodynamics is needed in future drug research. It is of interest to study the effect of new drugs as well as drugs already on the market, as lack of knowledge on the pharmacokinetics and pharmacodynamics of analgesic agents makes treatment of visceral pain a difficult task, and often far from optimal.

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GUIDELINES CLINICAL PRACTICE

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New technologies to investigate the brain-gut axis

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Abstract

Functional gastrointestinal disorders are commonly encountered in clinical practice, and pain is their commonest presenting symptom. In addition, patients with these disorders often demonstrate a heightened sensitivity to experimental visceral stimulation, termed visceral pain hypersensitivity that is likely to be important in their pathophysiology. Knowledge of how the brain processes sensory information from visceral structures is still in its infancy. However, our understanding has been propelled by technological imaging advances such as functional Magnetic Resonance Imaging, Positron Emission Tomography, Magnetoencephalography, and Electroencephalography (EEG). Numerous human studies have non-invasively demonstrated the complexity involved in functional pain processing, and highlighted a number of subcortical and cortical regions involved. This review will focus on the neurophysiological pathways (primary afferents, spinal and supraspinal transmission), brain-imaging techniques and the influence of endogenous and psychological processes in healthy controls and patients suffering from functional gastrointestinal disorders. Special attention will be paid to the newer EEG source analysis techniques. Understanding the phenotypic differences that determine an individual's response to injurious stimuli could be the key to understanding why some patients develop pain and hyperalgesia in response to inflammation/injury while others do not. For future studies, an integrated approach is required incorporating an individual's psychological, autonomic, neuroendocrine, neurophysiological, and genetic profile to define phenotypic traits that may be at greater risk of developing sensitised states in response to gut inflammation or injury.

Key words: Brain-gut axis; Central processing; Neuraxis; Neurophysiology

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INTRODUCTION

Pain is a complex multidimensional experience comprising sensory-discriminative, affective-motivational and cognitive-evaluative components[1]. The sensory-discriminative component represents the ability to localise pain, and assess its intensity whereas the affective-motivational component qualifies its unpleasantness and gives rise to emotional aspects such as fear and distress. The cognitive-evaluative component allows the evaluation and interpretation of the pain experience and is involved in attention, anticipation and memory of the experience[2].

Pain is an extremely common symptom in clinical practice[3] and often emanates from the intra-abdominal viscera. Visceral pain can be the manifestation of a myriad of underlying pathologies, occur with varying intensities ranging from mild discomfort to severe pain, be acute or chronic, and be referred to a variety of locations such as the chest, pelvis and skin. Understanding the complex mechanisms leading to the development and maintenance of visceral pain, in particular that which arises from

the gastrointestinal tract, requires an appreciation of the neuroanatomical structures and neurophysiological processes involved. The gastrointestinal (GI) tract has a complex innervation including sensory neurones (afferents), and the rich neuronal innervation closely regulates visceral function as well as providing sensory information to higher structures. The ability to dissociate specific neurophysiological mechanisms of aberrant gastrointestinal sensory processing has been the aspiration of an increasing number of gastrointestinal researchers. Improved access to brain imaging techniques has vastly increased our understanding of the central processing of gastrointestinal sensation and pain in both healthy volunteers as well as in patients suffering from functional gastro-intestinal disorders (FGID).

So how far are we now? As the episodical gastrointestinal pain still exploits different non-investigated aspects, the question is whether the newer brain-imaging techniques have provided the scientists with further understanding of the underlying pathophysiology and mechanisms in FGID? This review will focus specifically on the sensory pathways (peripheral, spinal and supraspinal) involved in these pain mechanisms and highlight the newer techniques in electroencephalogram (EEG) source analysis.

SENSORY INNERVATION OF THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract has a complex innervation with sensory neurones (afferents). As well as receiving dual sensory innervation from the central nervous system (CNS) referred to as extrinsic afferents, it has its own integrated network of intrinsic afferents (the enteric nervous system, ENS), that project locally. This rich neuronal innervation closely regulates visceral function as well as providing sensory information to higher structures.

Intrinsic sensory innervation (enteric afferent neurones)

The hollow intra-abdominal viscera have a rich sensory innervation with locally projecting afferent neurones, forming the enteric nervous system, whose cell bodies are located in the myenteric or submucosal plexuses[4]. This network of neurones and interneurones has a structural complexity and functional heterogeneity similar to that of the CNS, but mainly regulates local functions and reflexes such as secretion, motility, mucosal transport and blood flow[5,6]. Motor neurones located within the ganglia of the ENS coordinate these functions largely by regulation from local sensory neurones, although some also receive inputs from the CNS *via* autonomic (both sympathetic & parasympathetic) pathways[7]. Although the majority of enteric afferent axons are confined to the gut wall, some can project to the pre-vertebral ganglia of the sympathetic nervous system[8].

Extrinsic sensory innervation (primary afferent neurones)

The gastrointestinal tract has a dual sensory innervation from the CNS. In humans, visceral afferents project to the CNS mainly *via* the vagus nerve to the brainstem (vagal afferents) or through splanchnic nerves to the spinal cord (spinal afferents), and are described below.

Vagal afferent neurones

The vagus nerve innervates the majority of the GI tract apart from the distal third of the colon[9]. 70%-90% of the fibres in the vagal trunks are unmyelinated C-fibre neurones with their cell bodies located in the nodose ganglia situated just below the jugular foramen, although a minority lie more proximally within the jugular ganglia and contain afferents primarily from the oesophagus[10]. Around 80%-85% of nerve fibres in the vagus are afferent and project viscerotopically to the medial division of the nucleus of the solitary tract (NTS). Second-order neurones project from the NTS to sites in the brainstem, hypothalamus and amygdala including the vagal motor nuclei, the rostral areas of the ventrolateral medulla and the parabrachial nuclei[11,12]. Cortical projections from the brainstem include the orbitofrontal, infralimbic anterior cingulate and insula cortex, the latter having reciprocal connections with the secondary somatosensory cortex.

Vagal afferents are classically believed to mediate non-noxious physiological sensations such as satiety and nausea due to their low response thresholds and saturation characteristics that are within the physiological range[13-15]. However, animal experiments have suggested that vagal afferents may be involved in the central inhibitory modulation of pain. For instance, electrical stimulation of cervical vagal afferents inhibits the responsiveness of spinothalamic tract neurones to noxious stimuli[16].

Spinal afferent neurones

Spinal afferent neurones project from the viscera through the splanchnic nerves to the thoracic, upper lumbar and sacral spinal cord with their cell bodies located in the dorsal root ganglia (DRG). They constitute only 5%-10% of all afferent fibres in the thoracic and lumbar dorsal nerve roots with the majority traversing the pre- and paravertebral ganglia en route to the spinal cord. Collaterals to the prevertebral ganglia may mediate local autonomic reflexes[7].

Spinal afferents are contained within the cardiac (superior, middle and inferior) and splanchnic (thoracic, greater and lesser) nerves. These pass through the white rami to join spinal nerves before entering the DRG. The oesophagus is innervated craniocaudally by afferents from the DRG located between the first cervical and third lumbar segments. Retrograde labelling studies have shown the maximum distribution of spinal sensory neurons to be in the following DRG: C1-T8 (striated muscle); C5-L2 (smooth muscle), and T1-L3 (lower oesophageal sphincter)[17].

SPINAL PAIN PROCESSING

From the cell bodies within the DRG, spinal visceral afferents enter the spinal cord and ascend or descend one or two spinal levels in the dorsolateral fasciculus (Lissauer's tract) before terminating within the grey matter. In the

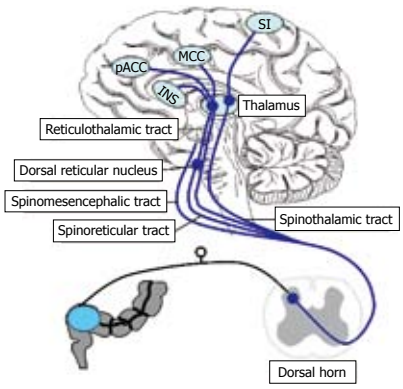

Figure 1 The principal visceral projections from the spinal cord to subcortial and cortical structures (blue lines). The spinothalamic tract terminates in the medial and posterior thalamus. Thalamocortical fibres then project to the primary somatosensory cortex. The spinoreticular tract terminates in the reticular formation to the medial thalamus. The spinomesencephalic tract projects to various regions in the brainstem, including the periaqueductal grey, locus coeruleus, and dorsal reticular nucleus in the medulla. Thalamocortical projections from the medial thalamus project to the cingulate cortex and insula which are involved in processing noxious visceral and somatic information. The brain regions innervated by these pathways that respond to painful visceral stimuli include the thalamus, insula, amygdala and anterior cingulate cortex (ACC). The ACC is comprised of two components, the perigenual ACC (pACC) involved in affect and the mid cingulate cortex (MCC) with behavioural response modification. Other pathways for transmission of noxious visceral stimuli (such as the dorsal column pathway), exist, but are not shown here.

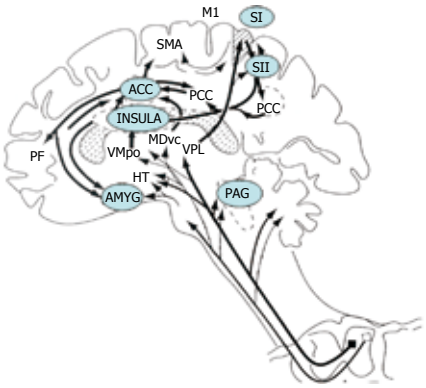

Figure 2 The subcortical and cortical structures that have been shown to be activated in response to visceral pain. PAG: Periaqueductal grey; PB: Parabrachial nucleus of the dorsolateral pons; VMpo: Ventromedial part of the posterior thalamic nuclear complex; MDvc: Ventrocaudal part of the medial thalamic dorsal nucleus; VPL: Ventroposterior lateral thalamic nucleus; ACC: Anterior cingulate cortex; PCC: Posterior cingulate cortex; HT: Hypothalamus; S1, S2: First and second somatosensory cortical areas, respectively; PPC: Posterior parietal complex; SMA: Supplementary motor area; AMYG: Amygdala; PF: Prefrontal cortex; M1: Motor cortex. (Adapted from Price DD. Psychological and neural mechanisms of the affective dimension of pain. *Science* 2000; 288: 1769-1772).

1950's Rexed divided the spinal grey matter into a system of ten laminae (LI-LX) which in turn divides the grey matter into four regions: the dorsal horn (LI-VI), the intermediate zone (LVII), the ventral horn (LVIII and IX) and the region of the central canal (LX)[18]. Second order neurones in the afferent pathway have a cell body in the dorsal horn of the spinal cord and relay signals to the brain *via* a number of ascending tracts.

The central pathways for processing nociceptive information begin at the level of the spinal cord dorsal horn. Spinal afferent projections terminate in distinct laminae of the spinal cord dorsal horn (mainly I and V, and occasionally to the contralateral laminae V and X) where they are organised in a segmental manner, but distributed over several spinal segments[19]. This diffuse termination pattern may explain the poor localisation of visceral sensation often seen in clinical practice, whereas the convergence of visceral and spinal afferents in the spinal dorsal horn may explain the phenomenon of viscerosomatic convergence, whereby visceral pain is often referred to nearby somatic structures[20,21].

ASCENDING SPINAL PATHWAYS

The ascending spinal tracts that convey sensory information to supraspinal structures are contained within the anterior lateral and posterior tract systems. The anterior lateral system comprises the spinothalamic, spinoreticular, spinomesencephalic, and spino-limbic tracts, illustrated in Figure 1. The medial and lateral subdivisions of the spinothalamic tract project to the medial/intralaminar and ventral/ventral posterior lateral (VPL) nuclei of the thalamus. respectively[22]. Third-order thalamocortical fibres then project to the somatosensory, insula and medial prefrontal cortices[23]. The spinothalamic tracts mediate sensations of pain, cold, warmth and touch and are also important for sensory discrimination and localisation of visceral and somatic stimuli[24,25].

The spinoreticular tract conducts sensory information from the spinal cord to the reticular formation in the brainstem. The reticular formation is mainly involved in the reflexive, affective and motivational properties of such stimulation[26]. Third-order reticulothalamic tract neurons project from the dorsal and caudal medullary reticular formation to the medial and intralaminar nuclei of the thalamus. From the intralaminar nuclei, ascending pain signals spread bilaterally to the prefrontal cortex (PFC), including the anterior cingulate cortex (ACC)[25]. The spinomesencephalic tract ascends the spinal cord with fibres to various regions in the brain stem, including the periaqueductal grey (PAG), locus coeruleus (LC), and dorsal reticular nucleus in the medulla[25].

The spinolimbic tracts project to areas such as the amygdala, medial thalamus, hypothalamus and other limbic structures, and are also believed to be important in mediating the motivational aspects of pain[25]. See Figure 2.

The posterior system comprises three synapsing tracts: first order dorsal column neurones, the post-synaptic dorsal column (PSDC) pathway and the spinocervical tract. These pathways were not believed to convey nociceptive information; however, recent studies have highlighted the importance of the dorsal column in viscerosensory processing. Al-Chaer demonstrated in primates that the responsiveness of neurones in the ventral posterior lateral nucleus of the thalamus to colorectal distension could be significantly attenuated by dorsal column lesions[27]. Lesions of other tracts had no consistent effects, thus, supporting the role of the dorsal column in conveying visceral nociceptive input to the thalamus.

PAIN PROCESSING IN THE BRAIN

Knowledge of how the brain processes sensory information from visceral structures is still in its infancy; however, our understanding has been propelled by technological imaging advances such as functional Magnetic Resonance Imaging (f-MRI), Magnetoencephalography (MEG), Positron Emission Tomography (PET), and EEG. Human studies have non-invasively demonstrated the complexity involved in pain processing, and highlighted a number of subcortical and cortical regions involved.

The pathways involved in the perception of visceral pain are highly complex. In addition, these pathways are dynamic and amenable to change in response to internal or external stressors. Numerous mechanisms can be engaged in response to stressors from the primary afferent level right up to the cerebral cortices, resulting in a high degree of plasticity in the nervous system. The ultimate outcome of pain perception is brought about by a delicate balance between facilitatory and inhibitory mechanisms. As pain is a conscious feeling, the ultimate goal in pain-imaging is to follow the pain stimulus throughout the neuraxis.

Imaging studies have been performed to explore normal brain processes involved in visceral perception, whether liminal or subliminal and its modulation by attention, conditioning and emotion[22,28-31]. Several studies have also looked at the role of visceral perception in emotions and cognitive processes such as learning[32,33].

Visceral pain has been contrasted with pain arising from superficial skin structures[34,35]. Recent reviews have summarized imaging findings in normal GI sensation[36-38].

Recently, a number of new technologies have emerged within imaging of the brain-gut axis, and in this review we focus on the EEG techniques where signal analyses have made it possible to follow the early and pain specific pathways to the brain with high temporal and spatial resolution.

IMAGING TECHNIQUES

Most commonly f-MRI is based on a technique using different paramagnetic properties of oxy- and deoxyhaemoglobin in the blood. These regional changes in blood flow, volume and oxygenation of haemoglobin derive from changes in neuronal activity and, thus, regions of activation may be identified by subtracting regional cerebral blood flow during a control condition from blood flow during a stimulus condition or by correlating regional blood flow with the intensity or time course of a stimulus or its perception[2]. A major advantage of f-MRI is that it is non-invasive and non-cumulative, allowing subjects to be studied repetitively. f-MRI has an excellent spatial resolution (2-5 mm), especially in the more superficial layers. Limitations are seen in the deeper structures, such as the brainstem and thalamus, due to pulsation artefacts. The temporal resolution is poor (1-3 s) and therefore f-MRI is not a specific tool for investigating the neuronal activity directly related to the painful stimuli. Since the exogenous brain activity takes place within the first 150 ms post stimulus, the response may miss the fast occurring activity and model, instead, the endogenous activity rather than brain responses due to pain. In contrast to PET studies a limitation in f-MRI studies is the lack of information regarding neurotransmitters or involved receptors[39]. A comparison between localization of visceral and somatic regions of the oesophagus in healthy subjects using fMRI has been done[40]. Distension of the distal oesophagus was represented bilaterally at the junction of SI and SII. Different activation patterns were also observed in the ACC, prefrontal cortex and cerebellum. Another recent study was carried out to determine whether behavioural differences are due to differences in the central processing of visceral and somatic pain[30]. It was demonstrated that visceral stimuli induced deactivation of the perigenual cingulate bilaterally with a relatively greater activation of the right anterior insula i.e. regions encoding affect. Kwan *et al*[41] used f-MRI as a diagnostic tool for demonstrating abnormal brain processing in Irritable Bowel Syndrome (IBS). They identified abnormal event-related sensations in five brain regions following rectal distensions. In the primary sensory cortex, there were urge-related responses in the IBS, but not the control group. In the medial thalamus and hippocampus, there were pain-related responses in the IBS, but not the control group. However, pronounced urge- and pain-related activations were present in the right anterior insula and the right anterior cingulate cortex in the control group, but not the IBS group. These findings conflict with the findings of Bonaz *et al*[42], who demonstrated significant deactivations within the right insula, the right amygdala, and the right striatum following rectal stimulations in patients suffering from IBS compared to healthy subjects.

PET

PET measures the cerebral blood flow after injection of a radioisotope. The most commonly used in gastrointestinal research is $H_2{}^{15}O$ labelled water. PET has excellent spatial resolution (2-5 mm) and allows the operator to tag important biological molecules that bind to targeted receptor groups or glucose metabolism in active neuronal tissue. PET is superior in imaging radiopharmaceuticals and/or other ligands as it offers the ability to study receptor distribution and explore the site of action[2]. However, the temporal resolution is poor (minutes), and as the subject receives a considerable dose of radiation, group analyses are needed for meaningful results, interpreting endogenous brain activity following pain rather than exogenous brain activity following painful stimulation. Another major disadvantage is the expense of a PET scanner.

Silverman *et al*[43] characterized the cerebral processing of visceral noxious events, by measuring the changes in regional cerebral blood flow. Healthy controls demonstrated a significant increase in anterior cingulate cortex activity following noxious stimuli, whereas no activity was seen in response to non-painful stimuli. In patients suffering from IBS, the ACC failed to respond to the same stimuli, whereas significant activation of the left prefrontal cortex was seen. In contrast, another study

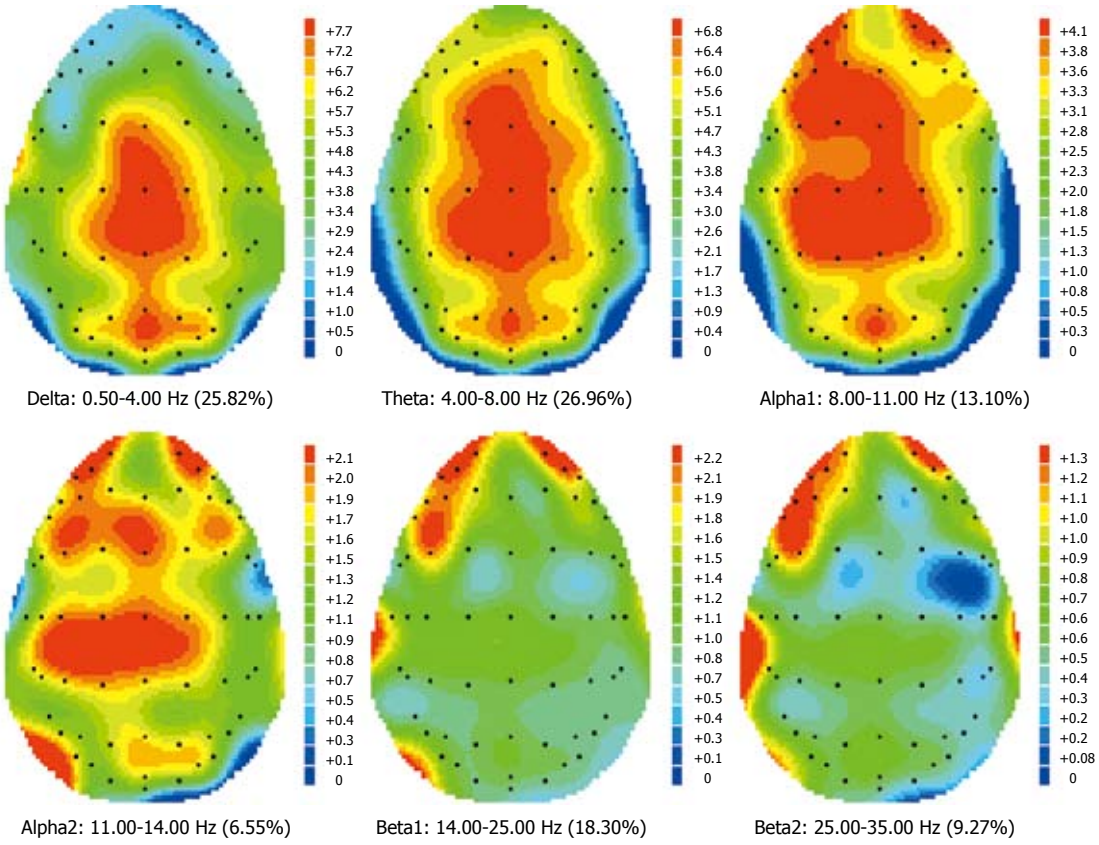

Figure 3 Example from painful CEP from the gut performed in a healthy volunteer. The figure shows the topographies at different frequency bands from one subject, and the percentage of the presence of each frequency band in the overall signal. The black dots represent electrodes. The colours represent how much power a particular frequency band holds at each electrode. The scales describing the colours are to the right of the topographies.

compared healthy controls and patients suffering from IBS, and found no group differences in anterior insula and dorsal anterior cingulate cortex (dACC) activity, two regions consistently activated by painful intestinal stimuli[44]. However, IBS patients showed greater activation of the amygdala, rostroventral ACC, and dorsomedial frontal cortical regions.

MEG

MEG is a non-invasive brain imaging tool, which allows detection of cortical neuromagnetic activity as opposed to metabolic changes, which are secondary. The spatial resolution is comparable to f-MRI and PET; however, MEG also has millisecond temporal resolution, and is suitable for both individual and group studies. MEG is not widely available; systems are only present in specialist centres. The technical limitation of MEG is that it is less able to resolve the radial current, and is not sensitive to deep sources; but it is especially sensitive to the tangential activity in the cortex.

EEG

EEG measures direct electrical brain activity, through non-invasive scalp electrodes. This electrophysiological tool is widely used. EEG can be used to investigate the activity in both health and disease, as it is non-invasive and completely harmless. While f-MRI and PET brain imaging techniques have excellent spatial resolution, their time resolution is poor. Thus, these methods do not directly show brain activity in time. The EEG signal is divided into five frequency bands: Delta: < 4 Hz, Theta: 4-8 Hz, Alpha 8-12 Hz, Beta: 13-30 Hz, and Gamma: greater than 30 Hz. Figure 3 shows an example of a presentation of different frequency bands present in a painful cortical evoked potential (CEP) in the oesophagus. Analyses like this can be used to compare frequency alterations and topographical appearance between different subject groups. Drewes *et al*[45] found significant differences in theta and delta bands in CEPs between healthy controls and patients with chronic pancreatitis (CP) following painful stimulation in the gut. The patients showed higher activity in the theta band and the main theta band components oscillated by 4.4 Hz in patients and by 5.5 Hz in controls. Furthermore, the energy in the delta band was higher in the controls, whereas patients only showed scattered delta activity.

EEG recordings can be used for CEP, which detect brain activity in real time, with temporal resolution on the millisecond scale. CEP is an electrical response in the brainstem or cerebral cortex following a stimulus, i.e. painful stimulation in the gut. CEP amplitudes are typically lower than the amplitudes of spontaneous EEG (less than a microvolt to several microvolts, compared to tens of microvolts for EEG); but, since the CEPs are time-locked to the stimulus and the background activity occurs randomly, the CEP amplitudes become higher during the averaging process, and most of the background noise cancels out. In order to extract the CEPs with a

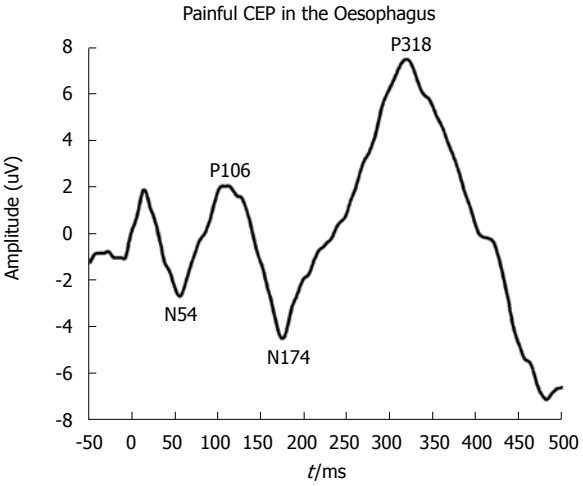

Figure 4 CEP at Cz electrode from a healthy volunteer. The subject was electrically stimulated in the oesophagus through a 6-mm nasal endoscope. Electrical stimulation was at the pain threshold and this CEP is an average of 35 such stimulations.

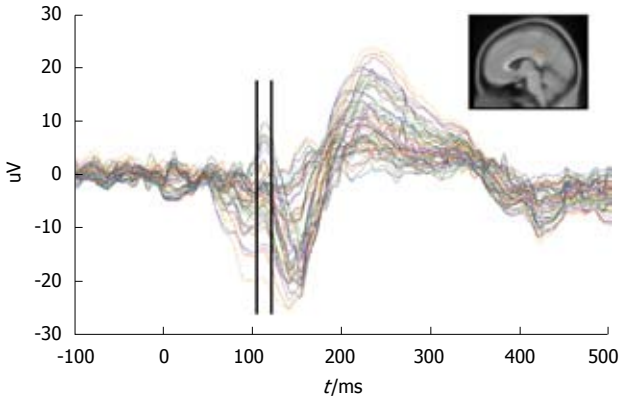

Figure 5 This is a butterfly plot of 62 channels (data of all 62 channels superimposed on each other). The vertical lines mark the time course of the peak that was used for analysis. The red dot on the MRI image on the top right corner represents where the activity was calculated to be by MUSIC.

good signal-to-noise ratio, a number of stimulations are presented at a certain frequency, and these stimulation trials are then cleaned for artefacts and averaged. An example of an averaged painful CEP from electrical stimulation of the gut is shown in Figure 4. Each peak in the CEP represents a synaptic event associated with the synchronous transmission of afferent information from one group of neurons to another. Several studies have examined the amplitudes and latencies of painful CEPs in the gut, and compared the results between a control group, and a study group (i.e. patients suffering from chronic pancreatitis, non-cardiac chest pain or patients treated with analgesics)[46-51]. Dimcevski *et al*[46] showed decreased early CEP latencies in patients with CP compared to healthy controls. Sami *et al*[48] showed decreased latencies in the first two positive peaks (P1 and P2) of CEPs following painful stimulation in the oesophagus after acid perfusion. Rossel *at al*[47] found that P1 had a shorter latency and smaller amplitude in patients with IBS compared to healthy controls. Furthermore, the group showed that the controls had a mid-latency positive component after 100 ms, which was absent in the patient group, and the healthy controls had a single late positive component (> 150 ms) whereas the IBS group had a late component which was biphasic. The demonstrated changes in latencies and frequencies most likely explain neuronal changes, such as plasticity, in the CNS.

INVERSE MODELLING OF CORTICAL EVOKED POTENTIALS

EEG is a mixture of signals from all over the brain due to the current generated by groups of neurons not only being produced at the source location, but also flowing to the surrounding tissue *via* volume conduction. Thus, by the time the signal arrives at the scalp electrodes it is distorted. Therefore, while CEPs have excellent time resolution on the millisecond scale, the spatial resolution is limited, and it is impossible to predict which sources in the brain are generating these potentials. However, methods using advanced mathematics and signal analysis to address these problems exist. This is known as "inverse modelling." Inverse modelling is based on the idea that groups of neurons generating the potentials at the scalp can be modelled by equivalent current dipoles. From multiple-channel recordings of CEPs, it is possible to mathematically calculate the locations of these dipoles. In order to do this, freeware and commercial software such as [EEGLAB, BrainStorm, Statistical Parametric Mapping (SPM), BESA, ASA and CURRY] are available. Some studies have performed inverse modelling on CEPs following painful stimulation in the gut. Dimcevski *at al*[46] found that dipolar activities corresponding to the early CEPs were located consistently in the bilateral insula, in the anterior cingulate gyrus, and in the bilateral secondary somatosensory area. Furthermore, they showed that in a CP patient group, the bilateral insular dipoles were localized more medial than in the healthy control group. They also showed changes in the cingulate cortex where the neuronal source was more posterior in patients than in controls. Drewes *et al*[52] showed two dipoles in the bilateral insular cortex, one dipole in the anterior cingulate gyrus and two dipoles in the bilateral secondary somatosensory area post the painful stimulus. Moreover, they found the anterior cingulate dipole to have a more posterior position in IBS patients than in healthy controls[53]. Inverse modelling algorithms, such as low-resolution brain electromagnetic tomography (LORETA) and multiple signal classification (MUSIC) have usually been applied to instantaneous CEP data by selecting a certain time frame in the data and calculating the location of dipole(s) generating the CEP at this time, see Figure 5.

Different inverse modelling algorithms and the ideas behind them are discussed in detail elsewhere[54-57]. The disadvantage of performing inverse modelling on instantaneous CEPs is the instability of algorithms when multiple sources are active and the interference of background electrical and physiological noise. For this reason, different signal decomposition methods have been used in order to separate the signal into a sum of

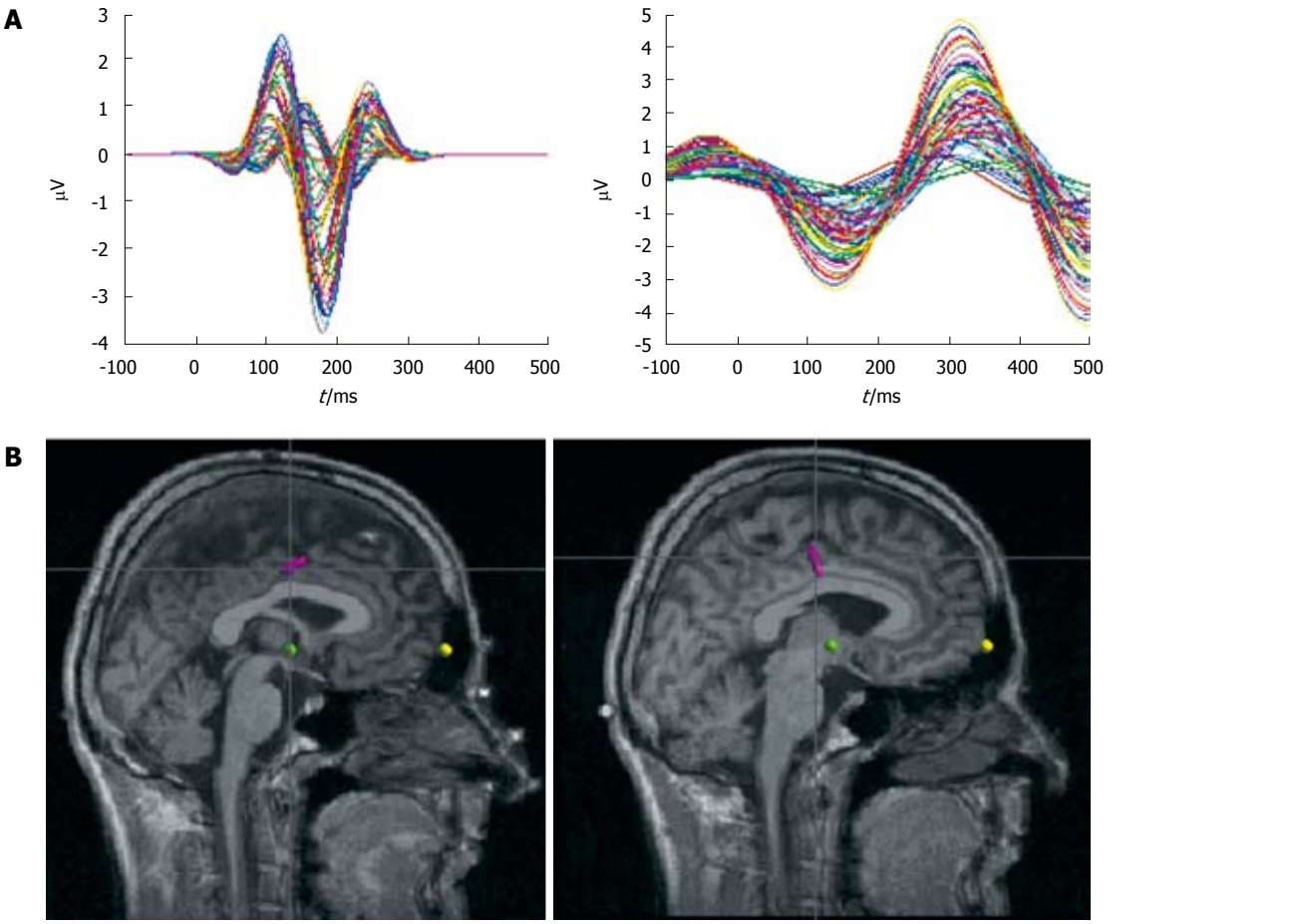

Figure 6 An example of two MMP atoms from painful CEPs in the oesophagus. A: Butterfly plots of the atoms; B: Dipole location of each atom.

waveforms, each having a single dipole generator. These methods make it possible to differentiate signals corresponding to brain activity from those corresponding to noise and artifacts. Once the signals are decomposed, inverse modelling can be completed on each waveform, and furthermore, it is possible to observe at which time and frequency this particular dipole is active, as shown in Figure 6.

Recently, Multichannel Matching Pursuit (MMP) was introduced, which decomposes the data into a sum of waveforms (usually termed atoms), each of them defined in time, frequency and space. We showed that decomposing the CEPs using MMP prior to inverse modelling (namely MUSIC) is superior to some blind source separation (BSS) methods, namely Independent Component Analysis (ICA) and Second-Order Blind Identification (SOBI), which are typically used for CEP signal decomposition prior to source analysis. These decomposition methods are described in detail elsewhere[58-66]. Additionally, we showed that MMP prior to MUSIC was much more accurate than MUSIC on the instantaneous data on both simulated and empirical CEPs[67]. MUSIC on MMP atoms was able to localize deep, superficial, and simultaneously active dipoles with high accuracy. The spatial resolution for MUSIC on MMP atoms was 3-20 mm compared to MUSIC on ICA components (5-27 mm for superficial dipoles, deep dipoles failed to localize), MUSIC on SOBI components (5-32 mm, deep dipoles failed to localize), and MUSIC on raw data (7-81 mm, simultaneously active dipoles typically did not localize correctly). Comparisons between different inverse modelling methods have been carried out in other studies[54,55]. We chose MUSIC because it has demonstrated an advanced ability to localize a restricted number of independent sources, and has the ability to reliably replicate temporal waveforms[57]. Furthermore, it is possible to combine an individual's MRI scan with the digitized locations of electrodes on their scalp in order to create a realistic head model, and use this head model to find the inverse solution for the individual's CEPs. These combinations of non-invasive methods allow us to study the sequence of cortical activations due to pain. Although combination of MMP, inverse modelling, and individual MRIs allows us to find new information regarding pain processing in the brain, one shortcoming of MMP is the lack of order in the atoms. This makes it difficult to compare between groups; hence, to distinguish which atoms from one subject correspond to the atoms of another subject and which atoms in one group are different/similar to the atoms in another group. For this reason, clustering of atoms/dipoles can be done. Delorme *et al*[58] have implemented such a method for clustering of ICA components and incorporated it into their EEGLAB toolbox. Currently, we are developing a toolbox to cluster MMP atoms based on time/frequency, topography, both time/frequency and topography, or dipoles. Furthermore, it is

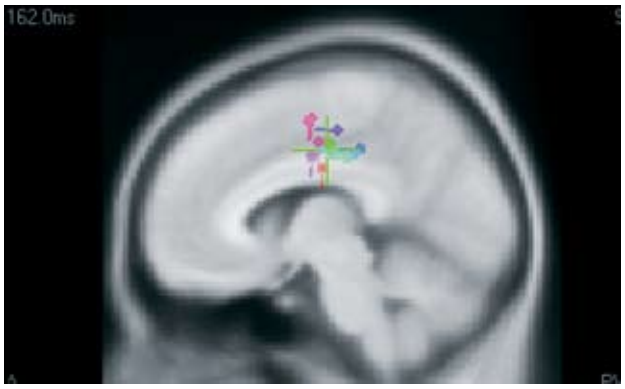

Figure 7 One of the clusters of cingulate dipoles generating CEPs following a painful stimulation in the gut in 10 subjects.

possible to take the Talairach coordinates of each dipole in individual clusters, and look up the anatomical location of the source in a Talairach atlas. For an example of clusters, see Figure 7. These dipoles were localized in the cingulate gyrus. In the future, we are aiming at clustering the dipoles after source localization has been performed using realistic head models for each individual (i.e. combining their individual MRI scans and digitized EEG electrode locations). These methods will allow for more precise source localization, automated separation of dominant sources during painful CEPs in different groups, and allow the study of the sequential order of activated centres post stimulus. These advancements will provide new insight into how different subject groups process pain.

THE BRAIN-GUT AXIS IN FUNCTIONAL GASTROINTESTINAL DISEASES

The ROME II multinational consensus has defined functional gastrointestinal disorders (FGID) as "a variable combination of chronic or recurrent gastrointestinal symptoms which are not explained by structural or biochemical abnormalities"[68]. Despite remarkable advances in our understanding and management of "organic" gastroenterological complaints such as peptic ulcer disease, IBS and even cancer over the last 30 years, our understanding of the mechanisms of pain in FGID patients remains far from complete. The lack of effective treatments for these disorders leads to chronic symptoms, recurrent attendances in hospital, poor patient satisfaction and significant morbidity. Health care costs are estimated to be around $34 billion in the 7 largest Western economies[69,70].

Although patients with FGID show marked heterogeneity in their clinical presentation and response to treatment, common features have become apparent as our knowledge of these disorders has increased. These patients often display a heightened sensitivity to experimental gut stimulation, termed visceral pain hypersensitivity (VPH) which is believed to be important in their pathophysiology and symptom generation. The hypersensitivity may be caused by peripheral and central factors relating to primary afferents as well as the autonomic and enteric nervous systems; however, in this review we will focus on the changes in the CNS which can be elucidated using the new imaging techniques described above.

Mayer *et al*[71] studied the perceptual responses to rectosigmoid distension in IBS patients and controls with functional brain imaging using $H_2{}^{15}O$ PET and found that following a train of repetitive sigmoid distensions, control subjects demonstrated greater activation of the PAG and thalamic regions compared to patients. This effect was seen both during actual rectal distension and during expectation of the stimulus, despite its absence. As has been outlined, the PAG is an important structure involved in the modulation of spinal pain processing, and the above finding suggests that a proportion of IBS patients have inadequate activation of brain regions involved with antinociception. Mayer *et al*[38] have recently reviewed imaging studies in FGID which has been critiqued by Hobson and Aziz[36,37,72].

"Visceral hypersensitivity" is a hallmark feature in IBS patients, who show an abnormal pattern of ACC activation during pain perception which is an interesting parallel to ACC activation relative to increasing pain perception in healthy subjects[43,73,74]; hemispheric preference, as well as cognitive style of information processing served as indicators of covert changes in brain functions in 21 adult IBS patients[75]; and abnormal cerebral processing of oesophageal stimuli was found in patients with noncardiac chest pain[50,51]. Drossman *et al*[76] found that alterations in brain activity were associated with resolution of emotional distress and pain in a case of severe IBS.

A recent longitudinal study in IBS found that there were significant decreases in amygdala, dACC and dorsal brainstem activation over a 12-mo period during anticipation for pain although pain-related activations and symptoms were stable[77]. Rectal pain induced significant activation of the perigenual ACC, right insula and right prefrontal cortex. Amitriptyline was associated with reduced pain-related cerebral activations in the perigenual ACC and the left posterior parietal cortex, but only during stress[78]. Taken together these findings strongly suggest that abnormalities in the brain-gut axis play a key role in our understanding of FGID, and future studies using the techniques described above will undoubtedly increase our understanding of these disorders.

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GUIDELINES CLINICAL PRACTICE

Asbjørn Mohr Drewes, Professor, MD, PhD, DMSc, *Series Editor*

New techniques in the tissue diagnosis of gastrointestinal neuromuscular diseases

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Abstract

Gastrointestinal neuromuscular diseases are a clinically heterogeneous group of disorders of children and adults in which symptoms are presumed or proven to arise as a result of neuromuscular (including interstitial cell of Cajal) dysfunction. Common to most of these diseases are symptoms of impaired motor activity which manifest as slowed or obstructed transit with or without evidence of transient or persistent radiological visceral dilatation. A variety of histopathological techniques and allied investigations are being increasingly applied to tissue biopsies from such patients. This review outlines some of the more recent advances in this field, particularly in the most contentious area of small bowel disease manifesting as intestinal pseudo-obstruction.

Key words: Enteric myopathy; Enteric neuropathy; Interstitial cells of Cajal; Intestinal pseudo-obstruction; Visceral pain

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INTRODUCTION

The term gastrointestinal neuromuscular diseases (GINMD) describes a clinically heterogeneous group of disorders of children and adults in which symptoms are presumed or proven to arise as a result of neuromuscular (including interstitial cell of Cajal) dysfunction[1,2]. Common to most of these diseases are symptoms of impaired motor activity which manifest as slowed or obstructed transit[3] with or without evidence of transient or persistent radiological visceral dilatation. Such diagnoses include primary and secondary disorders of the oesophagus to the colon e.g. achalasia, gastroparesis, intestinal pseudo-obstruction and severe constipation. Pathologic abnormalities of the sensorimotor apparatus have been demonstrated in such disorders by a variety of methods since the 1960s; however, this remains an area of evolving interest especially with the increasing availability of newer techniques and more critical appraisal of those more established techniques.

This review outlines some of the more recent advances in this field, particularly in the area of small bowel disease manifesting as intestinal pseudo-obstruction. The area of Hirschsprung disease diagnosis, although numerically important (this being by far the most common GINMD) is not covered here since, although some contention exists, in general the techniques for this diagnosis are long and better established. The review covers the safe acquisition of tissue and advances in histopathological and allied techniques.

SAFE TISSUE ACQUISITION

Tissue may be taken with deliberate diagnostic intent or alternatively come as the by-product of emergency or planned surgical interventions. On this basis, tissues may take the form of mucosal, deep submucosal, seromuscular or full-thickness biopsies or resection

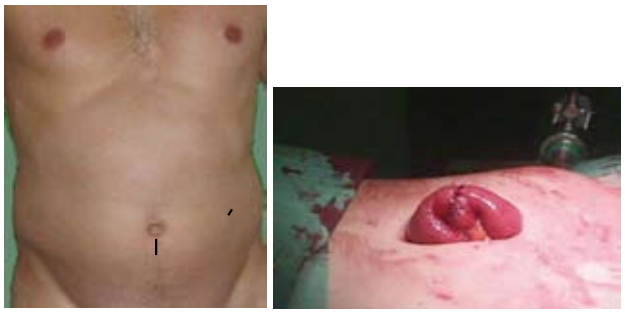

Figure 1 Laparoscopically-assisted full thickness jejunal biopsy. The port sites are shown. After finding a suitable proximal jejunal loop, the bowel is exteriorised by extending slightly the umbilical port incision and biopsy and suture closure performed extracorporeally (Courtesy of B Nyborg, Huddinge, Stockholm).

specimens. Of particular note are recent advances in minimally invasive surgery that have permitted safe access and biopsy of a variety of intra-abdominal tissues including full-thickness bowel biopsy[4]. In the context of GINMD, with some variations, the technique has now been applied to children with colonic dysmotility[5,6] and adults with small bowel dysmotility, predominantly those with proven chronic idiopathic intestinal pseudo-obstruction (CIPO)[7-10]. A very recent study reported on the safety and diagnostic yield of a predominantly laparoscopically-assisted approach (Figure 1) to biopsy the small and large bowel in a cohort of 124 adults with suspected GINMD from 3 European centres. Median operating time was 50 min, conversion rate was 2% and length of stay was 1 d. There was an 8% readmission rate for obstructive symptoms; however, other morbidity was minimal and there were no mortalities. Overall the specific diagnostic yield was 81%, being high for jejunal biopsies (89%), but low for a small number of ileal and colonic biopsies[10]. On this basis, an extracorporeal laparoscopically-assisted procedure appears safe and with acceptable yield if performed in the proximal small bowel for the indications in this study. Completely intracorporeal staple techniques may also be safe, but very little published data exists, at least for the jejunum[10]. Laparoscopic gastric biopsies may also now be taken at the time of gastric pacing[11], and may be important in predicting outcome from this procedure on the basis of ICC pathology (personal communication: Gianrico Farrugia).

The potential to increase yield with multiple biopsies must be balanced against the risk of complications. Clearly, whilst there is some evidence from colectomy and post-mortem small bowel that sections should be taken at fixed intervals to avoid missing 'patchy' abnormalities of muscle or nerve[12], extending this finding to suggest multiple biopsies, even with a small risk for each is not currently advised. On this basis, as well as the potentially increased risks of leakage, laparoscopic full-thickness colonic biopsy is currently not advised, although seromuscular biopsies have been shown to be safe in a large series of paediatric patients[6] and can also be used for determining the HSCR transition zone. The role of appendectomy as a diagnostic surrogate of GINMD has recently been suggested based on preliminary findings in diabetes[13], but needs further exploration[14]. The evolving technique of NOTES (natural orifice transluminal endoscopic surgery) will in the future (in the author's view) have an important role here, with proof of concept already demonstrated in the stomach[15]. Regardless of technique, because of regional differences, whenever full-thickness biopsies are taken, the corresponding intestinal segment(s) should be precisely indicated to the pathologist.

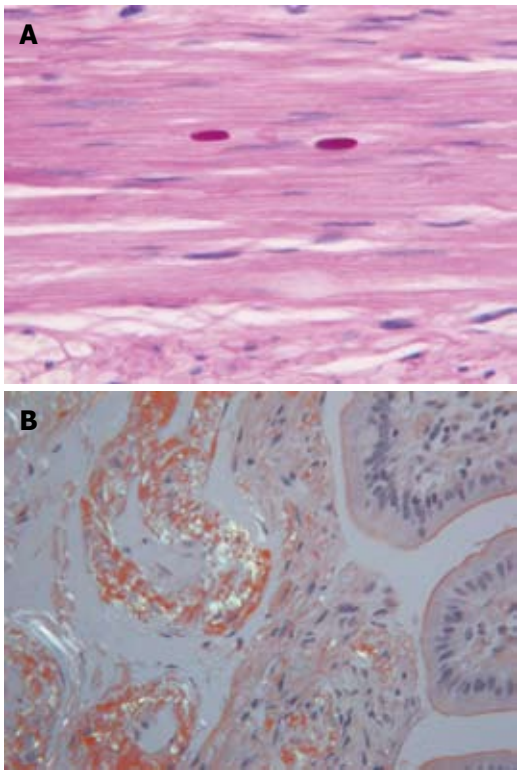

Figure 2 Tinctorial stains used in GI neuromuscular histopathology. A: Periodic acid Schiff staining showing polyglucosan bodies in a patient with intestinal pseudo-obstruction; B: Bifringence from amyloid visualised by Congo red staining (x 25-40).

HISTOLOGICAL TECHNIQUES

Although the histopathological diagnosis of GINMD (and exclusion of other disease) continues to be primarily based upon the analysis of H&E-stained sections with light microscopy, a number of other techniques can also be employed. A critical appraisal of the role of these techniques, particularly in comparison with the 'yield' of H&E, and guidelines for their use is currently being produced by an international working party: www.gastro2009.org/pdf/wp_project_descr07.pdf and is not covered here. Rather, descriptions of some newer diagnostic techniques are presented.

Tinctorial stains (Figure 2)

Although there is vast variation in current practice, tinctorial stains can supplement H&E with particular use in the assessment of specific structures and

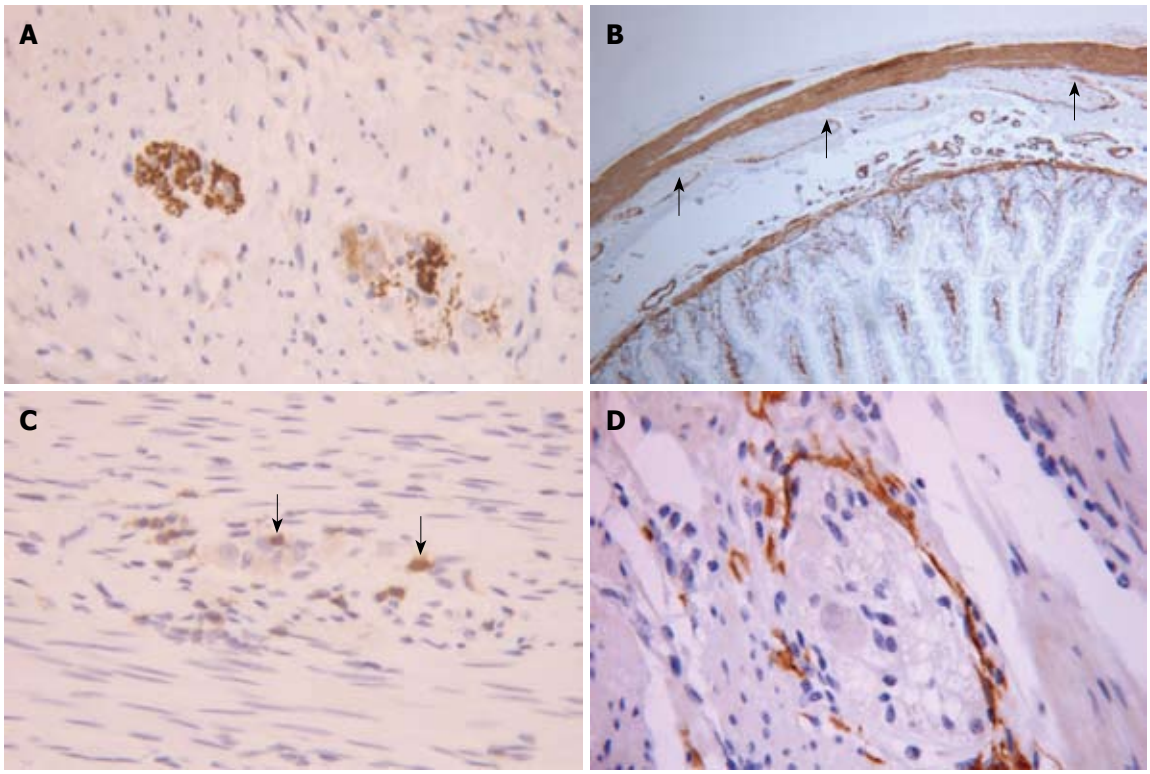

Figure 3 Immunohistochemistry using antibodies to. A: Neuron specific enolase allowing clear visualisation of myenteric ganglia, neuronal number and size; B: Smooth muscle alpha actin showing absent staining in the circular muscle layer of the jejunum (arrows) in a patient with enteric dysmotility; C: CD3 showing small numbers of periganglionic T lymphocytes (arrows) in numbers that most would deem abnormal and indicative of ganglionitis; D: CD117 staining showing normal myenteric plexus interstitial cells of Cajal (ICC-MP). (Original magnification x 40-100).

cell types. With periodic acid Schiff (PAS) staining, inclusion bodies e.g. polyglucosan, lipofuscin granules (secondary autophagic lysosomes), and glycogen can be observed, and PAS combined with diastase treatment can differentiate between glycogen and other structures (glycogen disappears after diastase pretreatment), which may be of value where a glycogenosis or related metabolic disorder are suspected. Polyglucosan inclusion body myopathy has recently been described in GINMD[16] and cannot easily be identified without use of PAS staining. Amyloid is a rare secondary cause of GINMD and can be detected with ease using Congo red staining. With Giemsa staining, mast cells and eosinophils can be seen easily, and the condition of the neuronal cytoplasm assessed (marginalization of the Nissl and chromatolysis). Various types of trichrome staining assist in the establishment of fibrosis and in differentiation from interstitial oedema (both cause increases in the distance between cells, and in early fibrosis this can be difficult to differentiate). Relevant to some rare cases of GINMD, Gomori trichrome staining is also used to diagnose mitochondrial neurogastro-intestinal encephalomyopathy (MNGIE) on the basis of finding 'ragged red fibres' on skeletal muscle biopsy[17].

Immunohistochemistry (IHC) (Figure 3)

The past thirty years has seen the use of IHC evolve in many areas of GI practice including that of GINMD diagnosis. With respect to mainly diagnostic rather than research applications, neuronal markers such as PGP9.5 and neuron specific enolase (NSE) may be employed to assist in the determination of neurons particularly if quantitation is considered important. This latter point is very contentious because heterogeneity of methods has meant that few normative data exist for any single method, especially when age and regional specificity are considered[18]. Nevertheless, diagnoses reliant on decreases in numbers of neurons and ganglia[19] have complemented findings made previously using the more laborious technique of silver staining[20]. Alpha smooth muscle actin deficiency has been demonstrated by IHC in some children[21] and adults[9] with GINMD, and stresses the importance also of regional specificity-this being a normal variant in the ileum[9]. Inflammatory neuropathies[8,10,22] and much less commonly leiomyopathies[23] may be best diagnosed by immunocyte IHC when large infiltrates (visible on H&E) are not apparent. This finding may prompt further autoimmune investigation (below) and dictate important changes in therapy[22-24]. Finally, c-kit (CD117) IHC has now become established in detecting changes in ICC numbers that certainly accompany, and may be causative of some GINMD[25].

Research applications of IHC have predominantly addressed disease mechanisms and pathways. In GINMD, many studies have attested to alterations in neurochemically-stained subsets of neurons allied to their differing functions. Changes said to underlie abnormal neuronal development[26], retarded colonic transit e.g. reduced substance-P[5], failure of sphincter

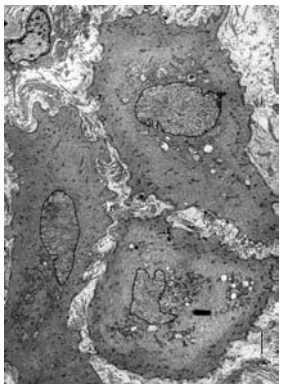

Figure 4 Electron micrograph of smooth muscle cells showing increased Golgi indicative of transition to a more secretory phenotype in a patient with enteric myopathy and pseudo-obstruction. (x 50 000).

relaxation e.g. decreased nitric oxide[27], or visceral hyperalgesia e.g. increased transient receptor potential channels[28] have been variously reported. Beyond mechanism and target identification, whether such changes may become clinical biomarkers of disease or guide treatment is the subject of much ongoing study, particularly if identifiable on endoscopic mucosal biopsy[29].

Electron microscopy (Figure 4)

Ultrastructural examination of neurons, muscle and interstitial cells of Cajal can be a useful adjunct to the above assessments in certain patients. These include some rare childhood myopathies where H&E findings are absent or equivocal (e.g. subtle fibrosis, atrophy of myocytes or myocyte vacuolation)[30], the identification of rare inclusion bodies[31] suggestive of mitochondrial disorders and some ultrastructural changes of ICC[32] and myocytes, including a transformation to more secretory phenotypes.

ADJUNCTIVE INVESTIGATIONS

Proteomic investigations

In cases characterized by clinical (adult onset, personal or family history of autoimmunity) and histopathological findings (especially inflammatory neuro or myopathies) an autoimmune pathogenesis may be suggested. A variety of antibodies directed to nuclear proteins[33,34] and, to a lesser extent, membrane-bound receptors[35,36] of the enteric neuromuscular compartment have been found in patients with secondary GINMP, especially of paraneoplastic origin. The presence of some of these autoantibodies in patients with idiopathic disorders affecting gut motility[22] has prompted their attempted identification in several recent studies[23,35,37,38]. In nearly all cases, proof of pathogenicity remains weak in comparison with established autoimmune diseases of the neuromuscular junction[39]. For a very recent full review, see Kashyap & Farrugia, 2008[40]. If clinically suspected, it is, however, reasonable to take a sample of serum for antibody testing. This should be sent to an established neuroimmunology unit where a variety of methods such as radioimmunoprecipitation assays may be employed[39] (Figure 5). Established antibody tests include those for anti-Hu[34] and anti voltage-gated calcium channels (particularly in paraneoplasia)[41], anti smooth muscle (particularly in myopathies and scleroderma), anti-ganglionic acetylcholine receptor (particularly if associated with dysautonomia)[35,39] and anti voltage-gated potassium channel antibodies[38,39]. One other blood-based investigation occasionally indicated in the investigation of pseudo-obstruction is the thymidine phosphorylase leucocyte activity assay[42] in patients suspected on the basis of clinical findings to have MNGIE[17].

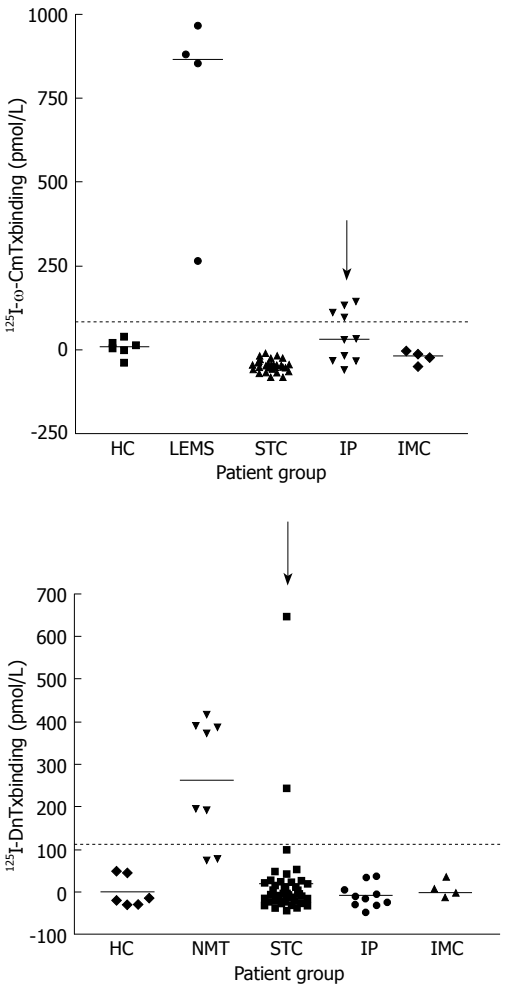

Figure 5 Radioimmunoprecipitation assays of sera from patients with GINMD and negative and positive controls. Assays for antineuronal antibodies directed to anti-voltage-gated calcium (anti-VGCC P/Q-type) and potassium channels (VGKC) are shown. Four IP sera are weakly positive for anti-VGCC P/Q-type antibodies, and 2 STC sera strongly positive for anti-VGKC (arrowed). Dotted line: mean + 3SD; HC: Healthy controls; LEMS: Lambert-Eaton myasthenic syndrome; STC: Slow transit constipation; IP: Intestinal pseudo-obstruction; IMC: Idiopathic megacolon; NMT: Neuromyotonia.

Genomic investigations

Recent history has witnessed a colossal expansion in data regarding the human genome in health and disease. In keeping with this, several studies have demonstrated molecular genetic changes that accompany, and in some instances, contribute to various forms of intestinal dysmotility. Whilst offering interesting research insights, few presently have great value in clinical practice, and these are in the most part limited

to quite characteristic clinical syndromes. Most utilised are candidate single gene approaches, and these have been applied to screening for RET mutations in patients with Hirschsprung disease[43] or suspected multiple endocrine neoplasia (MEN) 2 syndromes[44], and thymidine phosphorylase mutation analysis in patients with MNGIE[42]. A variety of tests may also be appropriate in patients in which GI dysmotility may accompany other systemic diseases such as muscular dystrophy, cystic fibrosis and neurofibromatosis. In most cases, the information delivered is used to guide genetic counselling, and prognosis rather than influence diagnosis (except prenatally) or treatment (except in MEN where prophylactic surgery may be required to prevent subsequent neoplasia[45]).

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GUIDELINES CLINICAL PRACTICE

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Mucosal blood flow measurements using laser Doppler perfusion monitoring

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Abstract

Perfusion of individual tissues is a basic physiological process that is necessary to sustain oxygenation and nutrition at a cellular level. Ischemia, or the insufficiency of perfusion, is a common mechanism for tissue death or degeneration, and at a lower threshold, a mechanism for the generation of sensory signalling including pain. It is of considerable interest to study perfusion of peripheral abdominal tissues in a variety of circumstances. Microvascular disease of the abdominal organs has been implicated in the pathogenesis of a variety of disorders, including peptic ulcer disease, inflammatory bowel disease and chest pain. The basic principle of laser Doppler perfusion monitoring (LDPM) is to analyze changes in the spectrum of light reflected from tissues as a response to a beam of monochromatic laser light emitted. It reflects the total local microcirculatory blood perfusion, including perfusion in capillaries, arterioles, venules and shunts. During the last 20-25 years, numerous studies have been performed in different parts of the gastrointestinal (GI) tract using LDPM. In recent years we have developed a multi-modal catheter device which includes a laser Doppler probe, with the intent primarily to investigate patients suffering from functional chest pain of presumed oesophageal origin. Preliminary studies show the feasibility of incorporating LDPM into such catheters for performing physiological studies in the GI tract. LDPM has emerged as a research and clinical tool in preference to other methods; but, it is important to be aware of its limitations and account for them when reporting results.

Key words: Laser Doppler perfusion monitoring; Gastrointestinal tract; Mucosal blood flow; Perfusion; Chest pain

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INTRODUCTION

Circulation and perfusion of individual tissues is a basic physiological process that is necessary to sustain oxygenation and nutrition at a cellular level. Ischemia, or the insufficiency of perfusion, is a common mechanism for tissue death or degeneration, and at a lower threshold, a mechanism for the generation of sensory signals including pain. Ischemia is a common cause of pain from the myocardium in coronary heart disease, ranging from reversible changes in angina pectoris to acute myocardial infarction with its multitude of complications. Myocardial ischemia is usually due to stenoses of the larger epicardial arteries; but, microvascular changes, such as those commonly seen in patients with diabetes mellitus, can cause ischemia and biochemical changes triggering pain signalling from the tissues. Ischemia can similarly cause pain from abdominal organs, including the intestines, when stenoses of the proximal mesenteric arteries limit perfusion in the distal vascular bed. It is also possible that abnormalities in smaller vessels, and in their regulation of blood flow may

cause similar biochemical changes in the intestinal wall and be an integral part of the pathogenesis of disease[1].

It is of considerable interest to study perfusion of peripheral abdominal tissues in a variety of circumstances. When studying the pathogenesis of various diseases, measurements of perfusion of the tissues affected may be essential to assess the relative contribution of ischemia to disease pathogenesis. Furthermore, in the surgical treatment of disease, assessment of perfusion may be important for assuring that anastomoses are established in well perfused segments of the gut. The beneficial or adverse effects of drug therapy might also be evaluated by monitoring perfusion of a segment of the gut.

MICROCIRCULATION IN THE GASTROINTESTINAL TRACT

Our knowledge of the peculiarities of the vascular bed of the gastrointestinal (GI) tract is still limited. There is considerable inter-individual variation in the anatomy of larger vessels, and the extent of collateral circulation, which may also explain differences in susceptibility to local ischemia. Perfusion is dependent on the arterial supply from the celiac, superior mesenteric and inferior mesenteric arteries. The watershed areas between these major arteries are likely to suffer from ischemia during acute or chronic arterial insufficiency.

The tissue volume occupied by moving blood cells is small; the average density of capillaries is about 50 capillaries per mm^2 of mucosal area, and on average 20% of capillaries are open under resting conditions, perfusion being mainly regulated by the opening and closing of precapillary sphincters. This autoregulation of perfusion is well established in several studies, including studies employing laser Doppler perfusion monitoring (LDPM) and is highly dependent on endothelial cell function[2].

MICROCIRCULATION AND THE SPECTRUM OF GASTROINTESTINAL DISEASE

Microvascular disease of the abdominal organs has been implicated in the pathogenesis of a variety of disorders, including peptic ulcer disease and inflammatory bowel disease (including both ulcerative colitis and Crohn's disease of the intestines). It has been suggested that apart from immunological and bacterial effects on the intestinal wall, changes in the microvasculature are essential for developing such key elements as mononuclear cell infiltration and fibrosis. Typically, the early stages of colitis show increased perfusion, whereas the later chronic stages of this disease show hypoperfusion of the mucosa. This was first shown in various animal models of acute inflammatory bowel disease (IBD), but also convincingly in patients with chronic disease with fibrosis[2]. Importantly, it has been found that the capacity for vasodilatation is decreased in chronic IBD, suggesting a mechanism for ischemia and pain[3]. It has been argued that in patients with Crohn's disease, chronic vascular changes may result in areas of microinfarction in the gut wall, leading to granulomatous inflammation and fibrosis[4].

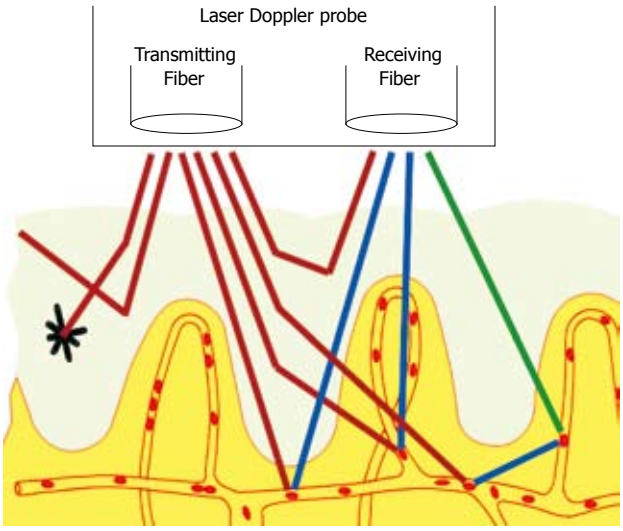

Figure 1 A schematic depiction of laser Doppler perfusion monitoring showing the probe with its emitting fibre bundle which applies monochromatic laser light to the tissue, and its receiving fibre bundle which returns reflected light for analysis. The light that has undergone a doppler shift due to moving blood cells in the tissues reflects the microcirculatory perfusion at a given time. Reproduced by permission of Perimed AB.

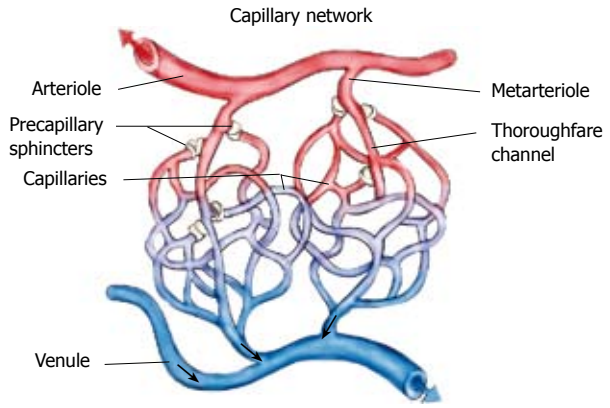

Figure 2 The capillary network showing also precapillary sphincters which regulate perfusion locally in response to metabolic needs, and shunts which participate in thermoregulation.

LASER DOPPLER PERFUSION MONITORING (LDPM)

The basic principle of laser Doppler perfusion monitoring (LDPM; laser Doppler velocimetry, or laser Doppler flowmetry) is to analyse changes in the spectrum of light reflected from living tissues as a response to a beam of monochromatic laser light emitted (Figure 1). LDPM reflects the total local microcirculatory blood perfusion including perfusion in the capillaries (nutritive flow), arterioles (thermoregulatory flow - such as in the skin), venules and shunts (Figure 2).

One of the earliest papers concerning this issue was a report by Stern *et al*[5] in 1975. They performed an ex-

periment to determine the feasibility of the method of coherent light scattering, in their case from a fingertip. They were able to demonstrate rapid microvascular reflexes which no other method was able to demonstrate at that time.

When a beam of light, carried by the fibre-optic probe, enters the tissues and hits moving blood cells in a random order, it undergoes changes in wavelength - a Doppler shift[6] - while the wavelength of light hitting static tissue structures is unchanged. The magnitude and frequency distribution of these changes in wavelength are directly related to the number of moving blood cells, but relatively unrelated to their direction of movement.

The tissue volume occupied by moving blood cells is generally small; the average capillary density is about 50 capillaries per mm^2 mucosal area, and most photons do not undergo a frequency shift, but are backscattered or absorbed[7]. The backscattered and Doppler broadened (extended) light carries information about the speed and concentration of blood cells traversing the scattering volume[8].

The quantity that is measured in LDPM is generally referred to as perfusion, and expressed in Perfusion Units (PU) which are arbitrarily chosen. In general it is not possible to change PU values into blood flow expressed as mL/min per g tissue; but, it can be done in specific preparations when calibration can be done. Perfusion is defined as the product of local velocity and concentration of blood cells[8]. Speed refers only to the magnitude (mm/s) of the velocity vector, and even though the majority (99%) of blood cells in the undisturbed microcirculation are red cells, LDPM does not selectively measure red cells.

Penetration into the tissue explored depends on the wavelength of the emitted light, and is regulated by differences in fibre diameter/separation. Penetration depth is also influenced, to a great extent, by factors such as structure and density of the capillary bed. The measuring depth is often defined as the depth below the tissue to which approximately 2/3 of the surface light penetrates, and returns back to the tissue surface. A typical probe today is designed using a solid-state laser with a wavelength of 780 nm, one transmitting and one receiving fibre and a fibre separation of 0.25 mm. This could lead to a sample depth of about 0.5-1.0 mm, and the sample volume could be estimated to 1 mm^3. This rather shallow measuring depth was the conclusion of several different studies published in the eighties[9-13]; but, other studies executed during the same period suggested that LDPM had a capacity for transmural measuring in the GI tract[14-17]. During the nineties a consensus was reached that LDPM monitors the microcirculation only in the mucosa and the upper submucosa of the GI tract.

Calibration generally has several purposes: to check the stability of the instrument; to establish the linearity of the instrument's response to blood flow; to establish a relationship between different instruments; and to relate the reading of the instrument to true perfusion, if possible. A gold standard for calibration of LDPM does not exist, and because the optical properties and distribution of blood vessels in the tissue are heterogeneous it is not realistic to calibrate the instrument to measure absolute blood flow. Therefore, the manufacturers of these instruments have provided a more simple calibration protocol, based on a two-point calibration, which makes it easy to calibrate the probes in a clinical or experimental situation. The motility standard is an aqueous suspension of polystyrene microspheres in Brownian motion. The method has some major shortcomings[8] regarding its dynamic properties, and the suspension induces Doppler shifts which give rise to a homodyne measurement. However, in living tissues, the opposite situation is seen and a heterodyne spectrum is produced because the majority of photons do not undergo a Doppler shift.

During the early years of LDPM, the method was validated against well established methods for measuring blood flow, such as the electromagnetic method. However, this method clearly measures total blood flow, not just blood flow in the microcirculation[9]. Later validation was performed using alternative methods known to selectively measure perfusion of the mucosal or muscularis layers i.e. local isotope washout[12], radioactive labelled microspheres[11], and H_2-clearance[11,13]. Generally, it is not easy to evaluate these validation studies because the single point laser Doppler probe is measuring from a different and much smaller tissue volume. However, carefully executed experiments performed on preparations of canine stomach and intestinal wall showed excellent linear correlation between the LDPM signal obtained and total blood flow measured by the electromagnetic technique[9,10,14,15,18]. One of these studies was also the first one to show that the gastric mucosa can autoregulate its blood flow, independent of other layers of the wall[10].

LDPM has emerged as a research and clinical tool in the absence of other methods, because it is a continuous, non-invasive and real time method for measuring microvascular blood flow, and it is also sensitive for detecting rapid changes in perfusion in the capillary circulation. LDPM is easily used in the clinical setting; but, to do so, one must be aware of its limitations. It is very important to ensure that the normal action and physiological responses of the microcirculation are not ignored when using this method. To get an optimal result there are both environmental and physical factors to take into consideration. These should be limited or accounted for when doing an investigation, in order to obtain reproducible data. It is also important to realise that it is impossible to say what the exact blood flow for any tissue is, and to remember that the optical properties and microvascular architecture cannot be determined in advance.

Physiological factors to be considered are temperature (thermoregulation has a significant effect on the microcirculation), the position and motion of the probe relative to the tissue surface, anatomical site and mental stress. Food and drugs also have effects on the microcirculation.

Technical limitations such as motion artifacts, multiple sequential Doppler shifts, variations in the specification of instruments from different manufacturers, lack

of exact knowledge of the depth of measurements, the instrument zero and/or biological zero[19] all have to be taken into consideration when analysing an investigation. There are several review articles published in recent literature describing these phenomena in more detail[8,20,21].

The laser Doppler probe is a sensitive motion detector, and many extraneous sources of noise e.g. respiratory movements cause mechanical vibrations in the same frequency range as the laser Doppler shifts produced by moving cells in the tissues (mucosa). Muscle fasciculation, vasomotion, respiration or any tissue movement relative to the laser Doppler probe may add noise to the laser Doppler signal. The GI organs are inherently motile, and motility-induced artefacts always occur during LDPM. One could argue that it is just noise which is recorded from the GI tract; but, Kiel *et al*[10] showed that this is not the case, and that true, perfusion can be measured from the GI tract.

Currently available instruments for LDPM generally also measure and display total backscattered light, of which Doppler shifted light makes up just a small fraction. The unit of measurement of backscattered light is EV, whereas that of Doppler shifted light is mEV. The significance of total backscattered light is that when this is detected as stable; it is an indication of minimal motion artifacts between probe and tissue. One can argue that only when backscatter is stable can we assume that LDPM actually measures perfusion in the adjacent tissues and is not simply dominated by artefacts.

THE APPLICATION OF LASER DOPPLER PERFUSION MONITORING TO STUDY DISEASE PROCESSES IN GASTROINTESTINAL TISSUES

During the last 20-25 years numerous studies have been done in different parts of the GI tract using LDPM. The majority of studies have been done on animals or humans during anaesthesia or surgery. This gives much better control of factors which potentially might influence the measurements. In a fully awake human, it is much more complicated to do LDPM, especially in the upper GI tract. A survey of the literature indicates that research employing LDPM has focussed on a limited number of questions, primarily those evaluating the influence of drugs or surgical procedures on mucosal perfusion, especially in the upper GI tract[22] or cardiovascular system[23], and the influence of septic shock[24], portal hypertensive gastropathy[25,26], or hepatic cirrhosis[27,28]. LDPM has certainly been used in some other clinical settings, but less systematically.

During recent years, we have been working with a multi-modal device (Figure 3) incorporating a laser Doppler probe, developing this device primarily in order to investigate patients suffering from functional chest pain of presumed oesophageal origin[29], an illness which is incompletely understood. Distending a bag in the oesophageal body typically reproduces the painful sensation in such patients but also elicits pain in a subset of healthy subjects[30-33]. The exact mechanism is unknown. We hypothesised that chest pain of presumed oesophageal origin could be due to a mechanical or an ischemic mechanism, leading to excitation of afferent nerves in the oesophageal wall.

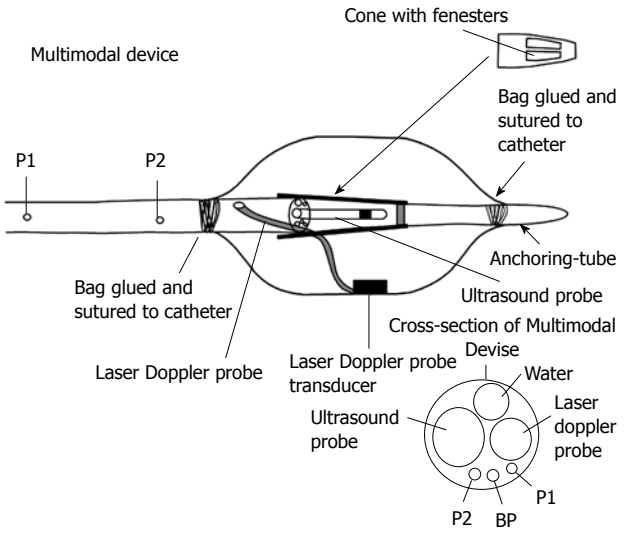

Figure 3 The multi-lumen PVC catheter (Outer Diameter = 6.0 mm) and a distal bag for acoustic coupling and symptom provocation. A water perfused manometric system measures pressures inside the bag (BP) and proximal to the bag at locations P1 and P2. The end of the multi-lumen catheter was attached to a fenestrated cone of polyethylene. The distal end of the cone was attached to a smaller end mounted catheter (anchoring-tube) for distal attachment to the bag. A 20 MHz ultrasound probe was placed in the centre of the bag and the transducer of the laser Doppler probe (780 nm) was fixed with double-sided tape to the inner surface of the bag. Modified from Hoff *et al*[34], 2006.

The multimodal catheter concept in gastroenterology was introduced in 2002 by Drewes and coworkers[32] who integrated technology for inducing electrical, mechanical, cold and heat stimuli into the same catheter device. We have developed the concept and technology further to include real time imaging with ultrasonography and LDPM[34]. As summarized in Figure 3, the device has a specially designed multi-luminal catheter as the central core, and a bag attached at its distal end. Inside the bag, as well as a sensor for measuring bag pressure, there is a radial 20 MHz miniature ultrasound probe (UM-3R, Olympus Corp, Tokyo, Japan) and a laser Doppler probe (LDP-415-253 (Perimed AB, Stockholm, Sweden). Its small size (10 mm × 6 mm × 4.5 mm, fibre diameter 140 μm, separation 250 μm, wavelength 780 nm) has facilitated its inclusion in the device. This is connected to a PF 5001 main unit with a PF 5010 LDPM Unit (Perimed AB).

In tests, high quality signals from manometry, LDPM and endosongraphy were obtained. The LDPM signal decreased moderately during bag distensions. Contractions characterized by high amplitudes and long duration were associated with a decrease in mucosal perfusion; but, minor fluctuations were also observed without contractile activity. During injection of 20 mg butylscopolamine bromide, fewer contractions were recorded and the LDPM signal fluctuated less.

LDPM can be obtained up to a depth of 1 mm with the type of equipment selected for our studies[8]. Hence, presumably at all degrees of bag distension, signals will originate primarily from the mucosa. Animal experiments have demonstrated residual compressive stresses in the mucosa-submucosa and tensile stresses in the muscle layers. This indicates more evenly distributed stress and strain throughout the oesophageal wall as also demonstrated in a study of the multilayered composite oesophagus by Liao and co-workers[35]. It is, therefore, likely that changes in perfusion throughout the wall are also evenly distributed. No current method can provide reliable flow data from the entire human oesophageal wall, and LDPM seems to be the best available choice, particularly for the multimodal device.

This is, to our knowledge, the first time LDPM has been included in a multimodal device for measuring perfusion in the oesophageal wall. Preliminary studies show the feasibility of the method; but, obviously the present material does not allow firm conclusions about whether GI pain is primarily of mechanical or ischemic origin. Future studies to look into ischemic- or strain-dependent pain mechanisms, may need to employ advanced distension protocols such as strain softening protocols.

CONCLUSION

Laser Doppler perfusion monitoring has emerged as a research and clinical tool in preference to other methods because it is non-invasive, and yields continuous and real-time measurements of microvascular blood flow. Furthermore, it is sensitive to rapid changes in perfusion in the capillary circulation. LDPM is easily used in the clinical setting; but, users have to be aware of its limitations and account for them when reporting results. LDPM can be included in multimodal devices, and we have demonstrated that simultaneous measurements of pressure, perfusion and ultrasound can be obtained from the oesophagus, when combined with bag distension. The quality of the data indicates that new insights can be obtained from studies in healthy volunteers and patients with functional chest pain. There are still some major challenges to face due to the fact that the method is highly motion-sensitive, and we cannot give the exact depth location from where perfusion measurements are obtained.

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BRIEF ARTICLES

Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates

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Author contributions: Torres LE and Rodríguez BL contributed equally to this work; Torres LE, Melián K, and Rodríguez BL designed the research; Torres LE, Moreno A, Hernández M, Bermúdez L collected and processed the samples and performed the research; Melián K, Alonso J performed the endoscopy and managed the patient data; Sabatier CA performed the histology; Torres LE and Rodríguez BL analyzed the data and wrote the paper.
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Abstract

AIM: To investigate the prevalence of vacuolating cytotoxin (*vacA)*, cytotoxin associated gene A (*cagA)* and blood adhesion binding antigen (*babA2)* genotypes of *Helicobacter pylori* (*H pylori*) isolates from Cuban dyspeptic patients.

METHODS: DNA was extracted from *H pylori*-positive cultures taken from 130 dyspeptic patients. Genotyping was performed by PCR, using specific primers for *vacA* (*s1*, *s2*, *m1*, *m2*), *cagA* and *babA2* genes. Endoscopic observations and histological examinations were used to determine patient pathologies.

RESULTS: *vacA* alleles *s1*, *s2*, *m1* and *m2* were detected in 96 (73.8%), 34 (26.2%), 75 (57.7%) and 52 isolates (40%), respectively, while the *cagA* gene was detected in 95 isolates (73.2%). One hundred and seven isolates (82.3%) were *babA2*-positive. A significant correlation was observed between *vacAs1m1* and *cagA* and between *vacAs1m1* and *babA2* genotypes ($P < 0.001$ and $P < 0.05$, respectively) and between *babA2* genotype and *cagA* status ($P < 0.05$); but, no correlation was observed between *vacAs1* and *babA2* genotypes. Eighty five (65.4%) and 73 (56.2%) strains were type 1 (*vacAs1-cagA*-positive) and "triple-positive" (*vacAs1-cagA-babA2*-positive), respectively, and their presence was significantly associated with duodenal ulcer ($P < 0.01$ and $P < 0.001$, respectively).

CONCLUSION: The distribution of the main virulence factors in the Cuban strains in this study resembled that of the Western-type strains, and the more virulent *H pylori* isolates were significantly associated with duodenal ulcer, ulcer disease being the worst pathology observed in the group studied.

Key words: Cuban dyspeptic patients; *Helicobacter pylori*; *vacA*; *cagA* and *babA*

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INTRODUCTION

Helicobacter pylori (H pylori), a spiral-shaped microaerophilic bacterium infects more than 50% of the world's population, the rate of infection being higher in developing countries[1]. *H pylori* is a major etiological agent in several gastroduodenal diseases, such as functional dyspepsia, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue lymphoma. The clinical outcome following infection with this pathogen has been related to environmental conditions, host

immunological factors and microorganism virulence[2].

Vacuolating cytotoxin (VacA), cytotoxin associated gene A (*cagA*), and blood adhesion binding antigen (*babA*) are the most commonly studied virulence markers of *H pylori*. However, there are other bacterial proteins with pathogenic potential, such as sialic acid-binding adhesin (SabA), outer inflammatory protein (*oipA*), and duodenal ulcer promoting gene (*dupA*); but, the influence of these proteins on *H pylori* pathogenesis is still under study[3].

The VacA protein induces vacuolation and apoptotic processes in epithelial cells, as well as immunosuppressive actions in immunological cells[4]. The *vacA* gene comprises two main regions: the signal zone (s1 or s2) and the middle region (m1 or m2)[5]. The *vacA s1m1* allelic combination exhibits the highest activity, while *s2m2* and the rare *s2m1* combinations are non-toxic[5]. Recently, a new polymorphic region in the *vacA* gene called the intermediate region (*i*) has been discovered and its *i1* active allele seems to be a better predictor of gastric cancer than the *s1* or *m1* allele[6].

Hydrophilic protein CagA contains the so-called EPIYA motifs[7], which interact with several eukaryotic proteins, promoting changes in the signal transduction pathway, cytoskeletal plasticity and IL-8 secretion in epithelial cells[8]. CagA-positive *H pylori* isolates are associated with a higher rate of gastric inflammation and damage, when compared with CagA-negative strains[8,9]. The *cagA* gene is located at the end of the cag pathogenecity island, a system that introduces CagA and a peptidoglycan into epithelial cells[10]. Several epidemiological studies have shown the correlation between *cagA*-positive strains and a higher risk of developing peptic ulceration, gastric atrophy and gastric cancer[8,9].

The blood group binding antigen mediates adherence of *H pylori* to human gastric epithelium[11]. This antigen is encoded by the polymorphic gene called *babA2*, while allele *babA1* is non-functional[11]. Some studies have suggested that BabA plays a crucial role in the development of severe functional dyspepsia, peptic ulcer and gastric adenocarcinoma[12,13]. Furthermore, the combined presence of *vacAs1* and *cagA* genotypes (type 1 strains) or even the "triple-positive" strains (*vacAs1*, *cagA* and *babA2*), has shown a higher correlation with the appearance of peptic ulcer, intestinal metaplasia and gastric cancer[14].

The clinical outcome of this bacterial infection seems to be influenced by the distribution of the above-mentioned pathogenic factors in *H pylori* strains[15]; but, complete genotyping of Cuban *H pylori* strains has never been carried out. Therefore, the aim of this study was to determine the frequency of the main virulence factor genes in Cuban *H pylori* isolates and establish their associations with the clinical outcome.

MATERIALS AND METHODS

Patients

H pylori isolates were obtained from 130 consecutive *H pylori*-positive patients (77 male and 53 female) with a mean age of 49.1 years (range, 18 to 88) who underwent routine endoscopy due to dyspeptic complaints at CIMEQ Hospital, Havana, Cuba. Endoscopic observation and histological confirmations were used to determine patient pathologies. This study was approved by the ethics committee at CIMEQ Hospital. All patients provided informed consent to participate in the study.

Microorganism culture

Antrum gastric biopsy specimens obtained from all patients were homogenized, inoculated into Columbia agar base plates with 7% human blood and SR0147E selective supplement (Oxoid, England, UK), and grown under microaerophilic conditions at 37℃ for 5 to 8 d. All *H pylori* isolates were positive for oxidase, catalase and urease. The reference strain J99[16] was kindly provided by Professor Francis Megraud from Pellegrin Hospital, Bordeaux, France.

DNA extraction and cagA, vacA and babA2 genotyping

Genomic DNA was extracted by CTAB methodology with phenol/chloroform and isopropanol precipitation as previously described[17]. Purified DNAs were stored at -20℃ until use. In all cases, PCR amplification was carried out in a 25 μL reaction mixture containing 2.5 μL 10X PCR buffer (Roche, Germany), 0.2 mmol/L of each deoxynucleotide triphosphate, 0.6 mM sense and antisense primers, 4 mmol/L magnesium chloride, 1.25 U Taq DNA polymerase (CIGB, Cuba) and 100 ng genomic DNA. The PCR had an initial step at 94℃ for 1 min, followed by 40 cycles at 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min, and a final extension at 72℃ for 5 min, using a Master Cycler apparatus (Eppendorf, Germany).

The primers used and their details are shown in Table 1. Primers to the *glmM* gene of *H pylori* were used to control DNA integrity and specificity. PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide. Images were taken through the Gene Genius system (Syngene, England, UK).

Statistical analysis

Differences among groups were tested using the χ^2 test. P values < 0.05 were considered to be significant. The statistic software, version 8 for Windows, was used for statistical analysis.

RESULTS

Detection of H pylori genotypes

H pylori was successfully cultured from 130 Cuban dyspeptic patients. DNA integrity and specificity was confirmed by *glmM* PCR, which rendered the expected 417 bp band from all isolates (data not shown). PCR product sizes of *vacA s* and *m* alleles were used to differentiate them in agarose gels (Figure 1, panel A). The most virulent *vacAs1* allele was predominantly present in Cuban *H pylori* isolates (Table 2), and

Table 1 Primer used for PCR genotyping of Cuban *H pylori* strains

Primer	Sequence (5'-3')	AT ℃	Size (bp)	Ref.
glmMF	CCCTCACGCCATCAGTCCCAAAAA	60	417	[18]
glmMR	AAGAAGTCAAAAACGCCCCAAAAC			
cagF1	GATAACAGGCAAGCTTTTGA	60	349	[7]
cagB1	CTGCAAAAGATTGTTTGGCAGA			
vacAsF	ATGGAAATACAACAAACACAC	52	*s1*-259/*s2*-286	[20]
vacAsR	CTGCTTGAATGCGCCAAAC			
vacAmF	CAATCTGTCCAATCAAGCGAG	56	*m1*-567/*m2*-642	[20]
vacAmR	GCGTCAAAATAATTCCAAGG			
bab7-F	CCAAACGAAACAAAAAGCGT	60	271	[21]
bab7-R	GCTTGTGTAAAAGCCGTCGT			
babA2F[1]	AATCCAAAAAGGAGAAAAAGTATGAAA	60	832	[13]
babA2R	TGTTAGTGATTTCGGTGTAGGACA			
babA2R607[2]	GTTTTCTTTGAGCGCGGGTAAGC	60	607	[14]

[1]Forward primer used with primer babA2R or babAR607 to amplify *babA2* gene; [2]Five nucleotides (GTTTT) were added to the original primer designed by Zambon *et al*[14] to increase specificity.

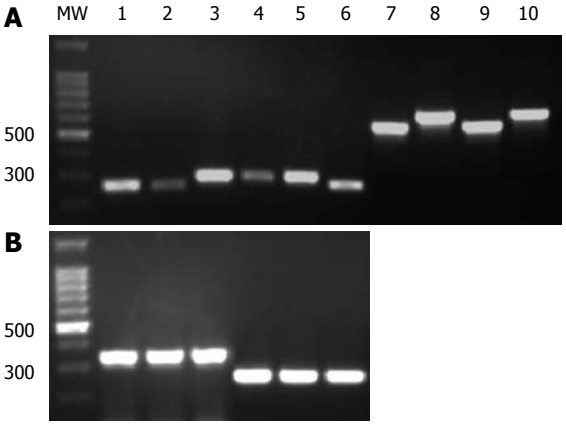

Figure 1 Genotyping of main virulence factor genes in Cuban *H pylori* isolates. The images shown are from a representative gel electrophoresis of two independent PCR amplification products of *vacA* (*s1*, *s2*, *m1*, *m2*), *cagA* and *babA2* genes from Cuban isolates and J99 control strain. A: Lanes 1 and 7, reference strain J99 (*vacAs1* and *m1* alleles, respectively); Lanes 2 and 6, *vacAs1* strains; Lanes 3-5, *vacAs2* strains; Lanes 8 and 10 *vacAm2* strains; Lane 9, *vacAm1* strain. B: Lanes 1 and 4, J99 strain (*cagA* and *babA2* gene, respectively); Lanes 2 and 3, *cagA*-positive strains; Lanes 5 and 6 *babA2*-positive strains. MW: 100 bp DNA Ladder (Promega, USA).

was visualized as a band of 259 bp on agarose gel electrophoresis (Figure 1, panel A), whereas 26.2% of isolates had the *vacAs2* genotype (Table 2). The middle region of the *vacA* gene was detected in only 127 of the 130 isolates, *m1* and *m2* genotypes were more equally distributed than s genotypes (Table 2). On the other hand, *s1m1* and *s2m2* genotypes were the most common allelic combinations of the *vacA* gene among Cuban isolates, and only one strain harbored the *s2m1* genotype (Table 2).

Amplification of the *cagA* gene was visualized as a band of 349 bp (Figure 1, panel B) and was present in 73.2% of the strains (Table 2). When primers babA7F/babA7R (Table 1) were used to amplify the *babA2* gene, over 80% of the Cuban strains carried this gene (Table 2). In contrast, a low prevalence of *babA2* genotype was observed among the Cuban isolates when using primers babA2F/babA2R and babA2F/babA2R607 (Table 1).

Table 2 Correlation between *vacA* alleles and *cagA* and *babA2* genotypes in 130 Cuban *H pylori* isolates

vacA	*s1m1*	*s1m2*	*s2m2*	*s2m1*	*s1m-*	Total (%)
cagA+	70	14	8	1	2	95 (73.2)
cagA-	4	5	25	0	1	35 (26.8)
babA2+	67	14	24	1	1	107 (82.3)
babA2-	7	5	9	0	2	23 (17.7)
Total (%)	74 (56.9)	19 (14.6)	33 (35.4)	1 (0.8)	3 (2.3)	130

Table 3 Correlation between virulence factor genotypes and disease outcome

Genotypes	Pathologies		
	FD *n* = 51 (%)	GU *n* = 33 (%)	DU *n* = 46 (%)
vacAs1m1	28 (54.9)	16 (48.5)	30 (65.2)
s1m2	5 (9.7)	9 (27.3)	5 (10.9)
s2m2	16 (31.4)	6 (18.2)	11 (23.9)
s1m-	1 (2)	2 (6)	-
s2m1	1 (2)	-	-
cagA+	36 (70.6)	19 (57.6)	40 (87)
babA2+	36 (70.6)	28 (84.8)	43 (93.5)[b]
Type 1	29 (56.9)	16 (48.5)	40 (87)[d]
Triple-positive	24 (47.1)	12 (36.4)	37 (80.4)[f]

FD: Functional dyspepsia; GU: Gastric Ulcer; DU: Duodenal Ulcer; *P* values were calculated with the χ^2 test; [b, d, f]Statistically significant differences (*P* values < 0.01).

Combinations of vacA, cagA and babA2 genotypes

On examining the association of the main virulence genes in each strain, a statistically significant correlation was observed between *s1m1* genotype and *cagA* status ($P = 0.00001$), between *s1m1* and *babA2* genotypes ($P = 0.047$), and between *cagA* and *babA2* genotypes ($P = 0.049$). A significant association was also observed between *vacAm1* allele and *cagA* status or *babA2* genotype ($P = 0.00001$ and $P = 0.035$, respectively), while most *s2m2* strains carried a *cagA*-negative genotype (Table 2). However, no correlation was observed between *vacAs1* and *babA2* genotypes ($P = 0.12$). Additionally, 85 isolates were classified as type 1 strains and 73 were triple-positive strains (Table 3).

Table 4 Worldwide distribution of main H. pylori virulence factors

World Area	*vacA* alleles prevalence (%)				References	*cagA* prevalence (%)	*babA2* prevalence (%)
	s1	*s2*	*m1*	*m2*		*cagA* +	*babA2* +
Europe	48-89	11-51	37	63	[14,23,26,27]	66-73[14,23,26,27]	34-72[13,14,34]
America	57-68	16-48	37-44	29-63	[19,20,24]	57-75[7,19,24]	46-69[24,32]
East Asia	100	0	41-94	5-55	[12,25,28]	90-100[12,25,28,35]	80-100[12,21,35]

Relationship between genotypes and gastric diseases

Of the 130 *H pylori* infected patients studied, 39.2% were diagnosed with functional dyspepsia, 35.4% had a duodenal ulcer (DU) and 25.4% had a gastric ulcer (GU). Table 3 shows that the *vacAs1m1* genotype was detected at a higher frequency in isolates from patients with DU, and in strains obtained from patients with functional dyspepsia; but, the presence of this genotype did not correlate with the presence of duodenal or gastric ulcer (P = 0.21 and P = 0.4, respectively). On the other hand, the *vacA s1m1* genotype had a higher frequency in DU patients; but, no association was observed between *s1m1* or any other *vacA* genotype, and the presence of severe pathologies in this study (Table 3). GU patients exhibited the highest frequency of *s2m2* strains, followed by patients with functional dyspepsia (Table 3). No correlation was found between the *cagA* genotype and duodenal or gastric ulcer (P = 0.051 and P = 0.22, respectively); but, an association between *cagA*-positive strains and DU may be assumed as a clear tendency (Table 3). Meanwhile, the *babA2* genotype was significantly associated with DU (P = 0.004), but not with GU (P = 0.13). Type 1 and triple-positive strains (Table 3) were also associated with DU (P = 0.001 and P = 0.0007, respectively) but not with GU (P = 0.45 and P = 0.33, respectively).

DISCUSSION

Several studies have shown that the incidence and/or severity of gastroduodenal pathologies related to *H pylori* may vary between geographic areas. This phenomenon is partly due to a different distribution of pathogenic markers in circulating strains[15]. Several pathogenic factors of *H pylori* have been described and their association with the clinical outcome studied[19-21]. Distribution of the main virulence factors around the world is summarized in Table 4, showing the high variation between geographic areas. This is the first report to examine the three main *H pylori* virulence associated genes, *vacA*, *cagA* and *babA2* in Cuban isolates.

vacA alleles

The *vacA s1* and *s2* leader sequences are different in a small insert, totaling 27 bp, carried by the *vacAs2* allele[20], which has a reduced capacity to secrete VacA toxin[22]. According to our results, the most virulent *vacAs1* allele was predominant in Cuban *H pylori* isolates (Table 2), a finding which has also been observed in other studies of Western strains (Table 4)[23,24]. In the present study, the prevalence of *vacAm1* and *vacAm2* were similar compared to that of the *s1* and *s2* allele; meanwhile, the *s1m1* and *s2m2* genotypes were the most common allelic combinations of the *vacA* gene from Cuban isolates (Table 2), a finding reported in several studies from various countries[19,20]. Furthermore, the middle region of *vacA* was not detected in three isolates, while only one strain harbored the *s2m1* genotype. Genotyping of the *vacA* middle region failed in three strains, probably due to heterogeneity in the *vacA* gene, a finding described previously[12,24]. Additionally, only one strain harbored the *s2m1* genotype, the *vacA* allelic combination relating to lower incidence in several studies[23,24]. On the other hand, the *vacA s1m1* genotype was noted at a higher frequency in DU patients; but, no significant correlation was observed between *vacA* genotypes and the appearance of peptic ulcer disease, which is in agreement with previous reports[19,25].

cagA genotype

H pylori cagA-positive strains have been associated with more severe gastroduodenal diseases[8,14,15]. Here, 73.2% of the *H pylori* strains were *cagA*-positive, a prevalence similar to that reported in many studies from Western countries (e.g. USA, 60%[7]; Spain, 66%[26]; and England, 68%[27]) but lower than that reported in some East Asian studies, which encountered over 90% of *cagA*-positive isolates (Table 4)[25,28]. In addition, a highly significant correlation was observed between *cagA* status and *vacAs1* and *vacAs1m1* genotypes (Table 2), which is commonly linked to an increase in *H pylori* virulence[13,14,29,30]. An association was also observed between the presence of the *cagA* gene and the *babA2*-positive genotype, due to the fact that most *cagA*-positive isolates carried the *babA2* allele. Our data support the relationship between *cagA* and *babA2* genes found in previous reports, which could be caused by selective pressure[13,14], although other authors, such as Mattar *et al*[24], did not find any correlation between these virulence factors in the isolates investigated. On the other hand, previous studies have shown a high association between the *cagA*-positive genotype and the appearance of DU[31,32]. In this study, however, no correlation was observed between *cagA* status and DU. Moreover, a high frequency of *cagA*-positive strains was observed in DU patients (Table 3), indicating that a statistical association could be reached by increasing the number of patients in future studies.

babA2 genotype

Adherence of *H pylori* to epithelial cells is a relevant step in the development of gastroduodenal pathologies. *BabA2* attaches *H pylori* to these cells, allowing delivery of VacA and CagA toxins near the gastric epithelium and therefore enhancing gastric tissue damage[3,11]. Here, Cuban *H pylori* isolates exhibited a high frequency (82.3%) of the *babA2* allele when the primers of Sheu *et al*[21] were used to amplify the gene. In contrast, a low prevalence of the *babA2* genotype was observed when the primers reported by Gerhard *et al*[13] and a variant of Zambon *et al*[14] were used, respectively (Table 2). Interestingly, these last two primers are located in a high polymorphic zone of the *babA* gene[33], which should lead to an underestimation of *babA2*-positive strains. Our results add new data to previous observations[24,34] that support the ineffectiveness of the Gerhard *et al*[13] primers to detect the *babA2* gene, and for the first time relate this to deficiencies in the primers used by Zambon *et al*[14]. Consequently, the low levels of *babA2* alleles reported in several previous studies[13,14,32] may be underestimated, due to the use of Gerhard primers[13]. However, the prevalence of the *babA2* gene was above 70% in Asian countries using the same primers[12,35], suggesting that underestimation due to allelic variation in the *babA* gene could have a variable impact in different geographic areas, as was previously suggested[34]. This study showed a high association between the presence of the *babA2* allele and DU disease (Table 3), which is in agreement with several reports which associate the presence of this gene with the appearance of severe gastric damage[13,14]. However, other studies have claimed no association between this genotype and more severe pathologies[24].

Combination of virulence genotypes

Of the 130 Cuban *H pylori* isolates, 65.4% and 56.2% were type 1 and triple-positive strains, respectively. Infection with these strains has been associated with a higher degree of inflammation and gastroduodenal lesions[14]. Similar percentages of both types of strains were found in this study and in a previous report[13], while Brazilian dyspeptic patients seem to have a lower rate (32.6%) of triple-positive strains[24]. Our data indicate that type 1 and triple-positive strains increase the risk of developing DU in Cuban dyspeptic patients, a finding consistent with other studies, in which these types of strains were mainly found in subjects with peptic ulcer disease[13], and in patients with intestinal metaplasia and gastric atrophy[14].

We hypothesize that the absence of a correlation between the virulence genes analyzed and the development of GU might be influenced by the small number of patients with this pathology in our study, although several other studies have not found any correlation between the presence of *H pylori* main virulence factor genes (alone or in combinations) and peptic ulcer disease[24,25].

It is interesting to note that despite the high rate of *H pylori* infection in Cuban dyspeptic patients[36-38], and the relatively high pathogenic potential of Cuban isolates found previously[37,38] and in the present study, a low incidence of gastric adenocarcinoma has been found in Cuban patients with dyspepsia[36-38]. This reflects a general tendency in the Cuban population towards low levels of gastric cancer; in fact, a gastric cancer death rate of 7.1/100 000 was observed in Cuba in 2007 (http://www.sld.cu/servicios/estadisticas/). Future studies are required to elucidate the above-mentioned investigative problem, including a full characterization of Cuban *H pylori* isolates.

In conclusion, this study has shown a relatively high prevalence of the main virulence factor genes in Cuban *H pylori* isolates, which is similar to that found in the Western-type strains. In addition, a significant association was found between the virulence genes in Cuban strains. Consequently, the presence of more virulent type 1 and triple-positive strains was relatively high in Cuban dyspeptic patients, and increased their risk of developing duodenal ulcer. On the other hand, more severe gastroduodenal pathologies, such as intestinal metaplasia, gastric atrophy and gastric cancer were not found in this study, or in other similar studies and which might merit further research.

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COMMENTS

Background

Cuba has a high incidence of *H pylori* infection. The presence and association of the main virulence factors VacA, CagA and BabA2 in *H pylori* strains influences the clinical outcome following infection with this pathogen. So far, no whole genotyping of Cuban *H pylori* strains has been carried out. This study addresses the frequency and association of the main virulence factor genes in *H pylori* isolates, and establishes their relationship to clinical outcome in a Cuban dyspeptic population.

Research frontiers

In a dyspeptic population in Cuba, the presence and association of the main virulence factor genes (*vacA*, *cagA* and *babA2*) in the infecting strains was significantly high, and their combined presence is a risk factor for duodenal ulcer (DU), but is not associated with gastric ulcer (GU). More severe pathologies, such as intestinal metaplasia, gastric atrophy and gastric cancer were not present in the group studied.

Innovations and breakthroughs

Studies of various populations have indicated an association between the presence of *vacA*, *cagA* and *babA2* genes in *H pylori* isolates and the appearance of more severe gastroduodenal pathologies. The distribution of these virulence markers in *H pylori* strains varies among populations. The present study showed a relatively high prevalence of the main virulence factor genes in Cuban *H pylori* isolates, similar to that found in the Western-type strains. In addition, the study demonstrated a significant association between the virulence genes in the strains studied, which was related to the risk of developing DU, but not GU in dyspeptic patients. Furthermore, despite the relatively high virulence potential of Cuban *H pylori* isolates, pathologies such as intestinal metaplasia, gastric atrophy and gastric cancer were not present in the dyspeptic population studied.

Applications

In developing countries with a high incidence of *H pylori* infection and dyspepsia, it is important to screen the isolates for main virulence factors. The information generated here may be used to develop a procedure to detect *H pylori* pathogenic factors in a given population from biopsy samples. Intervention may then be concentrated on subjects with a higher risk of severe pathologies.

Peer review

This study determined the prevalence of main virulence factor genes *vacA*, *cagA* and *babA2* in Cuban *H pylori* isolates and their association with gastroduodenal diseases.

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Role of bacterial and genetic factors in gastric cancer in Costa Rica

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Abstract

AIM: To evaluate several risk factors for gastric cancer (GC) in Costa Rican regions with contrasting GC incidence rate (GCIR).

METHODS: According to GCIR, 191 *Helicobacter pylori* (*H pylori*)-positive patients were classified into groups A (high GCIR, *n* = 101) and B (low GCIR, *n* = 90). Human DNA obtained from biopsy specimens was used in the determination of polymorphisms of the genes coding for interleukin (IL)-1β and IL-10 by PCR-RFLP, and IL-1RN by PCR. *H pylori* DNA extractions obtained from clinical isolates of 83 patients were used for PCR-based genotyping of *H pylori cagA*, *vacA* and *babA2*. Human DNA from gastric biopsies of 52 GC patients was utilized for comparative purposes.

RESULTS: Cytokine polymorphisms showed no association with GCIR variability. However, gastric atrophy, intestinal metaplasia and strains with different *vacA* genotypes in the same stomach (mixed strain infection) were more frequently found in group A than in group B, and *cagA* and *vacA s1b* were significantly associated with high GCIR (*P* = 0.026 and 0.041, respectively). IL-1β+3954_T/C (OR 2.1, 1.0-4.3), IL-1RN*2/L (OR 3.5, 1.7-7.3) and IL-10-592_C/A (OR 3.2, 1.5-6.8) were individually associated with GC, and a combination of these cytokine polymorphisms with *H pylori vacA s1b* and *m1* further increased the risk (OR 7.2, 1.4-36.4).

CONCLUSION: Although a proinflammatory cytokine genetic profile showed an increased risk for developing GC, the characteristics of *H pylori* infection, in particular the status of *cagA* and *vacA* genotype distribution seemed to play a major role in GCIR variability in Costa Rica.

Key words: Costa Rica; Gastric cancer; *Helicobacter pylori*; Host genetic factors

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INTRODUCTION

Costa Rica has one of the highest age-adjusted incidence and mortality rates for gastric cancer[1]. In fact, this country reported the highest age-adjusted gastric cancer mortality rate in males and females over the period 1983-1997, out of a total of 30 countries, including Japan and Chile[2].

Costa Rica has regions with contrasting gastric cancer incidence rates (GCIR). Topographically, the central part of the country is predominantly composed of regions with high GCIR while coastal areas are largely characterized by low GCIR[3]. Population density varies according to geographic area. While in coastal regions the population density is around 30 persons per square km, in the central regions of San Jose and Cartago, it ranges from 140 to 270 persons per square km. Cultural, behavioral and dietary patterns are very similar throughout the country, regardless of population density[3]. The pre-

dominant ethnic group is the criollo, which has Spanish ancestry. In spite of these homogeneous patterns, the GCIR in Costa Rica shows a distinctive regional variation[4]. Several environmental factors such as the components of drinking water, soil and nutrients have been compared in contrasting GCIR regions; however, none of these factors was significantly associated with GCIR variation in the country[4,5].

The cause of gastric cancer is thought to be multifactorial. A higher incidence of gastric cancer in blood type A subjects than in those with other blood types was reported as early as the 1950's[6,7]. Several decades later, after the discovery of *Helicobacter pylori* (*H pylori*), which is a Gram-negative microaerobic bacterium that persistently colonizes the human gastric mucosa, it was reported that *H pylori*-positive subjects are believed to have a two- to three-fold increased risk of developing gastric cancer when compared with *H pylori*-negative subjects[8-11]. The risk is even higher in subjects infected with strains co-expressing the *H pylori cagA*, *vacA s1* and *babA2* genes[12-15]. Recently, cytokine gene polymorphisms of the host, IL-1β, IL-1RN and IL-10, in response to *H pylori* infection, have been associated with an increased risk for developing gastric cancer[16-21]. Moreover, it has been suggested that an interaction between a host's immunological defenses, environmental and *H pylori* virulence factors play a main role in the development of gastric cancer[22,23].

We previously reported that the presence of serum CagA antibody was found to be significantly higher in high GCIR regions than in low GCIR regions in Costa Rica, despite the fact that no significant difference was found in the prevalence of *H pylori* infection between the regions, suggesting that the *H pylori cagA* gene was associated with the development of severe gastric injury, glandular atrophy and cancer, which probably influenced the GCIR variability in the country[24]. However, further investigation is needed to demonstrate a significant association of *H pylori* and/or host factors with GCIR variability in Costa Rica.

The aim of this study was to evaluate whether host genetic factors such as interleukin (IL)-1β (-511 and +3954), IL-10 (-1082 and -592) and IL-1RN intron 2 variable number of tandem repeat (VNTR) polymorphisms in response to *H pylori* infection, and/or *H pylori cagA*, *vacA* and *babA2* genotype distribution could be associated with the GCIR variability present in Costa Rica.

MATERIALS AND METHODS

Study population

The patients in this study attended a digestive center in San Jose, Costa Rica. Out of 402 continuous dyspeptic patients who underwent upper endoscopy from January to July 2005 and from January to July 2006, a total of 191 *H pylori*-positive patients (80 males, 111 females; age range 23-76 years) were enrolled for the determination of cytokine gene polymorphisms in *IL-1β*, *IL-1RN* and *IL-10*. Clinical isolates successfully obtained from both antrum and corpus specimens of 83 patients were eventually utilized for the PCR-based genotyping of the *H pylori cagA*, *vacA* and *babA2* genes. Informed consent was obtained from each patient and the study was approved by the Ethics Committee of the institution.

In addition, gastric tissue specimens obtained from 52 consecutive *H pylori*-positive gastric cancer patients (GC group) who underwent surgical treatment at a hospital in Cartago, Costa Rica between February 2006 and March 2007, were utilized in this study to determine cytokine gene polymorphisms of the host, and were used for comparative purposes.

Based on a previous study[4], dyspeptic patients were classified into either high or low GCIR groups. Group A (high GCIR) was composed of patients belonging to regions with a GCIR in the range of 24.7-48.5/100 000 persons, while in group B (low GCIR) the incidence rates ranged from 9.8-19.9/100 000 persons. Patients belonging to regions with a GCIR of 20.0-24.6/100 000 persons were removed from the study to further distinguish group A from group B. Information on age, gender, place of origin, symptoms and medication was collected. Patients with a recent intake of proton pump inhibitors, antibiotics, non-steroidal anti-inflammatory drugs, or any drug that could alter the state of the gastric mucosa were excluded from this study. Likewise, patients with *H pylori* eradication or previous attempted eradication therapy, previous gastric surgery as well as patients with Asian ancestry were also excluded from the study.

Endoscopic and histological evaluations

Endoscopy was performed with Olympus Evis Excera 160 and 180 videoendoscopes (Olympus America Inc., San Jose, CA, USA). From each patient, five biopsies (two from the antrum, two from the corpus and one from the cisura angularis) were collected for histological examination. Two more biopsies (one from the antrum and one from the corpus) were also taken to obtain the isolates following bacterial culture.

The five biopsy samples from each of the 191 patients were conventionally fixed in 100 mL/L aqueous formaldehyde, and embedded in paraffin. Serial 3- to 4-μm sections were stained with hematoxylin and eosin for histological observation. Each biopsy specimen was evaluated independently by two experienced pathologists blinded to the endoscopic and laboratory examinations. All discrepant diagnoses were re-examined by both pathologists together in order to reach a final consensus diagnosis. All five biopsies were examined for the presence of glandular atrophy and intestinal metaplasia and were scored into one of four grades (0: none, 1: mild, 2: moderate and 3: marked) for both the antrum and the body of the stomach, according to the updated Sydney System of classification and grading of gastritis[25]. Gastric glandular atrophy was defined as the loss of gastric glands, and its replacement with fibrosis or metaplastic epithelium. Intestinal metaplasia was defined as the presence of foci where at least three neighboring gastric pits containing two or more goblet cells (in each pit) were visualized in any part of the stomach.

Table 1 PCR primers for amplification of *cagA*, *vacA* and *babA2* genes

Region	Primer	Nucleotide sequence	Reference
cagA	D008	5'-ATAATGCTAAATTAGACAACTTGAGCGA-3'	[28]
	R008	5'-TTAGAATAATCAACAAACATCACGCCAT-3'	
	cagAFnz3	5'-AAAAGCGACCTTGAAAATTCC-3'	[29]
	cagA-seqR1	5'-TAGCATAATTGTCCAATTTCGC-3'	
vacA s1	VA1-F	5'-ATGGAAATACAACAAACACAC-3'	[30]
	VA1-R	5'-CTGCTTGAATGCGCCAAAC-3'	
vacA s1a	S1A-F[1]	5'-TCTYGCTTTAGTAGGAGC-3'	[30]
vacA s1b	SS3-F[1]	5'-AGCGCCATACCGCAAGAG-3'	[30]
vacA s1c	S1C-F[1]	5'-CTYGCTTTAGTRGGGYTA-3'	[13]
vacA s2	VA1-F[1]	5'-ATGGAAATACAACAAACACAC-3'	[30]
vacA m1	VA3-F	5'-GGTCAAAATGCGGTCATGG-3'	[30]
	VA3-R	5'-CCATTGGTACCTGTAGAAAC-3'	
vacA m2	VA4-F	5'-GGAGCCCCAGGAAACATTG-3'	[30]
	VA4-R	5'-CATAACTAGCGCCTTGCAC-3'	
babA2	babA-F	5'-AATCCAAAAAGGAGAAAAAGTATGAAA-3'	[31]
	babA-R	5'-TGTTAG TGATTTCGGTGTAGGACA-3'	
	babA2-Fnc1	5'-GAAAAAACATGAAAAAACACATCCTTTCAT-3'	
This study			
	babA2-Rmn2	5'-TCTGGGTTAATGGCTTGCC-3'	

[1]Used with primer VA1-F.

Determination of H pylori infection

H pylori infection was determined by either serum antibodies to *H pylori*, rapid urease test (RUT) or histological examinations of biopsy specimens obtained from the antrum, cisura angularis and body of the stomach. Patients were considered to be infected with the bacterium if either serum antibodies to *H pylori* were found, the biopsy specimen was positive for RUT or the bacterium was observed in any of the hematoxylin and eosin-stained sections.

Extraction of human DNA and genotyping of cytokine polymorphisms

Human DNA was extracted from biopsy specimens using a DNA extraction kit (QIAamp DNA mini kit; Qiagen K.K., Tokyo, Japan), according to the manufacturer's instructions. Cytokine gene polymorphisms in *IL-1β* (-511 and +3954) and *IL-10* (-1082 and -592) were examined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis, as described previously[26,27] and visualized by 50 mL/L ethidium bromide staining on 30 mL/L agarose gels. The IL-1RN variable number of tandem repeat (VNTR) polymorphism was detected by PCR and visualized on 20 mL/L agarose gels with alleles being classified conventionally according to El-Omar *et al*[18] as follows: allele 1, four repeats; allele 2, two repeats; allele 3, five repeats; allele 4, three repeats and allele 5, six repeats. Because alleles 3, 4 and 5 were very rare, the alleles were classified into short (allele 2: *2) and long (alleles 1, 3, 4 and 5: L) alleles for statistical analysis, as described previously[14].

Isolation of H pylori from biopsy specimens and DNA extraction

The homogenized biopsy specimens were placed on *H pylori* selective agar plates (Helico VI agar, E-MS70, Eiken Chemical Co., Ltd., Japan) and cultured at 37°C under microaerobic conditions (100 mL/L CO_2) for five to seven days. The presence of *H pylori* colonies was confirmed by typical morphology, Gram staining and a positive urease test. From 83 patients, a total of 166 clinical isolates obtained from both antrum and corpus specimens were subjected to genomic DNA (gDNA) extraction using a DNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Detection of H pylori cagA, vacA and babA2 genes by PCR

The genomic DNAs were subjected to PCR for *H pylori* genotyping analysis. Genotyping of the *cagA* gene was examined using primer pairs D008 and R008, and cagA-Fnz3 and cagA-seqR1[28,29] (Table 1). The analysis of the *vacA s* and *m* regions was carried out as previously described[13,30]. Genotyping of the *babA2* gene was examined using reported primers[31] and additional primers babA2-Fnc1 (5'-GAAAAAACATGAAAAAACACATCCTTT-CAT-3') and babA2-Rmn2 (5'-TCTGGGTTAATGGCT-TGCC-3') designed according to the following conditions: pre-heat for 2 min at 96℃, followed by 40 cycles at 96℃ for 30 s, 49℃ for 30 s, and 72℃ for 1 min. All discrepant results of *cagA* and *babA2* genotyping were confirmed by sequence analysis (Genetic Analyzer 3130 Applied Biosystems, Foster City, CA, USA) following PCR using a Big Dye Terminator v1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed using the Chi-square test and the Fisher's exact probability test (STATA SE (version 8) statistical software). A P-value of < 0.05 was regarded as statistically significant. Multivariate

Table 2 Characteristics of *H pylori*-positive Costa Rican patients

	Group A	Group B	*P*-value
Number of patients	101	90	
Gender (male/female)	49/52	33/57	0.81
Mean age (yr, ± SD)	50.4 ± 11.5	50.9 ± 13.6	0.99
AG-positive (%)	36 (35.6)	24 (26.7)	0.12
IM-positive (%)	17 (16.3)	6 (6.7)	0.02

analysis was performed by logistic regression (SPSS 13.0 Japanese version (SPSS Japan Inc., 2005) adjusting for gender and age. Odds ratios (OR) with 95% confidence intervals (CI) were used to study the influence of host and bacterial factors on the development of gastric cancer.

RESULTS

Comparison of gender and age of patients between groups A and B

Gender and age distribution in group A (101, 49 men, 52 women; mean ± SD, 50.42 ± 11.5 years) was not significantly different when compared with that in group B (90, 33 men, 57 women; mean ± SD, 50.87 ± 13.6 years) ($P = 0.81$ and $P = 0.99$, respectively; Table 2).

Gastric atrophy and intestinal metaplasia in groups A and B

The prevalence of gastric atrophy was higher in group A (35.6%, 36/101) than in group B (26.7%, 24/90), although the difference did not reach statistical significance ($P = 0.12$, Table 2). However, the prevalence of intestinal metaplasia was found to be significantly higher in group A (16.3%, 17/101) than in group B (6.7%, 6/90; $P = 0.02$).

Interleukin-1 and -10 polymorphisms in groups A and B

The analysis of cytokine gene polymorphisms including IL-1β-511 and +3954, IL-1RN intron 2, and IL-10-1082 and -592 did not reveal any significant difference between groups A and B (Table 3). However, when the role of cytokine polymorphisms on gastric cancer was evaluated, IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_A/A and IL-10-592_C/A were found to be individually associated with this cancer, irrespective of GCIR grouping (Table 3).

Mixed strain infection of H pylori colonized in the stomach in clinical isolates obtained from the antrum and corpus

Mixed strain infection of *H pylori* has been defined as the colonization of the same patient by *H pylori* strains harboring more than one *vacA* genotype in the same stomach[32]. The analysis of the *H pylori vacA* gene in terms of its presence/absence and genotype in each clinical isolate between the antrum and corpus in 83 patients, showed a mixed strain infection in only one patient belonging to group B and in six patients belonging to group A, of which five were diagnosed with either gastric atrophy or both gastric atrophy and intestinal metaplasia (Table 4). The *cagA* and *babA2* genes were also examined according to the same terms in those 83 patients. The prevalence of *cagA* did not differ in any of the patients while the prevalence of *babA2* differed in two patients without discordant *vacA* alleles, both belonging to group A.

H pylori cagA, vacA and babA2 genes in clinical isolates from a non-mixed infection

In the 76 clinical isolates obtained from a non-mixed infection, the prevalence of *cagA* and the prevalence of *vacA s1b* in group A (both 87.8%) were found to be significantly higher than those in group B (65.7% and 68.6%, respectively) (Table 5). A tendency for an association between *vacA m1* and GCIR variability was reported, while no significant difference was found in the prevalence of *babA2* between the groups.

Combination of cytokine polymorphisms and H pylori virulence factors in gastric cancer and non-gastric cancer patients

To investigate the influence of combined factors on the development of GC, we used the cytokine polymorphisms that were associated with GC in this study. The presence of a combination of IL-1β+3954_T/C, IL-1RN*2/L and IL-10-592_C/A slightly increased the risk of GC (adjusted OR 4.7, 1.7-13.0) when compared with patients carrying only one of the cytokine polymorphisms previously cited (Table 6). However, a combination of these polymorphisms with the addition of *H pylori vacA s1b* and *m1* genotypes, which were chosen due to their association with GC reported in a previous Costa Rican study[29], considerably increased the risk of GC (adjusted OR 7.2, 1.4-36.4). The risk was further increased when a combination of only IL-1 polymorphisms (IL-1β+3954_T/C, and IL-1RN*2/L) and *H pylori vacA s1b/m1* was evaluated (adjusted OR 9.8, 2.9-32.9).

DISCUSSION

The gastric cancer incidence rate in Costa Rica shows regional variation. Using *H pylori*-positive patients selected from high and low GCIR regions, the main objective of this study was to evaluate the potential impact of *H pylori* and/or host genetic factors on GCIR variability in Costa Rica.

The analysis of human genetic polymorphisms within the cytokine genes *IL-1β*, *IL-1RN* and *IL-10* (Table 3) as well as the ABO blood group status (data not shown) did not show any significant differences between groups A and B (high and low GCIR groups, respectively) indicating that the genetic profile of the host, including these evaluated factors, did not seem to be linked to GCIR variability in Costa Rica. It has been reported that the presence of proinflammatory cytokines induces a hypochlorhydric and atrophic response to

Table 3 Statistical analysis for several cytokine gene polymorphisms according to high gastric cancer incidence rate and gastric cancer in *H pylori*-positive Costa Rican patients

	High GCIR			Gastric cancer		
	Pos/Neg	OR (95% CI)	*P*-value	Pos/Neg	OR (95% CI)	*P*-value
Interleukin-1β-511						
T/T	28/19	1.9 (0.8-4.3)	0.136	18/47	1.6 (0.7-4.1)	0.283
T/C	50/43	1.4 (0.7-2.8)	0.317	24/93	1.2 (0.5-2.9)	0.629
C/C	23/28	1.0 reference		10/51	1.0 reference	
Interleukin-1β+3954						
T/T	2/0	-	-	-	0/2	-
T/C	56/45	1.4 (0.8-2.6)	0.237	39/101	2.1 (1.0-4.3)	0.049
C/C	43/45	1.0 reference		13/88	1.0 reference	
Interleukin-1RN intron 2						
*2/*2	20/11	2.2 (0.9-5.2)	0.078	4/31	0.7 (0.2-2.2)	0.494
*2/L	31/28	1.2 (0.6-2.3)	0.592	33/59	3.5 (1.7-7.3)	0.001
L/L	50/51	1.0 reference		15/101	1.0 reference	
Interleukin-10-1082						
A/A	52/49	1.3 (0.3-6.1)	0.766	35/101	3.2 (0.3-30.2)	0.304
G/A	46/37	1.6 (0.3-7.8)	0.551	16/83	1.8 (0.2-17.1)	0.617
G/G	3/4	1.0 reference		1/7	1.0 reference	
Interleukin-10-592						
A/A	11/12	0.7 (0.3-1.7)	0.406	10/23	3.1 (1.2-8.2)	0.022
C/A	34/31	0.9 (0.5-1.6)	0.668	26/65	3.2 (1.5-6.8)	0.002
C/C	56/47	1.0 reference		16/103	1.0 reference	

-: Unable to compute. Pos: Positive; Neg: Negative.

Table 4 Patients with discordant *H pylori vacA* and *babA2* genes from antrum and corpus biopsy specimens in the same stomach

Patient	Gene	Antrum	Corpus	Diagnosis	GCIR group
1	*vacA*	*s2/m1*	*s1b/m1*	AG	A (High GCIR)
2		*s1b/m1*	*s2/m2*	NAG	A
3		*s1b/m1*	*s2/m2*	AG	A
4		*s1b/m1*	*s2/m2*	AG + IM	A
5		*s1b/m1*	*s1b/m2*	AG + IMA	A
6		*s1b/m1*	*s2/m2*	AG	A
7		*s1b/m1*	*s1b/m2*	AG + IM	B (Low GCIR)
8	*babA2*	Pos	Neg	NAG	A
9		Neg	Pos	AG + IM	A

Table 5 Statistical analysis for the prevalence of *H pylori* genes or alleles in Costa Rican clinical isolates from groups A and B

Gene/allele	Group A (*n* = 41, %)	Group B (*n* = 35, %)	OR (95% CI)	*P*-value
cagA	36 (87.8)	23 (65.7)	3.9 (1.2-12.9)	0.026
vacA s1b	36 (87.8)	24 (68.6)	3.6 (1.1-12.1)	0.041
vacA m1	33 (80.5)	22 (62.9)	2.7 (0.9-8.0)	0.068
babA2	19 (46.3)	15 (42.9)	1.1 (0.4-2.8)	0.812

Table 6 Adjusted odd ratios with 95% confidence intervals and *P*-value for combinations of host and bacterial factors according to gastric cancer in Costa Rican *H pylori*-positive patients

Combination of factors	Gastric Cancer		
	Pos/Neg	OR (95% CI)	*P*-value
IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_C/A			
Pos	10/8	4.7 (1.7-13.0)	0.002
Neg	42/183		
IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_C/A, vacA s1b/m1			
Pos	9/2	7.2 (1.4-36.4)	0.017
Neg	40/74		
IL-1β+3954_T/C, IL-1RN*2/L, vacA s1b/m1			
Pos	18/4	9.8 (2.9-32.9)	< 0.001
Neg	31/72		
IL-1RN*2/L, IL-10-592_C/A, vacA s1b/m1			
Pos	14/9	3.0 (1.1-8.1)	0.028
Neg	35/67		
IL-1β+3954_T/C, IL-10-592_C/A, vacA s1b/m1			
Pos	21/9	4.7 (1.9-11.9)	0.001
Neg	28/67		

H pylori infection[18,20,21]. In particular, IL-1β is important in initiating and amplifying the inflammatory response to *H pylori* infection, resulting in severe inflammation possibly leading to atrophic and metaplastic changes in the gastric mucosa. An association between cytokine polymorphisms in *IL-1β* and *IL-1RN*, and gastric premalignant lesions was previously reported in a Costa Rican population[33], while carriers of IL-1β+3954_T/C and IL-1RN*2/L had an increased risk for developing gastric cancer in another Costa Rican study[34]. Likewise, our results showed that the prevalence of the proinflammatory genotypes IL-1β+3954_T/C and IL-1RN*2/L was significantly higher in gastric cancer cases than in non-cancer cases, supporting the association of polymorphisms within *IL-1β* and *IL-1RN* and gastric cancer in the Costa Rican population. Our results also showed that the carriage of IL-10-592_A/A or IL-10-592_C/A was also associated with an increased risk for gastric cancer, which has been reported previously[21]. This is the first time that polymorphisms within the cytokine gene *IL-10* have been associated with increased risk for gastric cancer in a Costa Rican population. Collectively, these

studies thus suggest that in Costa Rica, the proinflammatory cytokine genetic profile of the host is involved in the development of gastric malignancy; but, it does not seem to play a main role in GCIR variability between regions.

The evaluation of *H pylori* virulence factors revealed that all *H pylori* strains detected in gastric atrophy and/or intestinal metaplasia cases were positive for *cagA*, *vacA s1b* and *vacA m1*, supporting the association of *H pylori cagA* and *vacA* genotype distribution with gastric cancer and premalignant lesions reported in a previous Costa Rican study[29]. In addition, the prevalence of *H pylori cagA* and *vacA s1b* was significantly higher in the high GCIR group than in the low GCIR group, and a tendency for an association between *vacA m1* and GCIR variation was also detected, confirming the association between *H pylori* virulence factors, specifically *cagA*, and the GCIR variability in Costa Rican regions suggested in a previous study[24]. However, additional factors, especially not yet determined host and/or environmental and lifestyle factors could also be involved in GCIR variability in Costa Rica, as it seems unlikely that this phenomenon could be solely explained by the status of *H pylori* infection. The association between several cytokine polymorphisms and gastric cancer reported in this and past Costa Rican studies may support this possibility. Furthermore, this study also showed that carriers of IL-1β+3954_T/C, IL-1RN*2/L and IL-10-592_C/A and carriers of these polymorphisms together with the presence of *H pylori vacA s1b/m1* increased the risk of gastric cancer when compared with patients not carrying any of these factors, suggesting that a synergistic effect of a combination of bacterial and host genotypes may determine the severity of the gastritis and the final outcome of *H pylori* infection. Such a suggestion has been documented in previous studies[18,20,21].

A comparative analysis of the status of the *H pylori* genes in each clinical isolate between antrum and corpus specimens demonstrated that a mixed strain infection (discordant *vacA* genes in the same stomach) was observed in six patients from the high GCIR group, but in only one patient from the low GCIR group. Likewise, the prevalence of gastric premalignant lesions, including gastric atrophy and intestinal metaplasia, was found more frequently in the high GCIR group than in the low GCIR group. The reason for the contrasting prevalence of mixed strain infection and premalignant lesions between high and low GCIR regions is still unknown. One may speculate that during persistent infection by *H pylori* due to yet undetermined factors associated with high population density areas such as urban lifestyle stress or inadequate intake of nutrients, subjects from high GCIR regions develop more severe gastric mucosal injury with atrophic and metaplastic changes, leading to a high genetic diversity of the bacterium for adaptation to this harsh gastric microenvironment. In fact, in strains isolated from Costa Rican patients, a high frequency of recombinated *H pylori* genes (ten of ten strains) has been reported[35]. Alternatively, it does not exclude the possibility that the contrasting prevalence is caused by the difference in the frequency rate of superinfection by *H pylori* strains, which according to population density or yet undetermined factors, may occur more frequently in subjects from high GCIR regions, supposing a higher possibility of infection with the more virulent strains, which in fact have been linked with the development of gastric premalignant lesions. However, such development of premalignant changes and superinfection or genetic recombination within *H pylori* remains unclear as to which is cause and which is effect. Further investigation is essential to understand this issue, especially an investigation which includes an increased number of mixed strain infection-positive cases.

To summarize, our results demonstrated that although the carriage of proinflammatory IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_C/A and IL-10-592_A/A polymorphisms was associated with an increased risk for the development of gastric cancer, the characteristics of *H pylori* infection, in particular the status of *cagA* and *vacA* genotype distribution, seemed to play a major role in gastric cancer incidence rate variability in Costa Rican regions.

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COMMENTS

Background

Costa Rica has one of the highest age-adjusted incidence and mortality rates for gastric cancer. Costa Rica has regions with contrasting gastric cancer incidence rates (GCIR). The cause of gastric cancer is thought to be multifactorial. The risk is high in subjects infected with *Helicobacter pylori* (*H pylori*) and even higher in those infected with strains co-expressing the *cagA*, *vacA s1* and *babA2* genes. Cytokine gene polymorphisms of the host, IL-1β, IL-1RN and IL-10, in response to *H pylori* infection, have also been associated with an increased risk for developing gastric cancer.

Research frontiers

The research in this area is focused on the evaluation of host genetic factors such as interleukin (IL)-1β (-511 and +3954), IL-10 (-1082 and -592) and IL-1RN intron 2 variable number of tandem repeat (VNTR) polymorphisms in response to *H pylori* infection, and *H pylori cagA*, *vacA* and *babA2* genotype distribution on the association with the GCIR variability in Costa Rica. A total of 191 *H pylori*-positive patients were enrolled for the determination of cytokine gene polymorphisms. Clinical isolates from gastric specimens of 83 patients were used for the PCR-based genotyping of the *H pylori cagA*, *vacA* and *babA2* genes.

Innovations and breakthroughs

Cytokine polymorphisms showed no association with GCIR variability. However, gastric atrophy, intestinal metaplasia and strains with different *vacA* genotypes in the same stomach (mixed strain infection) were more frequently found in the high GC risk group than in the low GC risk group, and *cagA* and *vacA s1b* were significantly associated with high GCIR (P = 0.026 and 0.041, respectively). IL-1β+3954_T/C (OR 2.1, 1.0-4.3), IL-1RN*2/L (OR 3.5, 1.7-7.3) and IL-10-592_C/A (OR 3.2, 1.5-6.8) were individually associated with GC, and a combination of these cytokine polymorphisms with *H pylori vacA s1b* and *m1* further increased the risk (OR 7.2, 1.4-36.4).

Applications

Although a proinflammatory cytokine genetic profile showed an increased risk for developing GC, the characteristics of *H pylori* infection, in particular the status of *cagA* and *vacA* genotype distribution seem to play a major role in GCIR variability in Costa Rica.

Peer review

This study revealed that bacterial factors (i.e., *cagA* and *vacA*, but not *babA2*) are involved in regional differences in gastric cancer risk in Costa Rica, although host factors (IL-1B, IL-1RN and IL-10 polymorphisms) are associated individually with gastric cancer risk. There are interesting points found in this study in Costa Rica, where gastric risk and genetic distribution on *H pylori* are uniquely heterogeneous.

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Acute effects of *Helicobacter pylori* extracts on gastric mucosal blood flow in the mouse

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Abstract

AIM: To investigate the mechanisms underlying the reduction in gastric blood flow induced by a luminal water extract of *Helicobacter pylori* (HPE).

METHODS: The stomachs of isoflurane-anesthetized mice were exteriorized, and the mucosal surface exposed. Blood flow was measured with the laser-Doppler technique, and systemic arterial blood pressure monitored. C57BL/6 mice were exposed to water extract produced from *H pylori* strain 88-23. To investigate the role of a nerve- or iNOS-mediated pathway, we used intraluminal lidocaine and iNOS-/- mice. Blood flow response to the endogenous nitric oxide synthase inhibitor asymmetric dimethyl arginine (ADMA) was also assessed.

RESULTS: In wild-type mice, HPE decreased mucosal blood flow by approximately 30%. This reduction was abolished in iNOS-deficient mice, and by pre-treatment with lidocaine. Luminally applied ADMA resulted in reduction in blood flow similar to that observed in wild-type mice exposed to HPE.

CONCLUSION: A *H pylori* water extract reduces gastric mucosal blood flow acutely through iNOS- and nerve-mediated pathways.

Key words: Gastric mucosal blood flow; *In vivo*; Laser-Doppler flowmetry; Mice; *Helicobacter pylori*

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INTRODUCTION

Gastric ulcer and cancer of the stomach have been shown to be associated with the bacterium *Helicobacter pylori* (*H pylori*), which colonizes up to 50% of the human population[1]. It is not known how an infection with this pathogen causes these lesions; but, disruption of the gastric protection mechanisms is certainly involved. We have previously found that a water extract of *H pylori* reduces the mucosal blood flow in rats by a mast cell- and platelet activating factor (PAF)-dependent pathway[2].

PAF is a very potent vasoconstrictor, which also mediates early leukocyte recruitment, and can cause gastrointestinal microcirculatory hypoperfusion[3,4]. PAF is released from a number of inflammatory cells, including mast cells. Mast cells function as "alarm cells" in the gastric mucosa in reaction to infectious material, evoking an inflammatory response[5]. It has been suggested that nerves in the mucosa signal to the mast cells, and inhibition with lidocaine has been found to attenuate mast cell-mediated effects[6].

Gastric mucosal blood flow has a vital role in gastric mucosal protection. A high blood flow is considered good protection against injury, as it dilutes, neutralizes, and removes hazardous substances that have penetrated the gastric mucosal barrier[7,8]. In previous studies, we have shown that high concentrations of luminal acid alone induce hyperemia without any macroscopic lesions[9,10]. Furthermore, these results suggested that epithelial inducible nitric oxide synthase (iNOS) is involved in the hyperemic response to acid, possibly signaling to afferent nerves, leading to a blood flow increase.

It is well known that *H pylori* induces iNOS expression as part of the inflammatory process[11]. Among several other functions, nitric oxide (NO) has antibacterial properties; but, despite this, *H pylori* is able to survive in the presence of the vast amount of NO that is produced. Several explanations for the survival of these bacteria have been proposed, including the production of an L-arginine analogue, asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of NOS activity. In line with this, the formation of ADMA has been demonstrated in the duodenal mucosa on exposure to a water extract of *H pylori*[12].

The aim of this study was to further elucidate the acute effects of *H pylori* on the gastric mucosal blood flow and on distinct signaling pathways. We challenged normal and iNOS-deficient mice with water extracts of *H pylori*. Furthermore, lidocaine was administered intraluminally to investigate if the blood flow response to *H pylori* was nerve-mediated. In addition, we assessed the blood flow response to luminally-applied ADMA.

MATERIALS AND METHODS

All experimental procedures in this study were conducted in accordance with the guidelines of the Swedish National Board for Laboratory Animals and were approved by the Swedish Laboratory Animal Ethical Committee in Uppsala.

Mice

The animals were kept under standardized conditions of temperature (21-22℃) and illumination (12 h light/12 h darkness). They were housed in cages with mesh bottoms, and had free access to tap water and pelleted food (Lactamin, Kimstad, Sweden).

Male C57BL/6 mice (n = 29, B&K Universal, Stockholm, Sweden) were used for all experiments except for the iNOS deficient and wild-type (wt) mice (background C57BL/6 × 129SvEv). Breeding pairs of mice deficient in iNOS were kindly provided by J.S. Mudgett (Merck Research Laboratories, Rahway, NJ, USA) and JD MacMicking and C Nathan (Cornell University Medical College, New York, NY, USA). The mice were generated by gene targeting in embryonic stem cells as previously described[13]. Homozygous iNOS-deficient mutants were maintained by interbreeding the F2 generation (n = 11, Animal Department, Rudbeck Laboratory, Uppsala, Sweden). For wild-type controls male C57BL/6 × 129Sv was used (n = 6, Taconic Farms, Germantown, NY).

H pylori water extracts

The procedure for the preparation of HPE is a modification of that of Xiang *et al*[14], and has been described earlier[15]. HPE were produced from *H pylori* strain 88-23, wt (kindly provided by M. Blaser, Nashville, TN, USA). The concentrated HPE were diluted twice with a 1.8% saline solution in order to obtain a solution with isotonic properties.

Animal preparation

Anesthesia was induced by spontaneous inhalation of isoflurane (Forene®, Abbott Scandinavia AB, Kista, Sweden). The inhalation gas was administered continuously through a breathing mask (Simtec Engineering, Askim, Sweden) and contained a mixture of air, oxygen (total oxygen 40%) and about 2.4% isoflurane. Body temperature was maintained at 37-38℃ by means of a heating pad regulated by a rectal thermistor probe.

A catheter containing heparin (12.5 IU/mL) dissolved in isotonic saline was placed in the left carotid artery to monitor blood pressure. The jugular vein was cannulated for continuous infusion of a modified Ringer solution at a rate of 0.35 mL/h. In some experiments, infusion was performed intra-arterially through a Y-catheter.

The preparation of the mouse gastric mucosa for intravital microscopy has been described previously[16]. Briefly, exteriorization of the mucosa through a midline abdominal incision was followed by an incision along the greater curvature in the forestomach. The animal was placed on a Lucite table with part of the corpus of the stomach loosely draped over a truncated cone in the center of the table, with the mucosal surface facing upwards. A "mucosal chamber" with a hole in the bottom corresponding to the position of the cone was fitted over the mucosa, exposing approximately 0.13 cm^2 of the gastric mucosa through the hole. The mucosal chamber did not touch the mucosa, so as to avoid impairment of blood flow, and the edges of the hole were sealed with silicone grease. The chamber was filled with 3 mL of unbuffered 0.9% saline, maintained at 37℃ by circulation of warm water in a jacket in the bottom of the chamber. The saline was replaced at regular intervals of 10 min and the pH was measured.

Blood flow measurements

Blood flow was measured with laser-Doppler flowmetry (LDF) equipment (Periflux instruments Pf3 and Pf4001, Perimed, Stockholm, Sweden) which had previously been used to study the microcirculatory blood flow of the gastric mucosa in the rat model, as described by Holm-Rutili & Berglindh[17]. In brief, blood flow was recorded as changes in the frequency, that is, the Doppler shift, of monochromatic light from a laser probe (wavelength 633 nm; probe fiber separation 0.5 mm) illuminating a limited area of the tissue. Recorded Doppler-shifted light can be directly and linearly correlated to changes in erythrocyte flux. This flux has been shown to correlate well with gastrointestinal blood flow[17-19]. The laser probe was held in a fixed position in the chamber solution at a distance of 1-2 mm above the mucosa by a micromanipulator. With the type and position of the probe used in these studies, the laser light most likely penetrates through the entire thickness of the gastric wall[20]. However, the recorded blood flow is mainly mucosal, since the amount of back-scattered light decreases exponentially with the depth in the tissue and about 80% of the blood flow of

the stomach perfuses the mucosa. Blood flow was monitored continuously throughout the experiment.

Experimental protocol

The continuously measured blood flow was reported as percent of that during the control period, i.e. the 10 min period, prior to HPE or ADMA application, respectively. Before the experiments, the animals were allowed to stabilize for 45-55 min after surgery. The animals were divided into the following groups: Ⅰ, Control (n = 5); Ⅱ, HPE (n = 6): after a 10 min control period, HPE was applied for 2 × 20 min; Ⅲ, Lidocaine control (n = 6): lidocaine (0.5%) was applied for 3 × 20 min. The last 10 min of the first 20 min period was used as control level; Ⅳ, Lidocaine + HPE (n = 6): lidocaine (0.5%) was applied for 20 min (the last 10 min were used as control level), after which HPE mixed with lidocaine (final concentration 0.5%) was applied for 2 × 20 min; Ⅴ, iNOS control + HPE (n = 6): after a 10 min control period, HPE was applied for 2 × 20 min; Ⅵ, iNOS deficient mice + HPE (n = 6): after a 10 min control period, HPE was applied for 2 × 20 min; Ⅶ, ADMA (500 μmol/L) (n = 6): after a 10 min control period, the NOS-inhibitor ADMA was applied for 2 × 20 min; Ⅷ, iNOS deficient mice + ADMA (500 μmol/L) (n = 5): after a 10 min control period, the NOS-inhibitor ADMA was applied for 2 × 20 min.

The ADMA dose was selected from a previously published *in vivo* study[12], and the pH of the ADMA solution was adjusted with NaOH to that of the saline.

Chemicals

The modified Ringer solution was composed of 120 mmol/L NaCl (Fluka Chemie GmbH, Buchs, Switzerland), 2.5 mmol/L KCl, 25 mmol/L $NaHCO_3$, and 0.75 mmol/L $CaCl_2$ (Merck, Darmstadt, Germany). Other chemicals included heparin (Leo Pharma AB, Sweden), silicone grease (Dow Corning high vacuum grease, Dow Corning GmbH, Weisbaden, Germany), ADMA (N^G, N^G-Dimethylarginine hydrochloride, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and lidocaine (Xylocain, AstraZeneca, Södertälje, Sweden).

Statistics

All values are presented as mean ± SE. Vascular resistance was calculated as the ratio of mean arterial blood pressure (MAP, mmHg) to blood flow (perfusion units). Statistical significance was determined with ANOVA for repeated measurements, followed by Fisher' s protected least significant difference test. The level of significance was set at $P < 0.05$.

RESULTS

Groups Ⅰ and Ⅱ: Control and HPE

In control animals, the mucosal blood flow was stable during the entire measurement period (Figure 1). Water extract from *H pylori* was applied to the exposed gastric mucosa in group Ⅱ. HPE significantly decreased the blood flow to 68% ± 4%, and the resistance increased to 140% ± 12% of the pre-HPE control level (Figure 1). Mean arterial blood pressure was stable around 80 mmHg during the experiments.

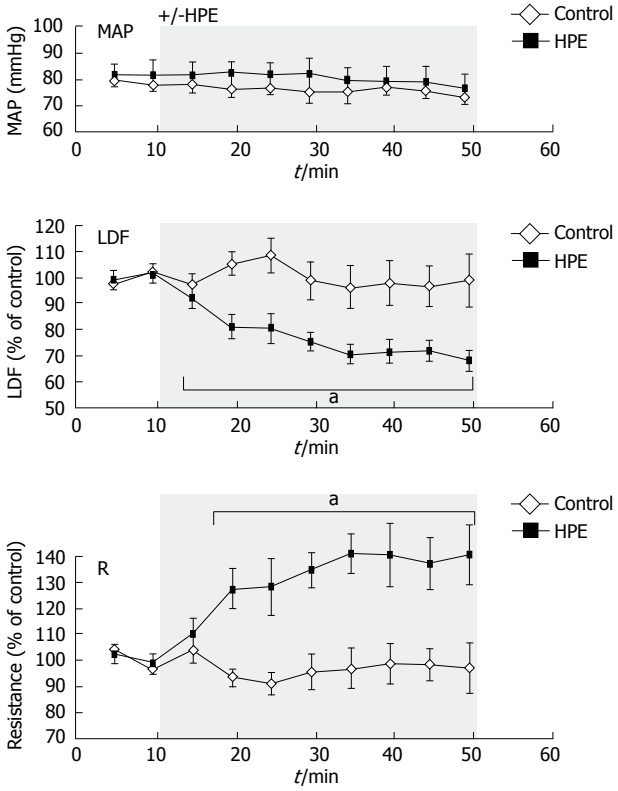

Figure 1 Blood flow measurements in control group (n = 5) and in animals exposed to HPE (n = 6). Mean arterial blood pressure (MAP) is expressed in mmHg, and blood flow (LDF) and vascular resistance (R) in percent of the control period at time-points 5-10 min. The mucosa was exposed to HPE for 40 min. Values are mean ± SE. [a]$P < 0.05$ compared with control period in HPE-treated animals.

Groups Ⅲ and Ⅳ: Lidocaine experiments

Upon application of lidocaine, LDF decreased slightly, but significantly, to 83% ± 5% of the first control value (last 10-minute period of the first 20 minutes with lidocaine) (Figure 2). The blood flow also decreased slightly, but significantly, to 87% ± 5% during the application of lidocaine + HPE. Thus, the slight blood flow reduction was similar in the two groups, i.e. independently of the application of HPE, suggesting a nerve-mediated blood flow reduction. Lidocaine did not influence blood flow during the first 10 min of application, when LDF was 97% ± 3% of the mean value observed 10 min before the lidocaine application (not shown in the figure). Mean arterial blood pressure was stable during the experiments, and not significantly different between the groups.

Groups Ⅴ and Ⅵ: iNOS wt and iNOS-/-

In iNOS wt mice, the blood flow decreased significantly to 71% ± 7% upon exposure to HPE, comparable to the reduction in the control group Ⅱ. In iNOS-/- mice blood flow did not decrease during application of HPE (99% ± 6%, Figure 3), indicating that iNOS is involved in the HPE induced mucosal blood flow reduction.

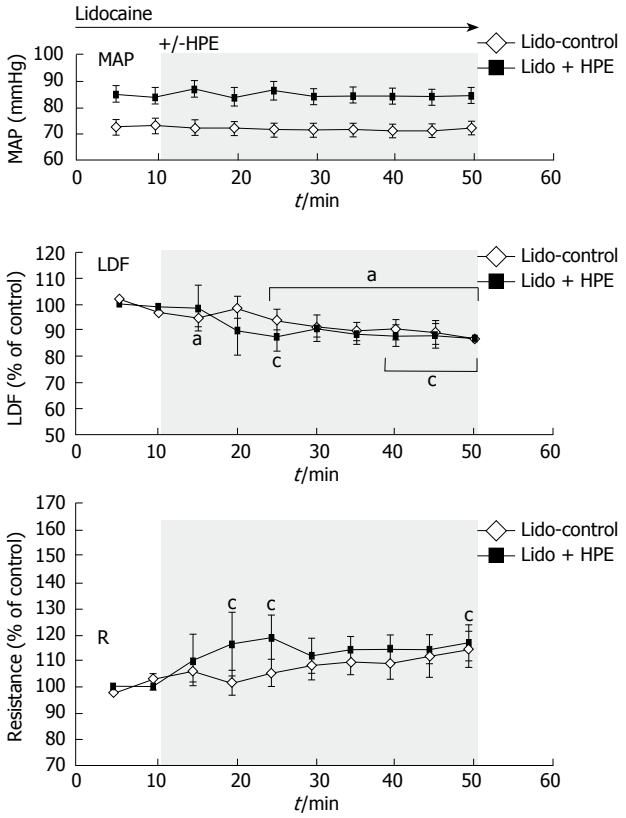

Figure 2 Blood flow measurements in the lidocaine control group (*n* = 6), and in animals treated with lidocaine and HPE (*n* = 6). Mean arterial blood pressure (MAP) is expressed in mmHg, and blood flow (LDF) and vascular resistance (R) in percent of the values of the control period at time-points 5-10 min. The mucosa was exposed to lidocaine for 10 min before the control period started, and then to lidocaine with or without HPE for 40 min. Values are mean ± SE. $^{a}P < 0.05$ compared with the control period in control animals, $^{c}P < 0.05$ compared with control period in HPE-treated animals.

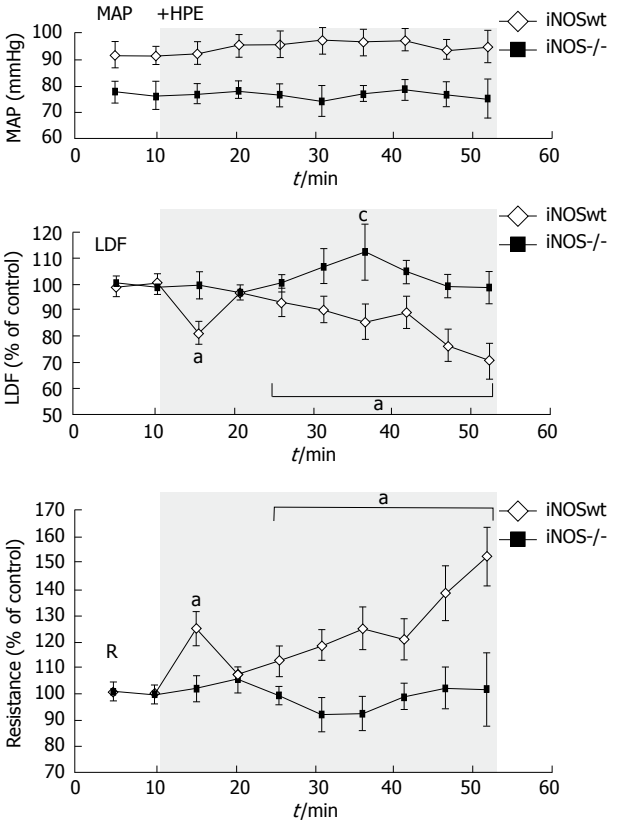

Figure 3 Blood flow measurements in iNOS wild type (*n* = 6), and iNOS-/- mice (*n* = 6), both exposed to HPE. Mean arterial blood pressure (MAP) is expressed in mm Hg, and blood flow (LDF) and vascular resistance (R) in percent of the values in the control period, at time-points 5-10 min. The mucosa was exposed to HPE for 40 min. Values are mean ± SE. $^{a}P < 0.05$ compared with the control period in control animals, $^{c}P < 0.05$ compared with control period in iNOS-/- animals.

Mean arterial blood pressure was stable during the experiments, and not significantly different between the groups.

Groups Ⅶ and Ⅷ: ADMA experiments

During application of the NOS inhibitor ADMA in normal control mice, the blood flow decreased significantly (to 79% ± 5% of the control level, Figure 4). In iNOS-/- mice, blood flow did not decrease during application of ADMA (102% ± 7%), indicating that ADMA and HPE have the same effect in this setting. Mean arterial blood pressure was stable during the experiments and not significantly different between the groups.

MAP is usually between 70 and 90 mmHg in the C57BL/6 mice anesthetized with isoflurane. There is a tendency to higher blood pressure values in the C57BL/6x129SvEv wt mice, which was not seen in the iNOS deficient mice of the same genetic background. However, we could not find any correlation between perfusion units (LDF signal) and the blood pressure, which could explain our results. Thus, the highest LDF signal was recorded in the mice with the lowest MAP (Group Ⅲ).

DISCUSSION

In this study, we have addressed the question of how the gastric pathogen *H pylori* influences the gastric mucosal blood flow and its regulation on first contact of the mucosa with water extract containing bacterial components.

When the *H pylori* water extract was applied luminally, the blood flow decreased, in conformity with our previous findings in rats. Results from the present study suggest that the HPE-induced blood flow decrease is nerve-mediated, as inhibition of local nerve activity by application of lidocaine inhibited the reduction in blood flow. In the earlier study in rats, we have also shown that the reduction in blood flow caused by HPE was inhibited both by a mast cell stabilizator, and a PAF receptor antagonist, indicating a possible effect of PAF released from degranulating mast cells[2]. A regulatory relationship between the mucosal nerves and the mast cells has been suggested, as the nerve endings are located in close proximity to the mast cells[21].

We have recently found that a constitutively expressed iNOS in the gastric surface epithelial cells is involved in the regulation of gastric mucosal blood

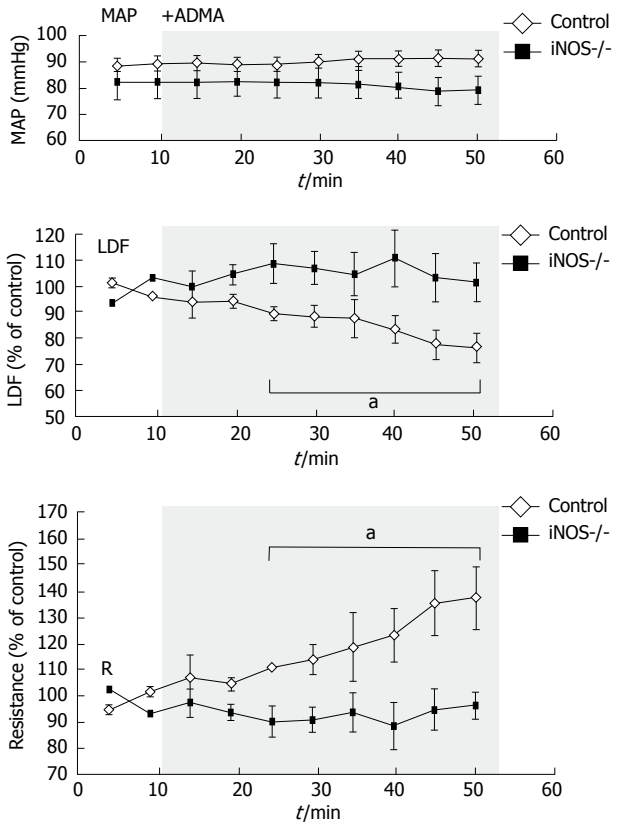

Figure 4 Blood flow measurements in the ADMA control group (*n* = 5-6), and iNOS-/- mice treated with ADMA (*n* = 5). Mean arterial blood pressure (MAP) is expressed in mm Hg, and blood flow (LDF) and vascular resistance (R) in percent of the control period at time-points 5-10 min. The mucosa was exposed to 500 μmol/L ADMA for 40 min. Values are mean ± SE. $^{a}P < 0.05$ compared with the control period.

flow[10]. The blood flow increase found upon gastric luminal application of acid was abolished in iNOS deficient mice. Holm *et al*[22] showed that iNOS played a role in the acid-stimulated HCO_3^- secretion in the duodenum. In the duodenum, HPE reduced acid-stimulated HCO_3^- secretion, and it was suggested that this effect was mediated through inhibition of the constitutively expressed iNOS by an endogenous NOS inhibitor, asymmetric dimethyl arginine (ADMA). ADMA is associated with oxidative stress[23] and the presence of HPE in the duodenum leads to increased levels of ADMA[12,24]. In the current study, the gastric mucosal blood flow reduction in response to HPE did not occur in iNOS-deficient mice. Furthermore, ADMA applied luminally caused a significant blood flow decrease in the same way as did HPE. However, when ADMA was applied luminally in iNOS-deficient mice no blood flow reduction was seen. Thus, the HPE-induced reduction in mucosal blood flow might involve inhibition of epithelial NO production. We have previously reported that the blood flow reducing effect by HPE is NO-independent. These studies were conducted on rats pretreated with the non-selective NOS inhibitor N-nitro-L-arginine (L-NNA). A reasonable explanation for the contradictory results is that L-NNA has a lower selectivity for iNOS compared with other NOS isoforms[25]. At the time of those experiments, the constitutively expressed epithelial iNOS had not been reported, and its role in blood flow regulation was consequently unheard of.

In addition to the constitutively expressed iNOS in the gastric epithelium, it has been found that *H pylori* infection induces further iNOS expression in the gastric mucosa[11], suggesting that excessive amounts of NO could be produced. Indeed, Elizalde *et al*[26] found an increase in the NO level and gastric mucosal blood flow in mice two weeks after *H pylori* infection. However, within four weeks of the infection, the NO concentration and blood flow had decreased to baseline levels[26]. Other studies have also shown lower or baseline levels of NO in infected patients[24,27]. Taken together, these results indicate that *H pylori* might alter the production of NO. The endogenous NOS inhibitor, ADMA, which is produced when HPE interacts with the mucosa, could inhibit the production of NO. Thus, even though iNOS expression is increased by *H pylori*, the epithelial NO production might be inhibited by the bacteria. An arginase produced by the bacteria has also been suggested as a strategy to reduce NO production, as it consumes the arginine-substrate for NOS[28].

In the present study, we investigated the effects of an acute exposure of products from *H pylori*. In a clinical situation it is probably of more interest to investigate the long-term effects of an infection. However, the physiological responses upon first contact with the pathogen are of great importance as it elucidates the early steps of the inflammation. We have recently investigated the effects of a chronic infection with *H pylori* in mice on different protective mechanisms in the stomach[29]. We found that the hyperemic response to luminal acid, earlier shown to be dependent on epithelial iNOS[10], was abolished in the *H pylori* infected mice. Thus, both *H pylori* infection and bacterial products have negative effects on gastric blood flow, which could be a contributing mechanism through which *H pylori* causes gastro duodenal injury.

In conclusion, we have shown that the reduction in gastric mucosal blood flow caused by a water extract of *H pylori* is mediated through iNOS- and nerve-dependent pathways. Our working hypothesis is that the epithelial iNOS is constitutively expressed and involved in the regulation of gastric blood flow in response to luminal contents. The NO produced by iNOS could, among other things, stabilize mast cells, be a signal to nerves or directly dilate blood vessels. HPE contains and/or produces ADMA, and when either of the solutions is applied onto the gastric mucosa, the NO production by iNOS is inhibited. This will remove the signal to maintain an adequate blood flow. Furthermore, reduction in NO-production will destabilize the mast cells, which can degranulate and release, for example, PAF, leading to vasoconstriction. The involvement of nerves is probably more complex, and could include both direct regulation of the blood flow and regulation

of the mast cells. Further studies are needed to elucidate which nerves are important in the blood flow effects induced by HPE.

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COMMENTS

Background

The stomach is frequently exposed to hazardous agents, and to resist this harsh environment, several protective mechanisms exist. Of special interest is the gastric pathogen *Helicobacter pylori* (*H pylori*), which causes gastritis, ulcers and cancer. However, the mechanism leading to these diseases is still unclear. It is very likely that *H pylori* negatively influences the protection mechanisms that exist in the stomach. The aim of the present study was to investigate the mechanisms underlying the reduction in gastric blood flow induced by a luminal water extract of *H pylori* (HPE).

Research frontiers

The authors studied the mechanism by which a water extract of *H pylori* reduces the gastric mucosal blood flow.

Innovations and breakthroughs

This study shows that a *H pylori* water extract reduces gastric mucosal blood flow acutely through iNOS- and nerve-mediated pathways.

Applications

The physiological responses upon the first contact with the pathogen are of great importance as they elucidate the early steps of the pathogen-associated inflammation.

Peer review

The authors showed that a *H pylori* water extract reduces gastric mucosal blood flow acutely through iNOS- and nerve-mediated pathways. The results of this research might be important for the understanding of the mechanisms of gastric inflammation.

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BRIEF ARTICLES

Adherence to surveillance guidelines for dysplasia and colorectal carcinoma in ulcerative and Crohn's colitis patients in the Netherlands

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Abstract

AIM: To study adherence to the widely accepted surveillance guidelines for patients with long-standing colitis in the Netherlands.

METHODS: A questionnaire was sent to all 244 gastroenterologists in the Netherlands.

RESULTS: The response rate was 63%. Of all gastroenterologists, 95% performed endoscopic surveillance in ulcerative colitis (UC) patients and 65% in patients with Crohn's colitis. The American Gastroenterological Association (AGA) guidelines were followed by 27%, while 27% and 46% followed their local hospital protocol or no specific protocol, respectively. The surveillance was correctly initiated in cases of pancolitis by 53%, and in cases of left-sided colitis by 44% of the gastroenterologists. Although guidelines recommend 4 biopsies every 10 cm, less than 30 biopsies per colonoscopy were taken by 73% of the responders. Only 31%, 68% and 58% of the gastroenterologists referred patients for colectomy when low-grade dysplasia, high-grade dysplasia (HGD) or Dysplasia Associated Lesion or Mass (DALM) was present, respectively.

CONCLUSION: Most Dutch gastroenterologists perform endoscopic surveillance without following international recommended guidelines. This practice potentially leads to a decreased sensitivity for dysplasia, rendering screening for colorectal cancer in this population highly ineffective.

Key words: Colorectal cancer; Crohn's disease; Dysplasia; Guidelines; Surveillance; Ulcerative colitis

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INTRODUCTION

Patients with longstanding ulcerative colitis (UC) and Crohn's disease have an increased risk of developing colorectal cancer. This severe complication of inflammatory bowel disease (IBD) generally develops in longstanding disease. If colorectal cancer has developed in patients with IBD, the mortality rate is higher than in patients with sporadic colorectal cancer[1,2]. The lifetime prevalence of colorectal carcinoma (CRC) in UC patients is estimated to be 2% after 10 years, 8% after 20 years, and even 18% after 30 years of extensive disease[3].

Surveillance of IBD for colorectal cancer is widely practiced, and is recommended by the American Gastroenterological Association (AGA), and the British

Society of Gastroenterology (BSG) guidelines[4-6]. These guidelines aim to detect dysplasia or surgically curable cancer, and are thought to improve the prognosis. However, the reduction in mortality in patients with IBD and colorectal cancer through surveillance has still to be proven in large prospective randomized controlled trials. Table 1 gives an overview of the key elements in screening patients with long-standing, extensive IBD as recommended by the AGA[6]. These recommendations are applicable to both UC and Crohn's colitis. Since dysplastic lesions in these patients often present as flat or depressed abnormalities, surveillance colonoscopies should be performed in combination with an extensive biopsy protocol. High-grade dysplasia (HGD) in flat mucosa or a Dysplasia Associated Lesion or Mass (DALM) is considered an indication for colectomy when the pathological findings are confirmed by a second experienced pathologist. There is still no consensus on management in cases of unifocal or multifocal low grade dysplasia (LGD) in flat mucosa. What complicates the issue, as earlier studies have indicated, is that there seems to be difficulty in confirming dysplasia by the pathologist[7,8]. The management of the different forms of dysplasia varies from no management or intensifying the screening program to immediate colectomy. When advising a patient on colectomy, other factors like age, a coexisting diagnosis of primary sclerosing cholangitis (PSC) or a family history of colorectal cancer should be taken into account.

As there are no current Dutch guidelines available regarding surveillance of IBD, we presumed that Dutch Gastroenterologists (GEs) would adopt the current AGA guidelines or other relevant guidelines. To investigate the effect of surveillance guidelines on the detection of dysplasia or colorectal cancer, the first step is to study adherence to these guidelines in clinical practice. This study was designed to assess whether screening programs and recommendations set by e.g. the AGA are used by Dutch GEs for patients with ulcerative or Crohn's colitis and whether the guidelines are followed correctly.

Table 1 Key elements in screening patients with long-standing, extensive colitis, adapted from the AGA and BSG guidelines

	Key element
Surveillance colonoscopy	Colonoscopy with systematic biopsies Perform surveillance every 1 to 2 yr After 8 to 10 yr of disease in those with pancolitis After 15 yr of disease in those with left-sided colitis
Biopsy protocol	Biopsies every 10 cm in all 4 quadrants. Additional biopsies of strictures and mass lesions other than pseudopolyps Polyps that appear potentially dysplastic remove by polypectomy with biopsy of adjacent flat mucosa
Dysplasia	If HGD or multifocal low-grade dysplasia is found in flat mucosa refer for colectomy Presence of low-grade dysplasia, particularly if it is unifocal: no consensus DALM is an indication for colectomy
Other factors of consideration to advise on colectomy	Ongoing colitis-related symptoms Life expectancy Duration, severity and extent of colitis A personal history of primary sclerosing cholangitis A family history of colorectal cancer Discussion around the time of surveillance of benefit, harms, and short comings of colonoscopy surveillance

MATERIALS AND METHODS

After reviewing the widely used guidelines of the AGA, the American College of Gastroenterology and the American Society for Gastrointestinal Endoscopy, in addition to the relevant literature, a questionnaire was developed. The questionnaire was focussed on the use, feasibility and ability to follow the screening guidelines, and contained 18 multiple choice questions and one open question. We asked the invited GEs if they practised surveillance in IBD patients, if they used one of the recommended guidelines and, if not, the reason why not. The other questions, all of a multiple choice design, were divided in four subgroups: (1) start of surveillance; (2) time interval between surveillance endoscopies; (3) biopsy protocol; and (4) management of dysplasia.

In the Netherlands, surveillance endoscopies are usually performed by gastroenterologists. As almost all gastroenterologists are also registered as members of the Dutch Gastroenterology Association, we only included registered gastroenterologists, with the exception of gastroenterologists that were still in training or did not work in Dutch hospitals (n = 34). To ensure the reliability of the answers provided, the questionnaire was anonymous, and to guarantee privacy of the hospitals involved, no questions were asked on the type of hospital (e.g. teaching, non-teaching). The questionnaire could be completed in less than 5 min. To increase the response rate, a reminder was sent to all GEs after 3 wk. Results were tabulated after the second letter. Data were statistically analysed using the Statistical Package for the Social Sciences or SPSS (version 12.0.2) using frequencies.

RESULTS

Of the 244 questionnaires, 153 were returned, yielding an overall response rate of 63%. Five GEs were excluded from further analysis: 2 were recently retired, 1 was currently working in another country and 2 stated they had no experience with IBD patients. The remaining 148 were analysed (61%).

Reasons for non surveillance

Seven (5%) GEs did not provide surveillance for their IBD patients. Four GEs indicated that they would only include IBD patients in a surveillance program in cases with a positive family history of CRC, while 2 considered the available evidence in the literature to be insufficient

to justify screening in this patient category. One GE did not explain his or her motivation.

Surveillance of patients

Of all responding GEs, 95% (n = 141) provided surveillance for their IBD patients. Of these GEs, 46% stated that they did not follow any of the recommended guidelines, 27% followed the AGA guidelines, and 27% used a local protocol. Only 2 GEs followed the British guidelines. All GEs performed surveillance in UC patients, and 65% performed surveillance in patients with Crohn's colitis.

All further results are based on the 95% (n = 141) of GEs who performed surveillance on IBD patients.

Start surveillance

The start of surveillance depends on which time point is taken as the starting point, i.e. the diagnosis of IBD. This can be crucial as there can be a substantial delay between the onset of symptoms and diagnosis of IBD. Sixty nine percent started surveillance from the moment a firm diagnosis of Crohn's disease or UC was made, whilst 31% started surveillance from the onset of IBD symptoms.

When asked how the duration of disease influenced their policy on commencement of surveillance for pancolitis or left-sided colitis, 53% of the GEs stated that they initiated colonoscopic screening for pancolitis after 8 to 10 years while 44% started screening after 10 to 15 years for left-sided colitis, which is in line with the AGA guidelines (Figure 1A).

The extent of colitis also plays a role[3,9]. Six percent of the GEs would screen patients with disease activity limited to the rectum, 68% would screen patients with left-sided colitis, and 26% would screen only in the case of pancolitis.

When asked what other factors would influence their screening protocol, 42% of the GEs mentioned PSC, 30% mentioned a positive family history of CRC, co-morbidity and general health, while 28% of the GEs did not take any factor into consideration.

Time interval between surveillance

Fifty-three percent of the GEs performed colonoscopic surveillance every 1 to 2 years, which is consistent with the AGA guidelines, whilst 22% performed surveillance once every 3 years in the second decade, once every 2 years in the third decade, and once a year in the fourth decade which is consistent with the British guidelines, 14% performed surveillance once every 3 years, and the rest of the GEs performed surveillance at different intervals without a specific protocol.

Biopsy protocol

Forty three percent of the respondents stated that they take biopsies every 10 cm in every quadrant. The remaining GEs took 2 to 4 biopsies from different bowel segments (cecum, colon ascendens, colon tranversum, colon descendens, sigmoid, and rectum). Only 27% of all the respondents obtained more than 30 biopsies per colonoscopy, as recommended by the guidelines and Rubin *et al*[10]. Overall, the mean number of biopsies taken was 24 (Figure 1B).

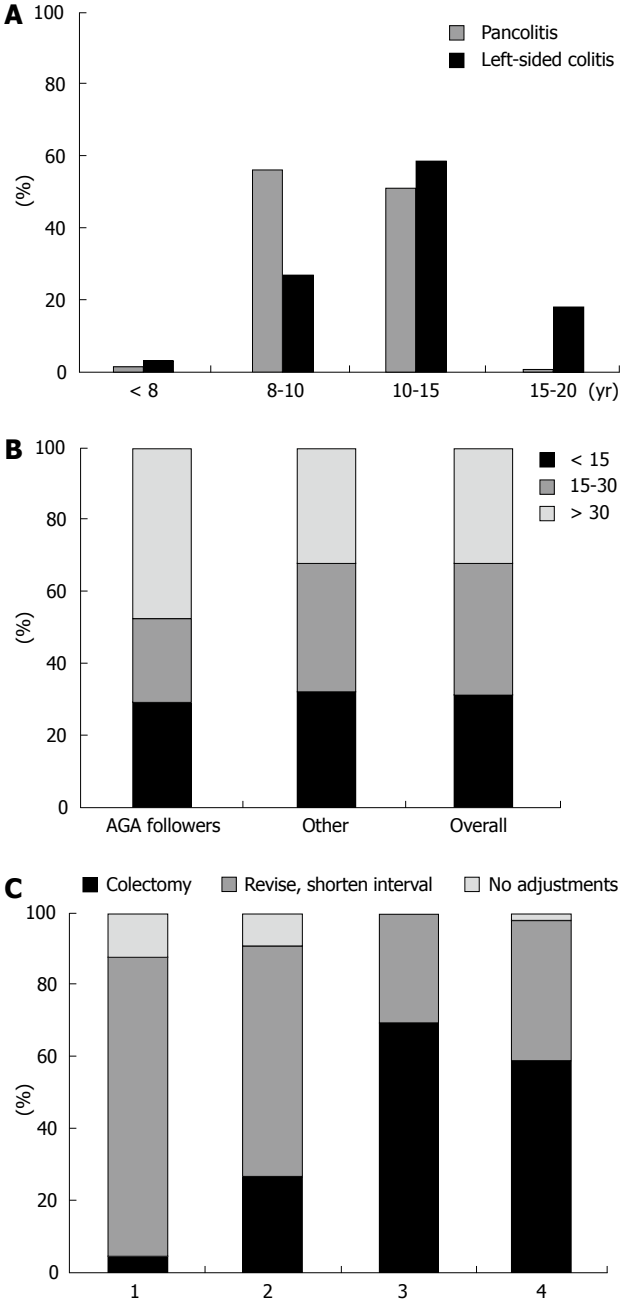

Figure 1 Different aspects of adherence to surveillance guidelines by Dutch GEs. A: Starting point of surveillance for colitis patients. Bars represent the percentage of Dutch Gastroenterologists who would start surveillance in patients with pancolitis (grey) or left-sided colitis (black) after different intervals following diagnosis (in years); B: Number of biopsies taken per colonoscopy. The first column represents clinicians claiming to follow the AGA guidelines in their clinic ("AGA followers"), the second column represents clinicians following other house protocols ("Other"). In the third column the overall results are depicted. Number of biopsies are shown per category (less than 15 biopsies in black, 15 to 30 biopsies in grey and 30 or more biopsies in pale grey); C: Management of Dysplasia. Columns represent unifocal LGD (1), multifocal LGD (2), HGD (3) and DALM (4). The percentages of doctors who would perform a colectomy (black), a revision of the pathology and/or shortening of the surveillance protocol (grey) or would not make any adjustment (pale grey) are shown.

Management of dysplasia

When asked which policy was adopted during the follow-up period when unifocal LGD, multifocal LGD, HGD, DALM or CRC was observed, the GEs responded as shown in Figure 1C. If dysplasia was confirmed, 47% of the GEs advised that they would revise the histopathology, while 40% advised that they would obtain new biopsies, and 13% advised that they would do both. In the case of a DALM, 60% of the GEs would take biopsies from the lesion and the surrounding area, 36% would take biopsies from the lesion only, and 4% would remove the lesion endoscopically.

If a subtotal colectomy was performed, 83% would screen the rectum, and 22% of the 83% would screen once every 4 to 5 years, 54% would screen once every 2 years, while 7% would screen, but not on a regular basis.

DISCUSSION

Most Dutch gastroenterologists perform endoscopic surveillance without following international recommended guidelines. Although, surveillance guidelines are widely used, there is no agreement on surveillance guidelines or a national surveillance protocol in Netherlands. We studied the surveillance practice of Dutch GEs using a postal questionnaire. Overall, 153 out of 244 questionnaires were returned, a response rate which was comparable to most questionnaire-based studies directed at physicians[11]. We, therefore, assume that this study gives a representative overview of the use, feasibility, and ability to follow surveillance guidelines in Netherlands and that the results reflect the practice of a representative number of Dutch GEs. It is possible that the GEs who answered this questionnaire were more in favour of surveillance than GEs that did not fill out the questionnaire, which might have led to information bias. All GEs who provided surveillance agreed that screening patients with UC is necessary. It appears from the literature that not only UC, but also Crohn's colitis is associated with an increased risk of colorectal cancer and, therefore, most experts recommend the use of the same guidelines for both UC and Crohn's colitis[4,6]. However, only 65% of Dutch GEs provide surveillance for patients with Crohn's colitis.

We compared our results with the guidelines set by the AGA. Firstly, we observed a large discrepancy between answers from GEs regarding the principle of surveillance in general, and the responses they provided related to their exact employment of surveillance in daily practice. Furthermore, although both pancolitis and left-sided colitis are associated with an increased risk of CRC[3], a quarter of the GEs do not provide surveillance for patients with left-sided colitis. On the other hand, a small group of GEs considered disease activity limited to the rectum an indication for screening, although there are no data to support the concept that proctitis increases the risk of CRC. All these inconsistencies could result in inefficient surveillance and missed dysplasia or even cancer.

The time between onset of symptoms and confirmed diagnosis of IBD can also differ substantially. Although there is no consensus on this subject, this difference in opinion might potentially lead to a delay in screening of months or even years.

Another important aspect of surveillance for CRC in IBD is adherence to the biopsy protocol. The median number of biopsies taken amongst Dutch GEs was 24 (range 10-40), while only 27% of the GEs approached the recommended number of 33 random biopsies. This number of biopsies was estimated to be necessary to detect possible dysplasia with a sensitivity of 90%[10]. A similar questionnaire-based study in New Zealand showed a median number of 17 biopsies[12]. This again, will inevitably lead to a pronounced decrease in sensitivity, rendering the surveillance tool ineffective.

If dysplasia is detected histopathologically, there seems to be uncertainty as to how to proceed with clinical decision-making. In the case of unifocal LGD, most of the Dutch GEs would have the histopathology revised, and would shorten the time interval to the next colonoscopy. If multifocal LGD is detected, Dutch GEs hesitate to recommend immediate colectomy, but prefer to revise the histopathology by consulting another pathologist or order a new colonoscopy with biopsies. Another suggested option was to shorten the time interval between screenings. Although controversy exists regarding the treatment policy which should be adopted after diagnosing dysplasia in patients with colitis, most experts agree that in all cases of confirmed dysplasia a colectomy should be recommended. Even the presence of LGD, which is associated with CRC in 21.4%-54%, can be considered an indication for surgery[4,6]. There is a disconcertingly low referral rate for colectomy amongst Dutch GEs, and even more so when findings are compared with 3 similar questionnaire-based studies in New Zealand, United Kingdom and Canada[12-14]. It is remarkable that the referral rate is higher for LGD and much lower for HGD and DALM compared with the other studies. The difficulty in confirming dysplasia, the lack of consensus for management of LGD, and the underestimation of the potential risk of LGD and HGD may contribute to the cautious management of LGD and HDG in Dutch GEs. Another reason could be that in the UK and the USA, the guidelines have already been implemented, which would explain the higher referral rate of cases with HGD in these countries.

In conclusion, 95% of Dutch GEs offer some form of surveillance, but most do not adhere to international guidelines. This leads to a decreased sensitivity for dysplasia, rendering this surveillance practice less effective. Furthermore, the management of dysplasia, even in cases of DALM, is inconsistent and will potentially lead to delays in the diagnosis of carcinomas. We suspect that this deviation from the guidelines is a general phenomenon in clinical practice, and is not only restricted to the Netherlands. Implementation of national guidelines and education of GEs concerning all aspects of colonoscopic surveillance is of great importance and will lead to a more consistent and efficient surveillance practice.

COMMENTS

Background

Patients with longstanding ulcerative colitis (UC) and Crohn's colitis harbour an increased risk of developing colorectal cancer. It is generally agreed that screening and surveillance is a rational strategy in these patients, although the optimal screening strategy and approach to managing outcomes is still being debated.

Research frontiers

Data from recent studies and new endoscopic techniques have changed the concepts on which surveillance guidelines have been built. Still, surveillance will depend on colonoscopy and requires commitment from both patients and gastroenterologists (GEs). Implementation of widely accepted guidelines is indispensable in realising optimal efficacy of a surveillance protocol. The challenge is to acquire nationwide support; only this will lead to a more consistent and efficient surveillance practice.

Innovations and breakthroughs

The authors report that most Dutch GEs offer some form of surveillance, although the majority do not follow international guidelines. This potentially results in the delayed diagnosis of advanced neoplasia in these patients, such as Dysplasia Associated Lesion or Mass (DALM), high-grade dysplasia (HGD) and colorectal carcinoma (CRC). The data are in line with studies from the UK, Canada and New Zealand, and call for more awareness on the level of national gastroenterology associations and GEs alike.

Applications

Implementation of national guidelines and education of GEs concerning all aspects of colonoscopic surveillance is of great importance and will probably lead to a more consistent and efficient surveillance practice.

Peer review

In this manuscript, authors describe the results of their questionnaire regarding the association between the risk of CRC in patients with inflammatory bowel disease (IBD) and endoscopic screening. They conclude that implementation of national guidelines and education of GEs concerning all aspects of colonoscopic surveillance in IBD patients is of great importance. The findings are of interest.

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BRIEF ARTICLES

Features of hepatocellular carcinoma in cases with autoimmune hepatitis and primary biliary cirrhosis

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Abstract

AIM: To characterize the clinical features of hepatocellular carcinoma (HCC) associated with autoimmune liver disease, we critically evaluated the literature on HCC associated with autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC).

METHODS: A systematic review of the literature was conducted using the Japana Centra Revuo Medicina database which produced 38 cases of HCC with AIH (AIH-series) and 50 cases of HCC with PBC (PBC-series). We compared the clinical features of these two sets of patients with the general Japanese HCC population.

RESULTS: On average, HCC was more common in men than in women with AIH or PBC. While many patients underwent chemolipiodolization (CL) or transcatheter arterial embolization (TAE) (AIH-series: P = 0.048 (*vs* operation), P = 0.018 (*vs* RFA, PEIT); PBC-series: P = 0.027 (*vs* RFA, PEIT), others refused therapeutic interventions [AIH-series: P = 0.038 (*vs* RFA, PEIT); PBC-series: P = 0.003 (*vs* RFA, PEIT)]. Liver failure was the primary cause of death among patients in this study, followed by tumor rupture. The survival interval between diagnosis and death was fairly short, averaging 14 ± 12 mo in AIH patients and 8.4 ± 14 mo in PBC patients.

CONCLUSION: We demonstrated common clinical features among Japanese cases of HCC arising from AIH and PBC.

Key words: Autoimmune hepatitis; Autoimmune liver disease; Hepatocellular carcinoma; Literature review; Primary biliary cirrhosis

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INTRODUCTION

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerotic cholangitis (PSC) form the triad of autoimmune liver diseases. As defined by Mackay *et al*[1], AIH is a chronic active hepatitis resulting from several distinct autoimmune phenomena. While the anti-inflammatory effects of steroid therapy for this disease may inhibit the promotion of liver carcinogenesis, hepatocellular carcinoma (HCC) does occur rarely in patients with this condition (in about 0.5% of AIH cases)[2,3].

In contrast to AIH, PBC results from an autoimmune mechanism causing chronic cholestasis and chronic non-suppurative destructive cholangitis in medium sized intrahepatic bile ducts[4]. Rare cases of HCC arising from PBC have been reported to date. However,

this association is rare (affecting between 0.3% and 4.22% of cases)[5-11], because a PBC patient's ability to produce regenerative nodules is weak[5-9,12]. Additionally, PBC is pathologically characterized in chronic non-suppurative destructive cholangitis (CNSDC), and the main inflammatory lesions associated with PBC are not hepatocytes, but cholangiocytes, which may be one of the reasons why the incidence of HCC with PBC is low, especially at the early stage when cirrhotic and fibrotic changes do not progress. Recently, reports have suggested that the prevalence of HCC arising from both AIH and PBC is higher than previously believed. In 2001, Caballeria *et al*[13] found that the incidence of HCC in patients with advanced PBC (Scheuer histological stage Ⅲ or Ⅳ) was 11.1%, approximating the 15% incidence in patients with HCV-related cirrhosis (RR 0.812, 95% CI 0.229-2.883). The clinical features of HCC associated with AIH and PBC, however, have not yet been extensively described. Here, we performed a systematic literature review of HCC cases associated with AIH and PBC in Japan, a country with a high burden of autoimmune liver disease. We conducted a critical analysis of case reports to find common themes in the demographic and clinical histories of patients with HCC associated with AIH and PBC.

MATERIALS AND METHODS

We performed a systematic literature review of case reports published in Japan and listed in the Japana Centra Revuo Medicina database, version 3 (systematic literature search system through a computer web site for Japanese literature), using the keywords "hepatocellular carcinoma", "autoimmune hepatitis", and "primary biliary cirrhosis". The database search was limited to the period between 1990 (when the hepatitis C virus was first detected) to the present. The quality of this database available for analysis is thoroughly well-documented. In total, 38 cases of HCC associated with AIH, and 50 cases of HCC associated with PBC were identified. No cases were duplicated, and patients were identified across multiple Japanese medical centers. Most patients in the series had been diagnosed with autoimmune liver disease before HCC was identified. Several cases also presented with co-factors of liver damage and HCC development other than AIH or PBC, such as excessive alcohol intake, HBV, or HCV infection. However, no cases had evidence of hemochromatosis or α1-antitrypsin deficiency. The demographics of these two groups were recorded based on gender, age, period of medical observation, and history of blood transfusion or excessive alcohol intake. Clinical data was also recorded to determine noncancerous pathologies of the liver, HBV or HCV infection status, serum α-fetoprotein (AFP) level, maximal tumor size, history of HCC therapy, clinical outcomes, and cause of death. Cases that did not include a description of alcohol intake were assumed not to have histories of excessive alcohol intake.

We confirmed that all 38 identified cases of HCC associated with AIH met generally accepted international criteria for diagnosis of AIH[14]. Scoring was performed prior to AIH therapy initiation; all scores were greater than 10, and thereby classified as either "probable AIH" or "definite AIH".

Because no internationally accepted diagnostic criteria yet exists for PBC, we utilized the Japanese standard criteria for PBC diagnosis, a standard first proposed in 1992 by a clinical study group supported by the Japanese Ministry of Welfare. According to this standard, PBC diagnosis requires that cases meet at least one of the following criteria: (1) pathologic evidence of CNSDC and positive anti-mitochondrial antibody (AMA) or anti-PDH antibody titers, (2) positive AMA or anti-PDH antibody titers and non-CNSDC pathology compatible with PBC, or (3) no liver biopsy, but, positive AMA or anti-PDH antibody titers and a clinical picture and clinical course compatible with PBC. We confirmed that all 50 identified cases of HCC associated with PBC met the above diagnostic criteria. Six of 50 (12.0%) HCC cases with PBC met the third criteria for PBC, and 44 of 50 (88.0%) cases met the first or second criteria for PBC. The third criteria for PBC remain ambiguous, and it is really hoped that internationally accepted criteria will be determined for PBC diagnosis.

If a case met both generally accepted international criteria for diagnosis of AIH, and the Japanese standard criteria for PBC diagnosis, we diagnosed the case as overlap syndrome. We had two cases of overlap syndrome, and excluded these cases from our analysis.

We did not include a control group, but used the general HCC population in Japan for comparison[15].

Statistical analysis

Intention-to-treat analyses were used throughout, and statistical analysis for categorical comparisons of the data was performed using the program ystat2006.xls for Windows/Macintosh (Igaku Tosho Shuppan Corporation, Tokyo, Japan). We used the χ^2 test and Fisher's exact test for categorical comparisons between patients with HCC associated with AIH or PBC and HCC patients without associated autoimmune disease[15]. The following variables were assessed: gender, HBV or HCV co-infection, history of blood transfusions, history of excessive alcohol intake, positivity for serum-AFP and clinical outcomes. Because the baseline male to female ratio of AIH and PBC was 1:7 and 1:9, respectively, we performed the χ^2 test for males and females separately. We also used the χ^2 test with or without the Yates correction for categorical comparisons of pathological findings of noncancerous lesions of the liver, HCC therapy choices, and cause of death. Where significant differences were noted, χ^2 tests or Fisher's exact tests were repeated with all categorical combinations, using Bonferroni corrections for multiple comparisons. Two tailed Mann-Whitney *U*-tests and *F*-tests were performed at the 5% significance level only for comparisons between HCC patients with AIH and PBC, as the following variables were unavailable for the general HCC

Table 1 Developement period of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Observation period (mean ± SD)	Total: 38 1 yr 1 mo-23 yr (10 yr 6 mo ± 6 yr 7 mo)	Total: 49 3 mo-24 yr (9 yr 4 mo ± 6 yr 4 mo)	NA	NA	NA	$P = 0.307$ ($P = 0.815$)
Interval between liver damage and HCC diagnosis (mean ± SD)	Total: 34 0-22 yr 9 mo (10 yr 2 mo ± 6y 5 mo)	Total: 40 0-24 yr (9 yr 9 mo ± 7 yr 0 mo)	NA	NA	NA	$P = 0.740$ ($P = 0.688$)
Period from HCC development to death (mean ± SD)	Total: 18 2 mo-3 yr (1 yr 2 mo ± 12 mo)	Total: 16 0-5 yr (8.4 ± 14 mo)	NA	NA	NA	$P = 0.047$[a] ($P = 0.401$)

The *P*-value above was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. [a]$P < 0.05$, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; SD: Standard deviation; NA: Not available.

Table 2 Analysis on gender and age of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Gender						
Actual number	Total: 38	Total: 50	Total: 16743			
Male	7 (18.4)	13 (26.0)	12025 (71.8)			
Female	31 (81.6)	37 (74.0)	4718 (28.2)	$P = 0.149$[1]	$P = 0.512$[1]	$P = 0.244$
Relative number	Total: 38	Total: 50				
Male	23.3 (61.3)	38.0 (76.0)				
Female	14.7 (38.7)	12.0 (24.0%)				
Age at HCC diagnosis	Total: 38	Total: 50	Total: 16743			
(mean ± SD)	(67.61 ± 8.58)	(68.54 ± 9.30)	NA			
< 40 s	0 (0)	2 (4.0)	761 (4.6)	NA	NA	$P = 0.410$
50 s	8 (21.0)	6 (12.0)	2818 (16.8)			($P = 0.614$)
60 s	16 (42.1)	21 (42.0)	6179 (36.9)			
70 s	9 (23.7)	14 (28.0)	5976 (35.7)			
< 80 s	5 (13.2)	7 (14.0)	1009 (6.0)			

The *P*-value above was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. [1]The *P*-value was calculated from the relative numbers. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; SD: Standard deviation; NA: Not available.

population: interval between liver damage and HCC diagnosis, interval from HCC diagnosis to death, age at HCC diagnosis, serum-AFP levels, maximum tumor size and number of HCC loci. Because the patient sample size in each group was greater than 20, we chose to use *P*-values calculated from the asymptotic distribution. The total number of cases in each patient group did not include cases for which categorical data were unknown (Table 1).

The statistical analysis for survival among HCC patients with AIH and PBC was performed on a personal computer with the statistical package SPSS for Windows (version II, SPSS Inc., Chicago, IL, USA). Because there were too few published cases of HCC arising from AIH or PBC, however, differences in survival between patient groups could not be calculated.

RESULTS

The intervals between HCC diagnosis and death for HCC patients with AIH (14 ± 12 mo) and PBC (8.4 ± 14 mo) was notably shorter than among general HCC patients in Japan (77.5% 1-year survival, 52.5% 3-year survival, and 35.4% 5-year survival)[15]. As shown in Table 1, the survival interval for HCC patients with PBC was also significantly shorter than that for patients with AIH ($P = 0.047$).

Among HCC cases associated with AIH, the actual male to female ratio was 7:31. Because AIH patients in Japan are predominantly female (7:1), the corrected risk ratio for HCC among male AIH patients was 1.6:1 relative to females, and the male to female ratio of the relative numbers was 23.3:14.7 (Table 2). The majority of Japanese PBC patients are also female, outnumbering

Table 3 Clinical status of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/General-HCC patients	PBC-series/General-HCC patients	AIH-series/PBC-series
History of blood transfusion	Total: 29	Total: 38	Total: 12602	$P = 0.040$[a]	$P = 0.581$	$P = 0.041$[a]
+	3 (10.3)	13 (34.2)	3633 (28.8)			
-	26 (89.7)	25 (65.8)	8969 (71.2)			
History of excessive alcohol intake	Total: 38	Total: 50	Total: 14694	$P = 0.812$	$P = 0.056$	$P = 0.352$
+	1 (2.6)	5 (10.0)	3271 (22.3)			
-	37 (97.4)	45 (90.0)	11423 (77.7)			
Co-infection	Total: 33	Total: 40	Total: 4121			
HBV (prior) +	2 (6.1)	10 (25.0)	2138 (51.9)	$P < 0.001$	$P < 0.001$	$P = 0.025$
HBV (prior) –	31 (93.9)	30 (75.0)	1983 (48.1)			
Co-infection	Total: 38	Total: 49	Total:16492			
HCV +	3 (7.9)	10 (20.4)	11488 (69.7)	$P < 0.001$	$P < 0.001$	$P = 0.044$
HCV -	35 (92.1)	39 (79.6)	5004 (30.3)			
Pathological findings of noncancerous lesion of the liver	Total: 31	Total: 44	Total: 4941	$P = 0.163$	$P = 0.007$[b]	$P = 0.489$
NL, CH, LF	13 (41.9)	15 (34.1)	2691 (54.5)			
LC	18 (58.1)	29 (65.9)	2250 (45.5)			

HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NL: Normal liver; CH: Chronic hepatitis; LF: Liver fibrosis; LC: Liver cirrhosis.

males by 9:1. The relative risk ratio for HCC among males with PBC was 3.2:1 relative to females, and the male to female ratio of the relative numbers was 38:12 (Table 2). No significant differences in male to female ratios were noted between the three patient groups ($P = 0.149$, $P = 0.512$, $P = 0.244$, respectively).

Among the HCC cases associated with AIH, only three (10.3%) had a history of blood transfusions, while 13 (34.2%) of the cases with PBC had such a history. Among all Japanese patients with HCC, 3633 (28.8%) had a history of blood transfusions[15]. The proportion of HCC cases associated with AIH having a history of blood transfusions was significantly lower than that of the general HCC cases in Japan ($P = 0.040$), and the proportion of HCC cases associated with PBC having a history of blood transfusions was significantly greater than that of the HCC cases associated with AIH ($P = 0.041$, Table 3).

Similarly, only one case (3.1%) of HCC associated with AIH had a history of excessive alcohol intake, while five (20.0%) cases associated with PBC had such a history ($P = 0.352$, Table 3). Among all Japanese patients with HCC, 3271 (22.3%) had a history of excessive alcohol intake[15].

While prior infection with HBV was relatively rare among AIH patients (6.1%), it was much more prevalent among patients with PBC (25.0%, $P = 0.025$). Similarly, 7.9% of AIH patients tested positive for HCV, as compared to 20.4% of PBC patients ($P = 0.044$). The population of Japanese HCC patients without autoimmune liver disease had significantly higher rates of both HBV and HCV co-infection ($P < 0.001$, Table 3).

Among the HCC cases associated with AIH, 18/31 (58.1%) were found to have cirrhosis on examination of liver biopsy samples or resected samples at operation. In contrast, 29/44 (65.9%) of the HCC cases associated with PBC were found to have cirrhotic liver tissue. Within the general HCC population in Japan, 2250 of the 4941 cases for which liver specimens were available (45.5%) showed evidence of cirrhosis[15]. While the proportion of liver cirrhosis among HCC cases associated with PBC was significantly greater than that in the general HCC population in Japan ($P = 0.007$), no statistical significance in the prevalence of cirrhosis was found between AIH-associated HCC and general HCC patients ($P = 0.163$, Table 3).

The numbers and positive ratios of the AIH-series, PBC-series and general-HCC patients were 22/37 (59.5%), 34/47 (72.4%) and 10075/15831 (63.6%), respectively. No significant differences in positive ratios of serum-AFP were noted between the three patient groups ($P = 0.597$, $P = 0.216$, $P = 0.214$, respectively, Table 4). AFP levels at diagnosis were 2340.2 ng/mL (range 1-49100 ng/mL) among patients with AIH, and 854.2 ng/mL (range 4.2-14646 ng/mL) among patients with PBC. The maximum size of the primary hepatic tumor at diagnosis was 3.97 cm (range 1.0-10.0 cm) among patients with AIH and 3.51 cm (range 1.0-8.8 cm) among PBC patients (Table 4). Due to lack of available data, we could not compare serum AFP levels, tumor sizes and numbers of HCC loci between the autoimmune-associated HCC cases and the general HCC cases in Japan. However, we found that serum AFP level did not vary widely, and that maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4).

Among both the AIH and PBC patient groups,

Table 4 Serum AFP levels, tumor sizes and number of HCC loci of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/PBC-series
Serum-AFP	Total: 37	Total: 47	Total: 15831			
AFP – (< 15 ng/mL)	15 (40.5)	13 (27.7)	5756 (36.4)	$P = 0.597$	$P = 0.216$	$P = 0.214$
AFP + (⩾ 15 ng/mL)	22 (59.5)	34 (72.3)	10075 (63.6)			
Serum-AFP (ng/mL)	Total: 37	Total: 47	Total: 15831			
(mean ± SD)	(2340.21 ± 8823.45)	(854.18 ± 2263.83)	NA			
< 15	15 (40.5)	13 (27.6)	5756 (36.4)			
15-199	12 (32.5)	16 (34.0)	5786 (36.5)	NA	NA	$P = 0.106$
200-399	1 (2.7)	6 (12.8)	902 (5.7)			($P < 0.001^b$)
400-999	3 (8.1)	2 (4.3)	907 (5.7)			
⩾ 1000	6 (16.2)	10 (21.3)	2480 (15.7)			
Maximum tumor size	Total: 36	Total: 48	Total: 15788			
of HCC				NA	NA	$P = 0.744$
(mean ± SD) (cm)	(3.75 ± 2.42)	(3.51 ± 1.69)	NA			($P = 0.028^a$)
< 2	14 (38.9)	11 (22.9)	5123 (32.4)			
2.1-5.0	16 (44.4)	29 (60.4)	7434 (47.1)			
⩾ 5.1	6 (16.7)	8 (16.7)	3231 (20.5)			
Number of HCC loci	Total: 38	Total: 49	Total: 16187			
(mean ± SD)	(1.58 ± 2.05)	(1.74 ± 1.97)	NA	NA	NA	$P = 0.418$
Single	33 (86.8)	38 (77.6)	9365 (57.9)			($P = 0.805$)
Double	2 (5.3)	7 (14.3)	2850 (17.6)			
Multiple	3 (7.9)	4 (8.1)	3972 (24.5)			

The *P*-value in the first row was calculated from the χ^2 test and Fisher's exact test. The *P*-value in the following row was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. $^aP < 0.05$, $^bP < 0.01$, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; AFP: α-fetoprotein; SD: Standard deviation; NA: Not available.

the most commonly selected forms of treatment were chemolipiodolization (CL) and transcatheter arterial embolization (TAE); other options included percutaneous ethanol injection therapy (PEIT) and radiofrequency ablation (RFA). Differences in the choice of therapeutic procedures were noted as follows, although no comparisons reached statistical significance following the Bonferroni correction: (1) The rate of CL or TAE among HCC patients with AIH was greater than the rate of operations among general HCC patients ($P = 0.048$), (2) The rate of CL or TAE among HCC patients with AIH was greater than the rate of PEIT and RFA among general HCC patients ($P = 0.018$), and (3) The rate of CL or TAE in HCC patients with PBC was greater than the rate of PEIT and RFA among general HCC patients ($P = 0.027$). Additionally, the frequency with which HCC patients with PBC chose to forgo treatment was significantly higher than the frequency with which general HCC patients chose to undergo PEIT or RFA ($P = 0.003$). Although not statistically significant, the frequency with which HCC patients with AIH refused therapeutic interventions was also higher than the frequency of PEIT or RFA in the general HCC population ($P = 0.038$, Table 5). Ideally, data on survival by treatment modality should be presented. However, the number of patients receiving each treatment modality who were able to be followed up to death was small. Hence, the mean period from HCC development to death was calculated from patient survival following all treatment options. Future prospective studies are needed to further analyze mean survival for each treatment alternative.

Across all three patient groups, we found that liver failure was the leading cause of death, followed by rupture of HCC. Among general HCC patients, neoplastic death was most common (1487/2700, 55.1%), although differences between causes of death did not reach statistical significance. Comparisons between patient groups showed that: (1) The rate of neoplastic death in general HCC patients was higher than the rate of variceal rupture in HCC patients with AIH ($P = 0.050$), (2) The rate of neoplastic death in general HCC patients was higher than the rate of gastrointestinal bleeding in HCC patients with AIH ($P = 0.013$), and (3) The rate of neoplastic death in general HCC patients was greater than the rate of variceal rupture in HCC patients with PBC ($P = 0.050$, Table 5).

DISCUSSION

While autoimmune liver disease is more common among women than men in Japan, HCC in our group of patients with autoimmune liver disease was more common in men than women (Table 2). Men with AIH had a 1.6-fold greater risk of HCC than women, while men with PBC had a 3.2-fold greater risk of HCC than women with PBC. Moreover, when we followed AIH and PBC patients during HCC surveillance, we noted that the rate of HCC development was higher in male patients with autoimmune liver disease than in female patients with autoimmune liver disease.

Cirrhosis was found in only 18/31 (58.1%) of HCC

Table 5 Therapy and outcome of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Therapy choices for HCC	Total: 38	Total: 47	Total: 17005	Operation *vs* CL or TAE $P = 0.048$[1]	RFA, PEIT *vs* CL or TAE	
CL or TAE	18 (47.4)	17 (36.2)	4636 (27.2)	RFA,PEIT,MCT *vs*	$P = 0.027$[1]	
Operation	8 (21.0)	16 (34.0)	5268 (31.0)	CL or TAE	RFA, PEIT *vs*	
RFA, PEIT, MCT	6 (15.8)	6 (12.8)	4890 (28.8)	$P = 0.018$[1]	No therapy	
Chemotherapy	0 (0)	0 (0)	765 (4.5)	RFA,PEIT,MCT *vs*	$P = 0.003$[2]	
Others	0 (0)	0 (0)	122 (0.7)	No therapy		
No therapy	6 (15.8)	8 (17.0)	1324 (7.8)	$P = 0.038$[1]		
Clinical outcome	Total: 37	Total: 49	Total: 16646			
Alive	20 (54.1)	31 (63.3)	13946 (83.8)	$P < 0.001$[b]	$P < 0.001$[b]	$P = 0.389$
Dead	17 (45.9)	18 (36.7)	2700 (16.2)			
Cause of death	Total: 16	Total: 18	Total: 2700	Neoplastic death	Neoplastic death	
Liver failure	8 (50.0)	8 (44.4)	581 (21.5)	*vs* variceal rupture	*vs* variceal rupture	
HCC rupture	3 (18.8)	4 (22.2)	172 (6.4)	$P = 0.050$[1]	$P = 0.050$[1]	
Variceal rupture	1 (6.2)	1 (5.6)	85 (3.1)	Cancer death *vs* GI		
GI bleeding	1 (6.2)	2 (11.1)	55 (2.0)	bleeding		
Neoplastic death	0 (0)	0 (0)	1487 (55.1)	$P = 0.013$[1]		
Others	3 (18.8)	3 (16.7)	320 (11.9)			

[b]$P < 0.01$, Statistically significant. [1]The calculated *P*-values did not reach statistical significance with Bonferroni correction; without the correction, however, *P*-values were below 0.05. [2]The calculated *P*-values reached statistical significance with Bonferroni correction. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; CL: Chemolipiodolization; TAE: Transcatheter arterial embolization; RFA: Radiofrequency ablation therapy; PEIT: Percutaneous ethanol injection therapy; MCT: Microwave coagulation therapy; GI: Gastrointestinal.

patients with AIH, 29/44 (65.9%) of HCC patients with PBC, and in only 2250/4941 (45.5%) of the general Japanese HCC population. We did not add cases with liver fibrosis (LF) to the incidence of liver cirrhosis (LC) in the general Japanese HCC population, which may be one of the reasons why the incidence of liver cirrhosis was surprisingly low. Additionally, we think that the HCC cases with PBC and AIH and with non-cirrhotic liver, in which sufficient examinations and successful treatments were performed because of their higher hepatic reserve, were likely to be reported and submitted for publication. The possibility of bias in the selection of the reported cases should be raised.

Another interesting finding was that the interval between HCC diagnosis and death was shorter for patients with autoimmune liver disease than for the general HCC population of Japan[15]. Furthermore, although we found that serum AFP level did not vary widely, the maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4). One explanation for this finding may be a selection bias, as cases which were detected earlier and treated successfully were more likely to be submitted for publication. Despite a smaller tumor size and a lower number of HCC loci in patients with HCC arising in the setting of autoimmune liver disease at the time of HCC diagnosis, a shorter reported survival was not attributed to late detection of HCC and failure to survey patients with autoimmune liver disease for HCC, but was more likely to be due to advanced liver disease and cirrhosis. Future prospective studies will be needed to verify or refute these findings.

Although CL and TAE were the most frequently selected treatment modalities across all patient groups (Table 5), many patients ultimately refused treatment due to advanced age or social circumstances. Medical treatments using CL or TAE may be common because HCC cases are often inoperable due to cirrhotic liver disease in these patients. While survival may be related to the choice of therapeutic options, inconsistencies in data reporting over multiple decades and across multiple medical centers made the calculation of survival data difficult.

Several mechanisms explaining the development of HCC from autoimmune liver diseases have been proposed: enhanced progression to cirrhosis through progressive autoimmune hepatitis, decreased antitumor immune responses caused by long-term administration of steroids and immunosuppressants, or virus-mediated hepatitis[16,17]. In this study, we found significantly higher rates of HBV and HCV among PBC patients with HCC than among AIH patients with HCC. This finding may be attributable to the higher rates of blood transfusion in HCC patients with PBC ($P = 0.041$, Table 3). This result is supported by the findings of Shimizu *et al*[18], who reported that 3/16 (19%) HCC patients with PBC tested positive for prior HBV and present HCV infections. Given the high rates of prior HBV infections among HCC patients with PBC, it is possible that prior HBV infection predisposes patients to HCC through HBV-DNA becoming integrated into hepatocyte DNA. It has been reported that even in patients who test negative for serum HCV-RNA and serum HBV-DNA (less

than the sensitivity of HBV-DNA), liver tissue samples frequently test positive for HCV-RNA or HBV-DNA. This suggests a possible role for positive HCV-RNA or HBV-DNA in hepatic tissue in the development of HCC[19-23]. In the present study, however, only six HCC patients with AIH and two HCC patients with PBC were found to have detectable HBV-DNA and HCV-RNA in liver tissue samples. Aggressive liver biopsies should be taken to allow genetic analysis for HBV and HCV in liver tissue, in order to further study HCC cases with non-B, non-C hepatitis.

We reported high rates of HBV or HCV infection among HCC patients with PBC (Table 3); however, this is less surprising because international diagnostic criteria for AIH allocate negative points for positive HBV or HCV diagnostic tests[14]. Furthermore there are no definitive histological features that allow a clinician or pathologist to distinguish AIH from chronic viral hepatitis. Thus, HBV or HCV infected patients are rarely classified as having AIH in the modern era. In contrast, the unique histological features of PBC and the relative specificity of AMA tests allow clinicians to diagnosis and report cases of concurrent PBC and chronic viral hepatitis with a greater degree of confidence.

It has been reported that HCC develops significantly more often in patients with concurrent PBC and HCV infection than in patients with AMA-positive PBC[9]. The incidence of HCC associated with PBC has been suggested to have increased recently due to prolonged periods of liver cirrhosis resulting from longer survival on steroid therapy, concurrence of the hepatitis virus or alcohol intake with HCC, and the administration of immunosuppressants which may disturb immunoregulatory function[24,25]. In a proportional hazards analysis of patients with PBC, Shibuya *et al*[11] found 3 factors to be independently associated with the development of HCC: age at time of diagnosis, male gender, and a history of blood transfusion. Our findings showed that HCC cases arising from PBC were more common in men and those with liver cirrhosis.

While the number of HCC cases arising from PBC is stated to be small, it has been reported that the calculated crude incidence of HCC was 492.4/100 000 person years, and that HCC has a relatively high prevalence in PBC[7]. Furthermore, there is a dramatically increased risk for development of hepatobiliary malignancies in patients with PBC, with a relative risk of 46 ($P < 0.0001$) in women and 55 ($P < 0.0001$) in men[26].

Finally, several questions remain for the clinician. Namely, should AIH and PBC patients be screened for HCC? Should screening be limited to cirrhosis? Does the clinical course after diagnosis differ from other HCC patients? Late-stage AIH and PBC patients should be screened for HCC just as in HCV-related cirrhosis, given the similar reported incidence of HCC development in late-stage PBC[13]. Furthermore, Suzuki *et al*[27], reported very recently that patients of older age, male sex, history of blood transfusion, and any signs of portal hypertension or cirrhosis should be considered for HCC screening. A prospective study or a case control study for AIH patients is needed similar to that conducted for PBC patients.

At present, HCC transformation in early-stage pre-cirrhotic AIH and PBC were thought to be very rare. However, a high incidence of HCC development was observed in AIH and PBC patients with overlapping HCV and HBV infection, including occult HBV infection[9,19,20]. These patients should be closely followed using ultrasonography, CT-scanning and MRI of the abdomen, as well as tumor markers for HCC. Reports of HCC cases arising from "pure" AIH and PBC (with no history of blood transfusion, excessive alcohol intake, immunosuppressant administration, and with negative HBV and HCV serotyping) are rare[2,27-31]. El-Serag *et al*[32], in a multivariate analysis reported that AIH itself is not significant; however, our study indicates that early-stage AIH and PBC patients also have the potential to develop HCC. We advocate that "pure" or "early-stage" AIH and PBC cases should also be regularly screened for HCC.

Our data also indicate that the clinical course after diagnosis of HCC with AIH and PBC differs from virus-associated HCC, although prospective studies are needed to confirm these results. Clinicians should note the common clinical features of HCC cases with AIH and PBC at diagnosis, treatment, and follow-up of these patients.

Lastly, our findings also beg the question of why HCC rupture is the second most common cause of death in both groups of patients examined. We have recently reported a pelioid-type HCC patient with PBC, who died from rupture of HCC[33]. A peliotic change was observed more frequency in large poorly-differentiated and encapsuled HCC[34], and the features of pelioid-type HCC were high blood flow into the HCC, high pressure in the tumor and fibrous capsular formation. It is unknown whether the ruptured HCCs in the present study had these features, as this study had severe limitations because it was retrospective. Tumors in such patients may grow rapidly, and pathophysiological factors shared by both patient groups may trigger the rupture of HCC. A prospective study on the cause of death and a pathologic study of ruptured HCC with AIH and PBC is awaited with great interest.

Further clinical and laboratory studies are needed to describe which pathological, biological and genetic features are common among HCC cases arising from AIH and PBC. How HCC in these patients relates to viral hepatitis also requires further clarification. The present study was retrospective; however, this is the first study to date that highlights the importance of these future research topics. Future prospective studies on these important subjects are required.

COMMENTS

Background

Hepatocellular carcinoma (HCC) development in autoimmune hepatitis (AIH) as well as in primary biliary cirrhosis (PBC) is a rare event. The common clinical features of HCC associated with AIH and PBC have not yet been extensively

described.

Research frontiers

In this study, we characterized these common features through a systematic review of the literature conducted using the Japana Centra Revuo Medicina database. We demonstrated common clinical features among cases of HCC arising from AIH and PBC in Japan.

Innovations and breakthroughs

We found common clinical features in HCC cases with AIH and PBC as follows, (1) HCC was more common in men than in women with AIH or PBC. (2) Many patients underwent chemolipiodolization (CL) or transcatheter arterial embolization (TAE). (3) Liver failure was the primary cause of death among patients in this study, followed by tumor rupture. (4) The survival interval between diagnosis and death was fairly short.

Applications

The present study was retrospective; however, this is the first study to date that highlights the common clinical features in HCC cases with AIH and PBC. Future prospective studies of these important subjects are required.

Peer review

This is a systematic literature review of HCC cases with AIH and PBC published throughout Japan. The review is clearly written and highlights a very interesting topic.

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BRIEF ARTICLES

Interaction of hepatitis C virus envelope glycoprotein E2 with the large extracellular loop of *tupaia* CD81

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Abstract

AIM: To further analyze the interaction of *tupaia* CD81 with hepatitis C virus (HCV) envelope protein E2.

METHODS: A *tupaia* CD81 large extracellular loop (CD81 LEL), which binds to HCV E2 protein, was cloned and expressed as a GST-fusion protein, and interaction of HCV E2 protein with a *tupaia* CD81 LEL was evaluated by enzyme-linked immunosorbent assay (EIA).

RESULTS: Although *tupaia* and human CD81 LEL differed in 6 amino acid changes, *tupaia* CD81 LEL was strongly recognized by anti-CD81 antibodies against human CD81 LEL conformation-dependent epitopes. Investigating LEL CD81-E2 interactions by EIA, we demonstrated that binding of *tupaia* CD81 LEL GST fusion protein to recombinant HCV E2 protein was markedly reduced compared to binding of human CD81 LEL GST fusion protein to recombinant HCV E2 protein.

CONCLUSION: These data suggest that the structural differences in-between the *tupaia* and human CD81 may alter the interaction of the large extracellular loop with HCV envelope glycoprotein E2. These findings may be important for the understanding of the mechanisms of binding and entry of HCV to PTHs.

Key words: Hepatitis C virus E2 protein; Tupaia; CD81, Bind; Enzyme-linked immunosorbent assay

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause for posttransfusion and community-acquired hepatitis worldwide[1-4]. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC)[5]. Virion contains an approximately 9.5 kb long positive-strand RNA genome encoding for a single polyprotein containing 3010-3030 amino acids[6-8]. The polyprotein is cleaved co- and post-translationally by host cellular and viral proteases into at least ten different products. The predicted structural components of the virus comprise the core and two envelope glycoproteins: E1 and E2. The E2 protein is responsible for initiating viral attachment to receptor(s) on potential host cells due to its ability to bind to human cells[6,9]. CD81 has first been identified as a HCV E2 binding molecule by expression cloning by Pileri and colleagues[10]. The E2 binding site of CD81 is located within the LEL domain[10-13]. Further studies then demonstrated a key role of CD81 as a host entry factor for infection of human hepatoma cells with recombinant HCV pseudoparticle and tissue-culture-derived HCV (HCVcc)[14-18]. Furthermore, one very recent study has demonstrated evidence that CD81 is also involved in HCV infection of primary human hepatocytes using serum-derived HCV[19]. However, there appear to be subtle differences regarding the role

of CD81 for productive infection of human hepatoma cells and human hepatocytes using serum-derived or recombinant virus[19-24].

Recently, a novel *in vitro* cell culture model system has been established for HCV with primary tupaia hepatocytes (PTHs)[25-27]. Using this cell culture system, we found that HCV E2 protein binding to PTHs and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81[25]. To further investigate the role of CD81 in the infection of PTHs with HCV, we cloned a tupaia CD81 large extracellular loop (LEL) and analyzed the interaction of *tupaia* CD81 with HCV E2 protein *in vitro*. The results indicate that changes occur in 6 amino acid residues of *tupaia* CD81 LEL and the ability of *tupaia* CD81 LEL to bind to HCV E2 is significantly decreased compared with human CD81 LEL.

MATERIALS AND METHODS

Reagents

Recombinant HCV E2 protein, and mouse anti-HCV E2 (3ES) were generously provided by M Houghton (Chiron Corp., Emeryville, CA). Human CD81 LEL (wild type and T163A mutant) and AGM CD81 LEL protein were kindly provided by Dr. S Levy (Department of Medicine, Stanford Medical School, Palo Alto, CA)[12]. Mouse monoclonal anti-CD81 antibodies 5A6 and 1D6 have been described elsewhere[11,12,28]. Glutathione S-transferase (GST) expression vector, PGEX-2T, was purchased from Amersham Pharmacia Bioteh Inc.

Cloning of tupaia CD81 LEL

Total cellular RNA was isolated from PTHs with RNeasy kits (Qiagen, GmbH, Hilden, Germany) according its manufacturer's instructions. cDNA was transcribed from cellular RNA using rTth reverse transcriptase. *Tupaia* CD81 (TupCD81) LEL was amplified by PCR using Pfu DNA polymerase (Stratagene) and human CD81-specific primers covering the LEL domain (sense: 5'-TAACGG ATCCAACAAGGACCAGATTGCCAAGGA-3', anti-sense: TAACGAATTCACAGCTTCCCGGAGAAGAG CTC-3'). The PCR products were then cloned into pCR-Blunt Ⅱ-TOPO (Invitrogen, Groningen, Netherlands). An EcoR I /BamH I fragment was subcloned into pGEX-2T (Amersham Pharmacia Biotech Inc.) with the same enzyme digestion. The resulting plasmid (named p2T-TupCD81 LEL) was sequenced using CD81-specific primers. Plasmids expressing African green monkey, and human CD81 LEL (both wild type and genetically modified T163A type) were kindly provided by Dr. S Levy and have been described previously.

Expression and purification of glutathione S-transferase (GST)-CD81 LEL fusion proteins

Expression and purification of GST-CD81 LEL fusion protein were performed as previously described[13]. Briefly, BL21 *E. coli* were transformed with plasmid DNA. Transfected *E. coli* were grown in 12 mL LB medium containing ampicillin (50 mg/L) at 30-32℃ until the optical density (OD) value of the culture medium reached 0.6-0.8 at *A*600. Protein expression was then induced by adding isopropyl β-D-thiogalactoside (IPTG, 0.75 mmol/L). After cells grew for additional 3 h, they were lysed by sonication and the fusion proteins were purified using the MicroSpin GST purification module (Amersham Pharmacia Biotech). GST protein expressed in control plasmid pGEX-2T was purified in parallel. The purified proteins were analyzed using SDS-PAGE under non-reducing conditions, quantified, aliquoted, and stored at -80℃ until use.

Test of HCV E2-CD81 LEL interaction

A 96-well microtiter plate was coated with GST-CD81 fusion protein (5 mg/L) at 4℃ overnight. After washing 5 times with PBS and blocking with 4% dry milk (in PBS) at room temperature for 1 h, 50 μL of recombinant HCV E2 protein (with reciprocal dilutions, starting from 2 mg/L) was allowed to bind to CD81 LEL at 4℃ overnight. The bound E2 was detected using anti-E2 (3E5; diluted at 1:2000 in PBS containing 1% Tween 20 and 1% BSA) and HRP-conjugated anti-mouse IgG (diluted at 1:5000 in PBS containing 1% Tween 20 and 1% BSA). GST protein and human and AGM CD81 LEL were tested as controls. For GST test, goat anti-GST (in dilution starting at 1:1000) (Amersham Pharmacia Biotech) was added, and incubated at room temperature for 1 h. The bound anti-GST was detected using HRP-conjugated anti-goat IgG. The bound HRP-conjugated second antibodies were quantified by colorimetric reaction with an Abbott OPD reagent kit (Abbott Laboratories, North Chicago, IL), and the OD value was measured at 490 nm on a Bio-Rad plate reader (Bio-Rad, Hercules, CA).

RESULTS

Tupaia CD81 LEL amino acid sequence

It has been demonstrated that the E2 binding site of CD81 is located within the LEL domain[10,12,13]. To study the E2-CD81 LEL interaction *in vitro*, *tupaia* CD81 LEL was cloned and sequenced. The deduced amino acid sequence of *tupaia* CD81 LEL cDNA showed mutations in 6 amino acid residues when compared with human CD81 LEL as previously showed[25]. These mutations were clustered around the E2 binding head subdomain (Figure 1), according to the 3D structure of human CD81 LEL[29].

Analysis of tupaia CD81 LEL-E2 interaction in vitro

To analyze the interaction between HCV glycoprotein E2 and *tupaia* CD81 LEL *in vitro*, *tupaia* CD81 LEL was expressed as a GST-fusion protein in *E. coli*. The correct expression and folding of *in vitro*-synthesized *tupaia* CD81 LEL were confirmed by analyzing the purified protein with non-reducing SDS-PAGE and immunoblot using anti-CD81 antibodies against conformation-dependent epitopes. As shown in Figure 2A and B, *tupaia* CD81 LEL was recognized by defined anti-human CD81

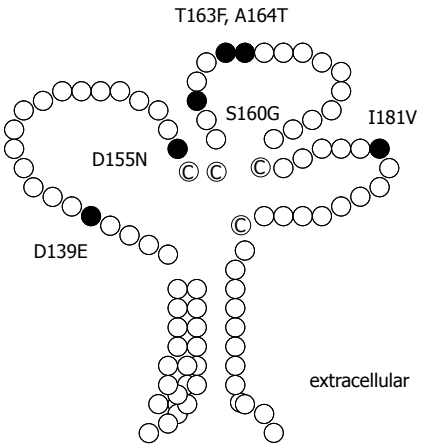

Figure 1 Putative structure of *tupaia* CD81. *Tupaia* CD81 LEL cDNA was cloned and sequenced as described in Materials and Methods. Deduced amino acid sequence was compared with that of human. The *tupaia* CD81 secondary structure was drawn according to the three-dimensional structure of human CD81 LEL, and changes in six amino acids of *tupaia* CD81 LEL were illustrated by black color.

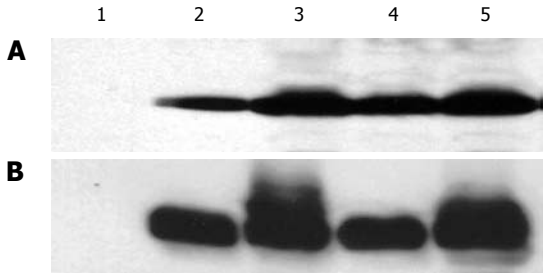

Figure 2 Expression and purification of soluble tupaia CD81 LEL using mouse monoclonal anti-human CD81 5A6 (A), and 1D6 (B) antibodies. *Tupaia* CD81 LEL was expressed and purified in *E. coli* as a GST fusion protein as described in Materials and Methods. Purified proteins were subjected to SDS-PAGE, and immunoblot using mouse monoclonal anti-human CD81 5A6 and 1D6 antibodies. Tup: *tupaia*; h: human; AGM: African green monkey; hCD81 LEL T163A: human CD81 containing a mutation of T to A at amino acid residue 163; 1: GST; 2: TupCD81 LEL-GST; 3: hCD81 LELT163A-GST; 4: AGMCD8 LEL-GST; 5: hCD81 LEL-GST.

antibodies (5A6 and 1D6), similar to human or African green monkey CD81 LEL. The data suggest that folding of CD81 LEL-GST fusion protein is comparable to that of the native molecule, while slight differences might exist in the 3D structure of human and *tupaia* CD81 LEL since the staining intensity of TupCD81 was week compared with that of human CD81 (both wild and T163A mutation type) using antibody to human CD81 conformation-dependent epitopes. Interaction of *tupaia* CD81 LEL with recombinant E2 protein was analyzed with EIA, and compared with that of human- or African green monkey-derived CD81 LEL. Binding of *tupaia* CD81 LEL to HCV E2 protein was markedly reduced compared with the native or mutant human CD81 LEL (Figure 3).

DISCUSSION

Recombinant HCV E2 could bind to *tupaia*, but not to rat hepatocytes in a dose-dependent manner and PTHs could be infected with HCV *in vitro*[25,26]. Furthermore,

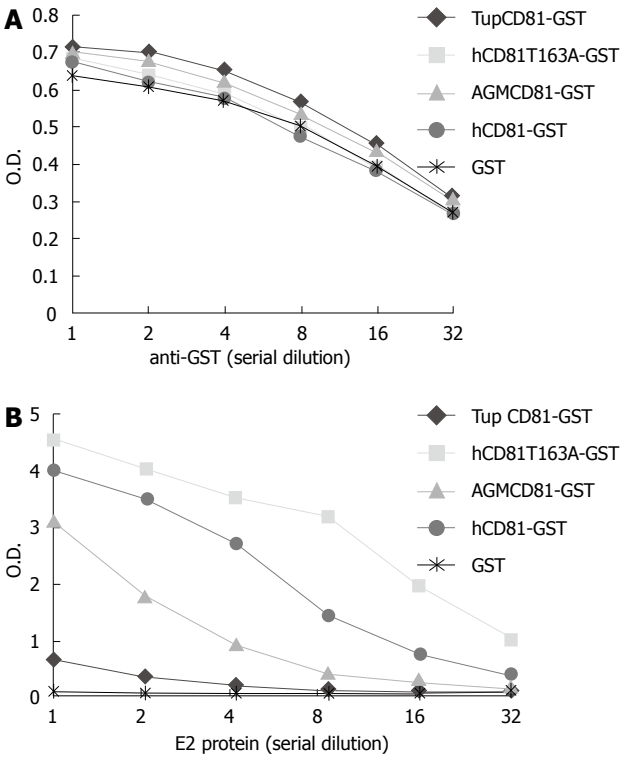

Figure 3 Interaction between *tupaia* CD81 LEL and HCV E2 protein. Plates were coated with 100 μL of recombinant GST or GST-CD81 LEL fusion proteins (5 mg/L), reciprocally diluted anti-GST antibody (A) (starting dilution at 1:1000) or HCV E2 protein (B) (starting concentration 2 mg/L) was added to the plates. Binding of anti-GST or HCV E2 protein was assessed as described in Materials and Methods. OD: optical density; Tup: tupaia; h: human; AGM: African green monkey; hCD81T163A: human CD81 containing a mutation of T to A at amino acid 165.

we demonstrated that both the binding of HCV E2 to PTHs, and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81 exhibiting the blocking ability of HCV E2 to bind to lymphoma cell lines, indicating that binding of E2 to PTHs and infection of *tupaia* hepatocytes with HCV might require additional or other molecules besides CD81. To further characterize the HCV E2-*tupaia* CD81 interaction, we cloned the HCV E2 binding domain of CD81, the CD81 LEL, and investigated the reaction of HCV E2 with *tupaia* CD81 *in vitro*.

CD81, a member of the super-family of tetraspanins, comprises 4 transmembrane (TM1-4) and two extracellular loops. The HCV E2 binding domain locates in the large extracellular loop (LEL), which folds to form a mushroom-like 3D structure, and is stabilized by a number of specific interactions within the defined amino acid residues[29]. In the present study, the deduced amino acid sequence of *tupaia* CD81 LEL showed changes in only 6 amino acid residues compared to human CD81 LEL, while all the residues necessary for the 3D structure-stabilization were conserved, suggesting that soluble *tupaia* CD81 LEL folds in a manner comparable to human CD81 LEL. This hypothesis was confirmed by the cross interaction of *tupaia* CD81 LEL with anti-human CD81 antibodies against conformation-dependent epitopes (Figure 2).

It has been reported that HCV E2 binds to the

head subdomain of CD81 LEL consisting of about 60 amino acid residues[29]. Distinct amino acid mutations can affect CD81-E2 interaction. African green monkey (AGM) CD81 LEL contains only 4 amino acid residues compared with human CD81 LEL, and shows reduced E2 binding[10-12]. Reduced E2-binding of AGM CD81 is due to a mutation of phenylalanine to leucine at the amino acid residue 186 (F186L)[12]. Interestingly, mutation of threonine to alanine at the amino acid residue 163 (T163A) can enhance E2-CD81 binding[12]. In this study, all the six mutant residues of *tupaia* CD81 LEL were clustered at its head subdomain which is the binding site for HCV E2, while the phenylalanine at residue 186, and the 4 cysteine residues, which are pivotal for human CD81-HCV E2 binding, were conserved in *tupaia* CD81. When purified *tupaia* CD81 LEL was tested for its binding to recombinant HCV E2 protein, only a mild E2-CD81 interaction was observed. By contrast, human CD81 LEL (both wild-type and genetically modified CD81 containing a T163A mutation) bound firmly to E2 protein. Interestingly, threonine at residue 163 of TupCD81 LEL changed into phenylalanine. The reduced E2 binding ability of *tupaia* CD81 was not due to the amount of protein coating the plates, since the GST activity of those fusion proteins was nearly similar. From the perspective of the 3D structure of CD81 LEL, mutations at amino acid residues 155 and 181 of *tupaia* CD81 LEL may be responsible for the reduced binding in *Tupaia* CD81 to HCV E2 protein[29]. The binding of HCV E2 to AGMCD81 LEL in this study different from a previous study[12]. This discrepancy might be due to the differences in the recombinant HCV E2 proteins. E2 used in this study is C-terminally truncated at amino acid 715, whereas other investigators used E2 C-terminally truncated at amino acid 661.

Although our studies were limited to evaluate CD81 LEL-E2 interaction with EIA, and need to be confirmed in model systems expressing full-length CD81 in transfected mammalian cell lines, the differences in HCV E2 binding between *tupaia* CD81 LEL and human CD81 LEL are consistent with our previous functional data assessing E2 binding to PTHs and HCV infection of PTHs in the presence of anti-CD81 antibodies. Taken together, these results indicate that although CD81 may play a functional role as a co-factor for entry of HCV into PTHs, it is likely that other or additional molecules besides CD81 play a key role in HCV entry into PTHs. These may include other identified HCV host factors including SR-BI[27] or Claudin-1[30]. Alternatively, other not yet identified host entry factors may mediate HCV-PTH interaction.

COMMENTS

Background

CD81, a member of the superfamily tetraspanins, comprises four transmembrane (TM1-4) and two extracellular loops. The HCV E2 binding domain locates in the large extracellular loop (LEL). The LEL folds to form a mushroom-like 3D structure, which is stabilized by a number of specific interactions within the defined amino acis residues.

Research frontiers

Hepatitis C virus (HCV) is a major cause for posttransfusion and community-acquired hepatitis worldwide. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC). The predicted structural components of HCV comprise the core and two envelope glycoproteins: E1 and E2. The E2 protein is responsible for initiating viral attachment to receptor(s) on potential host cells due to its ability to bind to human cells. HCV E2 could specifically bind to cell surface molecule CD81 expressed in lymphoma cells. Anti-HCV antibodies from chimpanzees that are protected against homologous HCV challenge by vaccination with envelope glycoproteins inhibit E2 binding to CD81, suggesting that CD81 represents a candidate receptor for HCV infection. Whether CD81-E2 interaction can mediate virion entry into host cells is unknown and has not been tested in a suitable model.

Innovations and breakthroughs

A novel *in vitro* cell culture model system has been established for HCV with primary tupaia hepatocytes (PTHs). Using this cell culture system, we found that HCV E2 protein binding to PTHs and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81. To further investigate the role of CD81 in the infection of PTHs with HCV, we cloned a tupaia CD81 large extracellular loop (LEL) and analyzed the interaction of *tupaia* CD81 with HCV E2 protein *in vitro*. The results indicate that changes occur in 6 amino acid residues of *tupaia* CD81 LEL and the ability of *tupaia* CD81 LE to bind to HCV E2 is significantly decreased compared with human CD81 LEL.

Applications

Tupaia CD81 has a reduced ability to bind to HCV E2 protein. HCV entry and infection of PTHs with HCV might occur through receptor(s) besides CD81.

Peer review

The manuscript is well written, but needs clarification of the importance of the found amino acid changes with regard to the interaction of *tupaia* CD81 and HCV E2 binding.

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CASE REPORT

Perigastric extraskeletal Ewing's sarcoma: A case report

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Author contributions: The surgery and clinical care of the patient was provided by Colovic RB, Grubor NM, and Matic SV; Micev MT performed the histological and immunohistochemical analyses; The paper was written by Colovic RB, Atkinson HDE and Grubor NM; Latincic SM did the literature analysis; Grubor NM carried out all the manuscript corrections and is the corresponding author.
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Abstract

Ewing's sarcoma (ES) is a neoplasm of undifferentiated small round cells, which occurs in the bones and deep soft tissues of children and adolescents. We present a rare case of a 44-year-old woman with gastric ES presenting with epigastric pain and weight loss. Ultrasound and computed tomography scans indicated a solid/cystic mass in the pancreatic tail. At laparotomy, the tumor was found attached to the posterior surface of the stomach, completely free from the pancreas, with no lymphadenopathy or local metastases. The polynodal, partly pseudocystic, dark-red soft tumor was excised. Histopathology revealed an anaplastic small-round-cell tumor with strong membranous CD99 immunoexpression. Additionally, there was patchy immunostaining for S-100 protein, vimentin, protein gene product (PGP) 9.5 and neuron-specific enolase, and weak focal CD117 cytoplasmic immunoreactivity. The patient had no adjuvant chemotherapy; her postoperative recovery was uneventful, and she remains symptom-free, and without any sign of recurrence at 20 mo. To the best of our knowledge, this is only the third ever case of gastric ES.

Key words: Stomach; Extraskeletal; Ewing's sarcoma

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INTRODUCTION

Ewing's sarcoma, or primitive neuroectodermal tumour (ES/PNET), is a neoplasm of undifferentiated small round cells that occurs in children, adolescents and young adults[1,2]. Although predominantly affecting the bones and deep soft tissues[2], these sarcomas are also being described as affecting the visceral organs, with increasing frequency. These extraskeletal ES/PNET sarcomas are histologically indistinguishable from the bony type[3], and have been documented in the pancreas[4,5], vagina[6], rectovaginal septum[7], small bowel[8,9], prostate[10], ovaries[11], esophagus[12], and kidney[13]. Two cases of gastric ES/PNET have been previously reported: the first in a 14-year-old boy[14] and the second in a 66-year-old woman[2]. We describe the third ever case of gastric ES.

CASE REPORT

In January 2006, a 44-year-old obese woman presented with a 6-mo history of epigastric pain and weight loss of 5 kg. On examination, she had only mild tenderness in the epigastrium. All standard laboratory data were within normal limits, though fasting cholesterol was elevated at 6.80 μmol/L (normal range 3.1-6.4 μmol/L), and fasting triglyceride was 2.30 mmol/L (normal < 1.95 mmol/L); erythrocyte sedimentation rate was 14 mm/h. Ultrasonography (US) and computed tomography (CT) scans (Figure 1) confirmed a solid/cystic mass measuring 66 mm × 46 mm in the tail of the pancreas.

The patient underwent laparotomy for the tumor at the tail of the pancreas; but, at operation, the only pathology found within the abdomen was a mass along the posterior wall of the stomach (Figure 2). There was no lymphadenopathy in the vicinity of the tumor or the upper retroperitoneum. The tumor was dark-red in color, moderately soft, covered by a thin light-grey membrane,

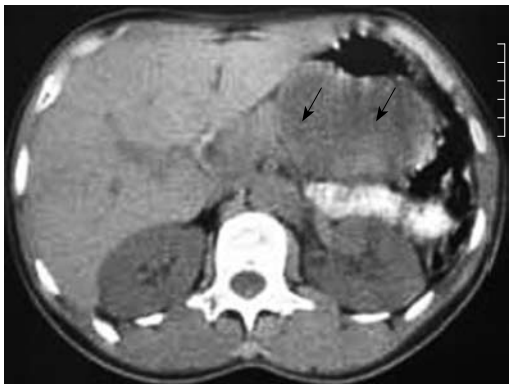

Figure 1 CT scan indicating a tumor at the tail of the pancreas (arrows).

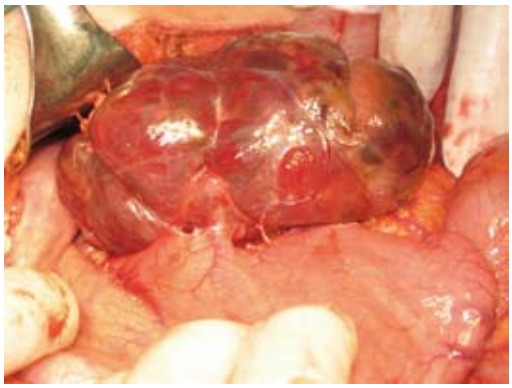

Figure 2 Photograph showing the tumor on the posterior wall of the stomach.

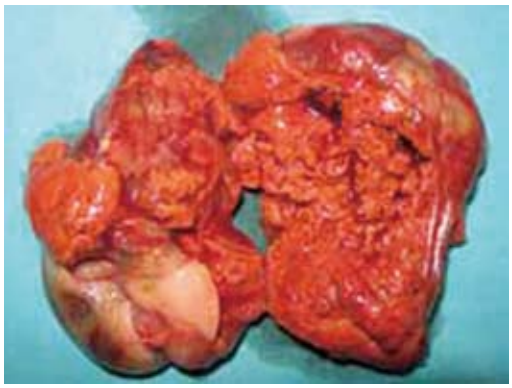

Figure 3 Photograph showing a cross section of the tumor.

and adherent to the stomach along a surface of 3 cm × 3 cm. Intraoperative frozen section biopsy failed to clarify either the tumor type or the eventual malignant potential. Thus, when the tumor was completely excised, and no significant damage was seen on the stomach wall, the decision was made to not proceed to a gastric resection.

The pathological specimen measured 10 cm in its greatest diameter. On gross inspection, the cut surface showed large central hemorrhagic and necrotic changes and pseudocystic degeneration. The tumor tissue was largely light grey and solid, with some softer and more friable, reddish congested areas (Figure 3). Microscopic examination revealed a hypercellular diffuse, and rather monotonous proliferation of small round cells with clear

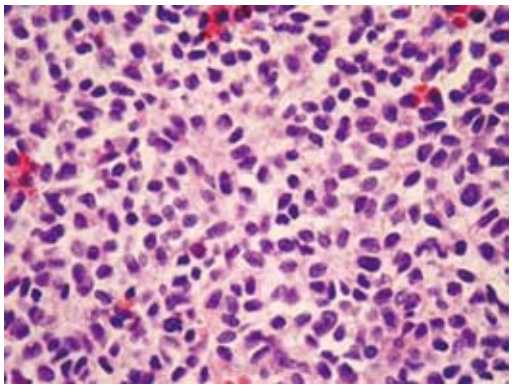

Figure 4 Histological appearance of the diffuse neoplastic infiltration showing rather uniform diffuse small round cells and abortive pseudorosette formation (HE, x 112).

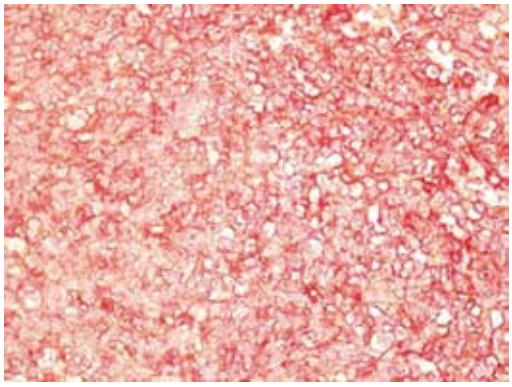

Figure 5 Tumor cells showing strong diffuse membrane immunohistochemical reactivity with CD99 antibodies [labeled streptavidin biotin (LSAB+) method, 3-amino-9-ethyl carbazole (AEC) visualization, x 112].

cytoplasm and relative nuclear uniformity. However, there were also areas showing nuclear atypia and abortive pseudorosette formation (Figure 4). Microcystic and hemorrhagic changes were seen on most of the sections, as well as sharply demarcated borders frequently covered by intact serosa. Tumor necrosis was minimal, although atypical mitotic figures were seen with a mitotic index of 14 per 50 high power fields.

Immunohistochemical examination with monoclonal antibodies excluded most of the differential diagnoses. Immunostaining was negative for pancytokeratin AE1/AE3, epithelial membrane antigen (EMA), leukocyte common antigen, chromogranin A, synaptophysin, desmin, muscle-specific antigen, the α subunit of smooth muscle antigen (SMA), glial fibrilar acidic protein, neurofilament protein, α-inhibin, melanin A and CD34. The diagnosis of ES/PNET was established by the presence of strong membranous CD99 immunoexpression by the vast majority of tumor cells (Figure 5), in addition to clear, but patchy S-100 protein, vimentin, protein gene product (PGP) 9.5 and neuron-specific enolase (NSE) immunostaining, as well as weak focal CD117 cytoplasmic immunoreactivity in very few neoplastic cells. Fluorescent *in situ* hybridization (FISH) was not available to us.

The patient did not receive any adjuvant chemotherapy or radiotherapy; her postoperative recovery was unevent-

ful, and she remains symptom-free and without recurrence on US or CT scan 20 mo later.

DISCUSSION

Histologically, ES/PNET is composed of small round cells that are usually rich in glycogen, and the neuroepithelial morphologic differentiation is confirmed by pseudorosette formation. Immunohistochemically, the neuroendocrine phenotype is confirmed by positivity to CD99, and to NSE and S-100 to a lesser extent, as these are also found in a number of other small-round-cell tumors[2]. FISH testing for the presence of the t (11;12) translocation can be particularly useful when the tumor occurs in older patients or in an unusual site[2,15], as well as to differentiate from desmoplastic small round cell tumors (DSRCT)[15]. The ultimate diagnosis should be based on both histology and immunohistochemistry[16].

We considered several other small-round-cell tumors in our differential diagnoses. Mesenchymal chondrosarcoma, and small-cell osteosarcoma were excluded as no chondroid or osteoid differentiation was found. Embryonic rhabdomyosarcoma was excluded as all muscular markers were negative. Intra-abdominal desmoplastic round-cell tumor was excluded as cytokeratin and EMA markers were negative, and nodular dissemination was absent. Hemangiopericytoma, glomus and other perivascular tumors were excluded on the basis of CD34 and SMA negativity.

The results of surgery alone for extraskeletal ES are poor in most cases, while patients receiving multimodal chemotherapy and radiotherapy have a much better prognosis[17]. Through the combination of local surgical treatment and systemic chemotherapy, long-term survival has improved from 10% to 50%-60% or greater[18,19], although the pathologist and oncologist will need to decide whether treatment regimens for tumors are better based on their phenotype or their genotype, when these two profiles are seemingly in conflict[16].

The two previously published cases of ES of the stomach both had poor outcomes. A 14-year-old boy with gastric ES was also found to have a diffuse metastatic lesion in the liver. He underwent a subtotal gastrectomy and lymphadenectomy followed by chemotherapy with a tyrosine kinase inhibitor because of intense expression of CD117 (c-kit), but died[14]. A 66-year-old woman with ES within the antropyloric area of the stomach underwent a distal gastrectomy and lymphadenectomy, but despite adjuvant chemotherapy, she also died 10 mo postoperatively[2].

Following postoperative pathological analysis, our patient was presented to the oncologists, who decided not to give any adjuvant chemotherapy. Despite this, she remains clinically well, and without recurrence to the present day (20 mo later).

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CASE REPORT

A case report of endocrine cell carcinoma in the sigmoid colon with inferior mesenteric vein tumor embolism

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Abstract

We report a case of endocrine cell carcinoma in the sigmoid colon with inferior mesenteric vein (IMV) tumor embolism. A 79-year-old woman was admitted to our hospital with narrowing of the stools. We performed colonoscopy, computed tomography and positron emission tomography, which disclosed sigmoid colon cancer with IMV tumor embolism. She underwent sigmoidectomy and lymph node dissection. The tumor was diagnosed as endocrine cell carcinoma (type 4, pSS, med, INFα, v3, n1, stage Ⅲb). Immunohistochemically, chromographin A, synaptophysin, cytokeratin 20 and mucicarmine showed partial staining, and CD56 was totally reactive. Three months after operation multiple liver metastases appeared. She was treated with chemotherapy of cisplatin (CDDP) + irinotecan (CPT11). This case highlights the aggressiveness of endocrine cell carcinoma with tumor embolism, and it is essential to establish an accurate diagnosis and effective treatment.

Key words: Enteroendocrine cells; Tumor embolism; Carcinoid tumor; Colon cancer, Cisplatin; Irinotecan

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INTRODUCTION

Endocrine cell carcinoma of the colon and rectum is uncommon, and accounts for less than 1% of colorectal cancer[1]. It is well known that colorectal carcinoma of common pathology has a relatively good prognosis, whereas endocrine cell carcinoma of the colon and rectum has a very poor prognosis. Many patients with endocrine cell carcinoma have liver and lymph node involvement at the time of diagnosis.

Tumor embolism is an uncommon complication in digestive system cancers. The majority of cases of tumor embolism are related to renal carcinoma and liver carcinoma, not colon and rectum carcinoma. In fact, there has never been a report of endocrine cell carcinoma of the colon and rectum complicated by tumor embolism. Treatment of endocrine cell carcinomas of the colon and rectum are very difficult. We, herein, report the malignant potential, and the treatment of colorectal endocrine cell carcinoma with tumor embolism of the inferior mesenteric vein (IMV).

CASE REPORT

A 79-year-old woman experienced narrowing of the stools that lasted for more than a few days. She had been well since she complained of intermittent abdominal pain. She had no remarkable past medical history. Laboratory findings showed no abnormalities. Colonoscopy disclosed a circumferential type 2 lesion in the sigmoid colon (Figure 1). However, biopsy of this tumor revealed no malignant lesion, only regenerative colon mucosa. Abdominal computed tomography (CT) and positron emission tomography (PET) showed hypertrophy of the sigmoid colon wall with fusiform enlargement of the IMV extending up to the confluence with the splenic vein and a few lymph node metastases in the mesocolon (Figure 2). There were no liver metastases. These findings suggested that she had sigmoid colon cancer (cT3N2M0 Stage ⅢB) with IMV tumor embolism. Considering the possibility of progressive cancer obstruction and tumor embolism, we decided to perform radical sigmoidectomy and lymph node dissection without preoperative chemotherapy. Perioperatively, we paid special attention to make an incision in the IMV first before the tumor was isolated. The specimen of tumor was a 11.5 cm × 3.5 cm, type 4 cancer (Figure 3A), pSS, med, INFα, v3, PM0,

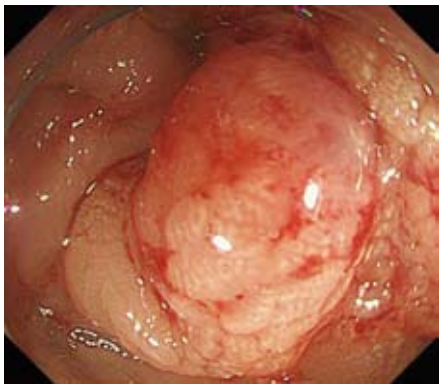

Figure 1 Colonoscopy showing a type 2 shaped tumor mainly located in the sigmoid colon.

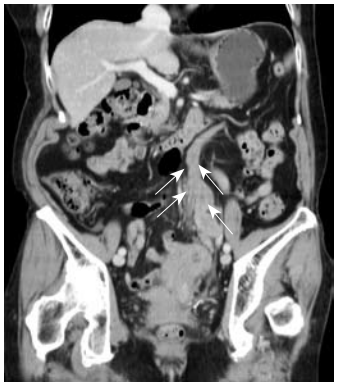

Figure 2 Computed tomography showing a hypertrophic colon wall in the sigmoid colon and dilation of IMV (arrows).

DM0, RM0, DX with tumor embolism in the IMV, and the clinical stage was stage ⅢB pT3N1M0 in the UICC TNM classification. The tumor embolism in the IMV was a solid lesion 14 cm long and 2 cm across in transverse section (Figure 3A and B). Microscopically, it was seen that the tumor had invaded the vein wall and expanded into the extra space (Figure 4).

Histological features included an irregular pattern of the nuclei in size and mitotis (Figure 5). The tumor was diagnosed pathologically as an endocrine cell carcinoma histochemically, using CD56, chromographin A, synaptophysin, cytokeratin 7, cytokeratin 20 and mucicarmine. Chromographin A, synaptophysin, cytokeratin 20 and mucicarmine were partly stained and CD56 was totally reactive (Figure 6).

The postoperative course was mostly uneventful. However, three months after operation, multiple liver metastasis appeared. Now, she is being treated with chemotherapy of cisplatin (CDDP) + irinotecan (CPT11) and still alive after 14 mo.

DISCUSSION

This case is the first report of a sigmoid colon endocrine cell carcinoma invading into IMV as a tumor embolism. Endocrine cell carcinomas of the colon and rectum are uncommon, comprising less than 1% of colon and rectal cancer[1]. Endocrine cell carcinomas do not include carcinoid tumors that have a benign course compared to adenocarcinoma and endocrine cell carcinoma. Iwafuchi *et al* have proposed four classifications of endocrine

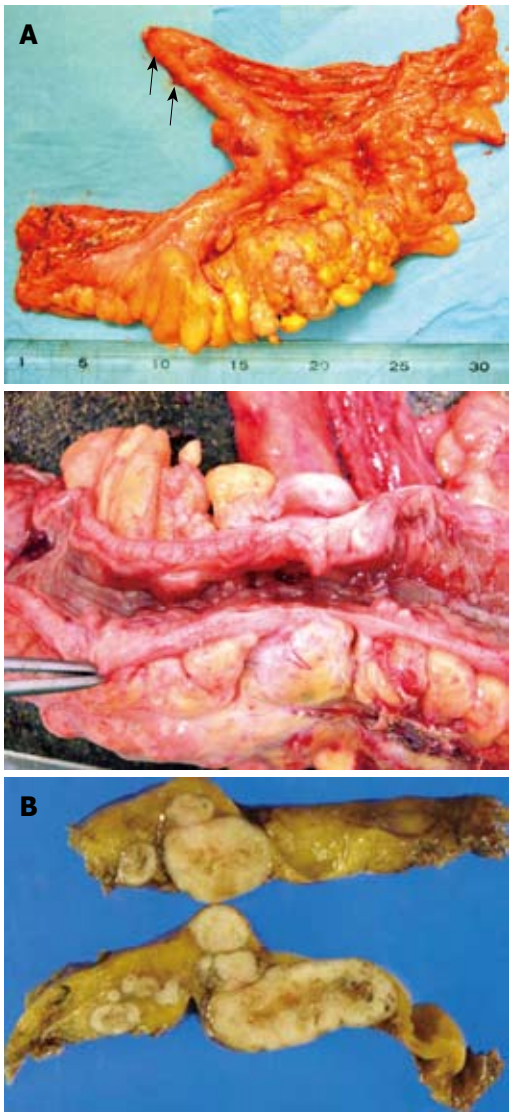

Figure 3 Resected specimen. A: A type 2 shaped tumor was located in the sigmoid colon with tumor embolism of IMV which was 14 cm long (arrows); B: In transverse section, the tumor embolism was 2 cm long.

cell carcinoma derived from: (1) preexisting general-histological adenocarcinomas; (2) preexisting carcinoids; (3) nonneoplastic multipotential stem cells and (4) nonplastic immature endocrine cells[2]. Recently, it has been thought that endocrine cell carcinomas predominantly arise from endocrine precursor cell clones occurring in preexisting adenocarcinoma components, transforming into endocrine cell carcinomas during rapid clonal expansion under the influence of *p53* gene alteration[3].

From 1986 to 2007, a total of 87 cases of endocrine cell carcinomas of the colon and rectum were reported in Japan (Table 1). The average patient age was 60.8 years (range, 34-89 years). There were 44 males and 43 females. Tumors were located as follows: 2 in appendix, 9 in cecum, 17 in ascending colon, 9 in transverse colon, 2 in descending colon, 2 in sigmoid colon and 47 in rectum. It suggests that endocrine cell carcinomas arise in any part of the colon, but particularly in rectum. The lesions invaded into subserosa or pericolic tissue (51/66; 77%). The rate of vascular invasion (32/44; 72%) was also very high. Endocrine cell carcinomas are aggressive

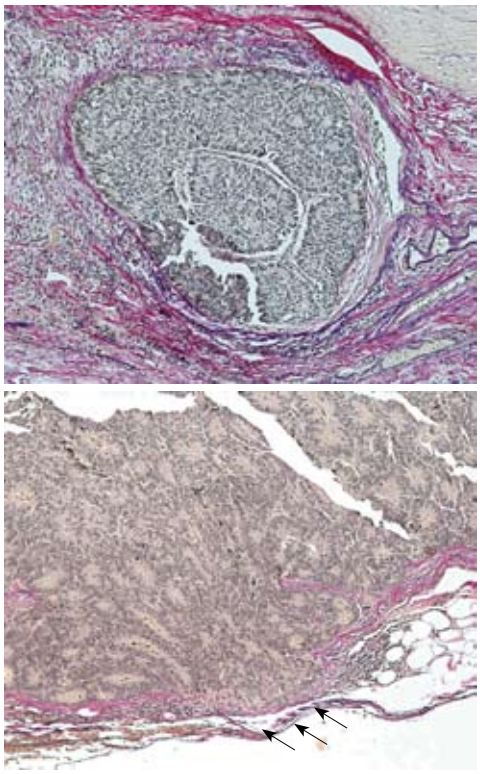

Figure 4 Histological features. Nuclei of the endocrine cell carcinoma cells were irregular in size, and mitotis was frequently seen with extensive vein invasion partially penetrating the vein wall (arrows).

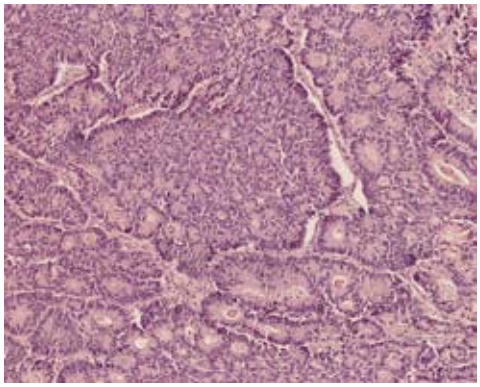

Figure 5 Histological features. Nuclei of the endocrine carcinoma cells were irregular in size, and mitosis was frequently identified.

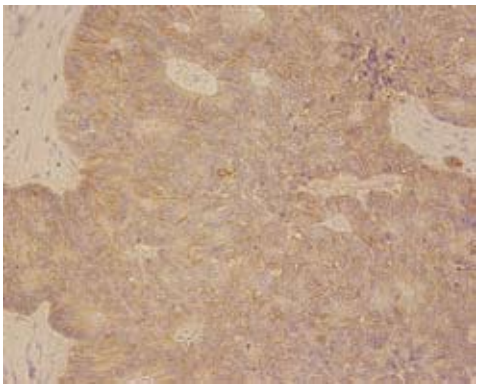

Figure 6 Immunohistochemical features. The endocrine carcinoma cells were immunoreactive for chromographin A, synaptophysin and CD56 (NCAM).

Table 1 Review of the literature

Clinical features of 87 patients with endocrine cell carcinoma	
Characteristic	
Sex M/F (n = 87)	44/43
Average age (Yr, n = 87)	60.4 (34-89)
Location (n = 87)	Appendix: 2
	Cecum: 9
	Ascending colon: 17
	Transverse colon: 9
	Descending colon: 2
	Sigmoid colon: 1
	Rectum: 47
Depth (n = 66)	m-sm: 5
	Mp: 10
	a1/ss: 20
	a2/se: 23
	ai/si: 8
Lym (n = 44)	Positive 38
	Negative 6
V (n = 44)	Positive 32
	Negative 12
N (n = 59)	Positive 46
	Negative 13
H (n = 56)	Positive 11
	Negative 45
Prognosis (death n = 56)	> 6 mo: 22
	> 1 year: 19
	⩾ 1 year: 15

V: Venous invasions; N: Lymph node metastases; H: Liver metastases.

neoplasms easily invading and expanding, which are reported to occur frequently with metastasis and carry a poor prognosis. It was reported that the liver metastasis rate of 52.1% was higher compared to general colon and rectal carcinoma which had a rate of 10%[4]. Actual 1-year survival rates were 39%[5]. Therefore, one would predict the presence of vascular invasion to be associated with an increased incidence of liver metastasis and cause poor prognosis.

The phenomenon of tumor embolism of colon and rectal carcinoma is rare. As regards our research on tumor embolism of colon and rectum cancers in the mesenteric vein, we could find only 14 case reports including our case in Japanese papers (Table 2). In these, it was considered that histologically immature carcinomas were more likely to be complicated by a tumor embolism; but, there have been no reports of endocrine cell carcinoma showing mesenteric vein tumor embolism.

Little is known about the causes of tumor embolism of colon and rectum carcinomas; but, we consider that they involve vessel invasion. The biological and pathological processes of tumor emboli formation consis of several phases: (1) tumor growth in the primary lesion; (2) invasion of the primary tumor into the surrounding vessels; (3) detachment of tumor cells from the primary lesion; (4) dissemination of tumor cells by blood flow; (5) adhesion of tumor cells to endothelial cells or tumor embolism formation. In these processes, the metastatic potential of tumor cells depends in part on the ability to undergo cell aggregation, leading to the embolization of tumor cells in the microcapillaries, and

Table 2 A summary of 14 colon and rectal cases complicated by tumor emboli in mesenteric veins

Age	Sex	Location	Histology	Depth	Size (mm)	H	Recurrent	Prognosis
50	F	A/SMV	Poor	-	10 × 50	-	Liver, lung	4 mo
53	M	S/IMV	Mod	-	-	+	Liver, local	-
77	F	T/SMV	Poor	-	15 × 40	-	-	-
62	M	A/SMV	Poor	se	8	-	None	-
65	F	A/SMV	Mod	si	-	-	None	7 mo alive
68	M	S/IMV	Mod	a2	5 × 50	-	None	24 mo alive
75	M	A/SMV	-	-	40	-	None	-
82	F	A/SMV	Mod	-	-	-	-	-
67	M	A/SMV	Mod	se	-	+	Liver	-
56	F	A/SMV	Muc	si	-	-	-	-
78	F	R/IMV	Well	se	10 × 185	-	None	-
47	F	T/SMV	Mod	-	-	-	-	5 mo alive
78	F	A/SMV	Poor	ss	-	-	-	5 mo
79	F	S/IMV	End	se	-	-	Liver	9 mo alive

A: Ascending colon; T: Transverse colon; S: Sigmoid colon; R: Rectum; H: Liver metastases; SMV: Superior mesenteric vein; IMV: Inferior mesenteric vein. Poor: Poorly differentiated; Mod: Moderately differentiated; Well: Well differentiated; Muc: Mucinous; End: Endothelial.

additionally in macro-vessels. A strong correlation has been demonstrated between *in vitro* aggregation and *in vivo* metastatic potential[6]. Poorly differentiated cell carcinomas including endocrine cell carcinomas which are well known to have strong aggregation potential related to adhesion factors, for example integrin or CD56, are thought to form a tumor embolism easily.

Because of this invasion propensity, endocrine cell carcinomas develop distant metastasis, particularly in the liver. It is not clearly known whether such metastasis can be prevented or not; but, it has been suggested that early ligation of the tumor invading the vein in the operation is useful.

The treatment strategy for patients with endocrine cell carcinomas is to detect the origin and metastases at an early stage, and to monitor carefully after the operation because of its malignant potential. Surgical resection remains the mainstay of treatment, with modest impact on survival. In cases of metastasis or cases of adjuvant therapy, the literature suggests that treatment be initiated using CDDP + CPT11 based on lung small cell carcinoma[7,8]. A response rate of 41.5%[9] and median survival range of 10.4 mo have been reported[10]. In another report, four of 11 patients with endocrine cell carcinomas who were treated with S-1 survived for over 2 years after surgery[11,12].

In conclusion, this case highlights the aggressiveness of endocrine cell carcinomas of the colon and rectum. We showed that endocrine cell carcinomas are clinically more aggressive than colorectal adenocarcinomas, and they are capable of rapid distant spread; so, the prognosis is generally worse. So far, many studies have emphasized prognostic features, immunohistochemical characteristics, and pitfalls in diagnosis and treatment of endocrine cell carcinoma[13]. Practically, there are uncharted territories left. Further improvement in diagnosis and treatment is awaited.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
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ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

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Snowmass, CO, United States
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Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
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Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
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November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
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Patent (list all authors)

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/14.doc.

Abbreviations

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H pylori*, *E coli*, *etc.*

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