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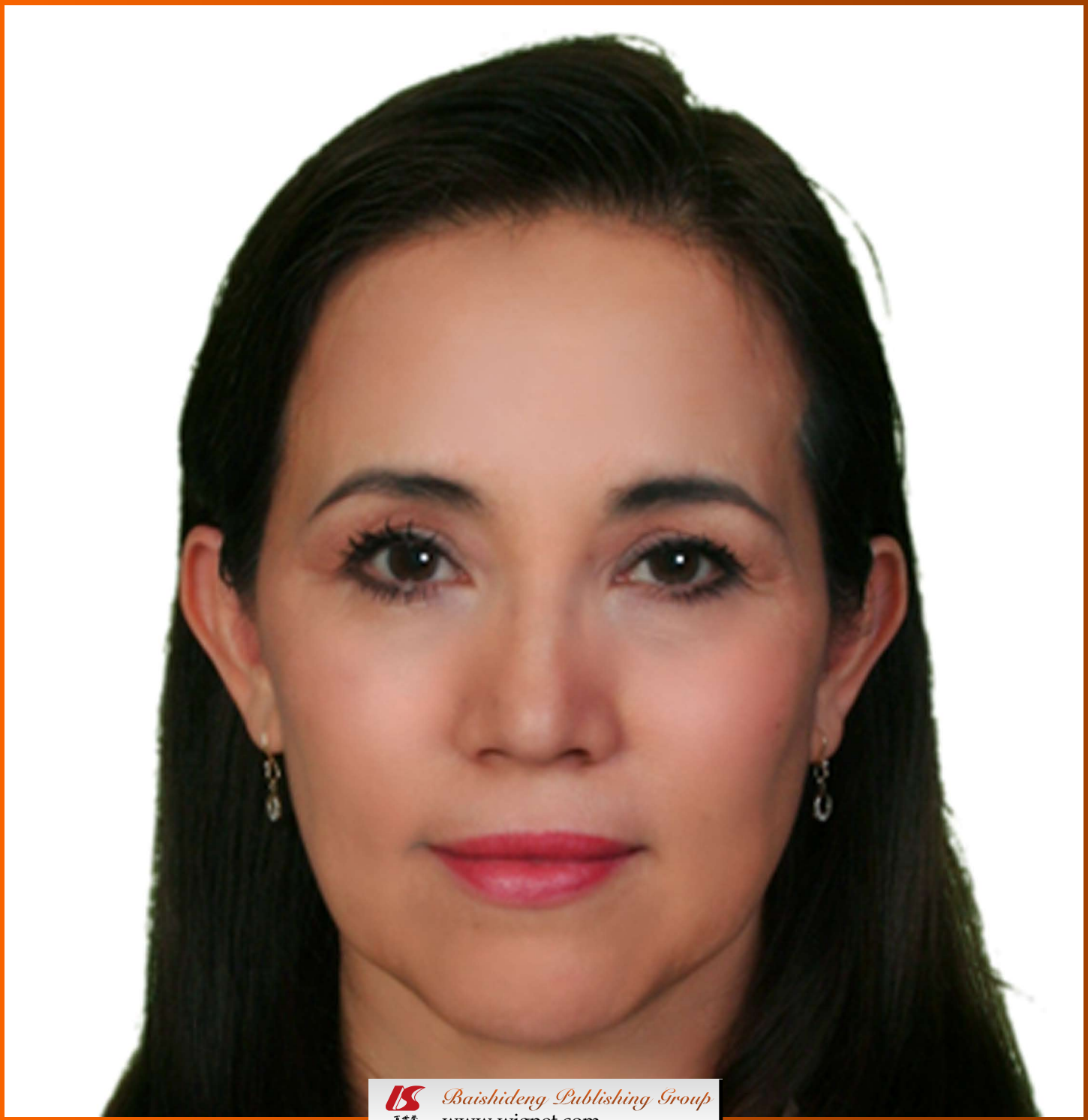

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WJGP is to report rapidly the most recent results in basic and clinical research on gastrointestinal pathophysiology, including all aspects of normal or abnormal function of the gastrointestinal tract, hepatobiliary system, and pancreas. *WJGP* specifically covers growth and development, digestion, secretion, absorption, metabolism and motility relative to the gastrointestinal organs, as well as immune and inflammatory processes, and neural, endocrine and circulatory control mechanisms that affect these organs. This journal will also report new methods and techniques in gastrointestinal pathophysiological research.

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EDITORIAL

Genetic contribution to motility disorders of the upper gastrointestinal tract

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Abstract

Motility disorders of the upper gastrointestinal tract encompass a wide range of different diseases. Esophageal achalasia and functional dyspepsia are representative disorders of impaired motility of the esophagus and stomach, respectively. In spite of their variable prevalence, what both diseases have in common is poor knowledge of their etiology and pathophysiology. There is some evidence showing that there is a genetic predisposition towards these diseases, especially for achalasia. Many authors have investigated the possible genes involved, stressing the autoimmune or the neurological hypothesis, but there is very little data available. Similarly, studies supporting a post-infective etiology, based on an altered immune response in susceptible individuals, need to be validated. Further association studies can help to explain this complex picture and find new therapeutic targets. The aim of this review is to summarize current knowledge of genetics in motility disorders of the upper gastrointestinal tract, addressing how genetics contributes to the development of achalasia and functional dyspepsia respectively.

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Key words: Achalasia; Functional dyspepsia; Genetic predisposition; Hypertrophic pyloric stenosis; Motility disorder

Core tip: Achalasia, functional dyspepsia and hypertrophic pyloric stenosis represent the main motility disorders of upper gastrointestinal tract. All these diseases have a less known pathophysiology and a presumable genetic predisposition in common. This review outlines the current knowledge on genes involved in the onset of these pathologies in order to promote further association studies which can help to explain this complex picture and find new therapeutic targets.

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INTRODUCTION

Digestive motility is a highly coordinated process which enables mixing, absorption and propulsion of the ingesta through the gastrointestinal tract up to expulsion of residues. This function depends on a finely balanced integration between smooth muscle contractility and the related pacemaker activity evoked by the interstitial cells of Cajal (ICCs) that are finely regulated by intrinsic and extrinsic innervations [*i.e.*, enteric nervous system (ENS) and sympathetic and parasympathetic nerves, respectively][1,2]. A disturbed digestive motility can occur as a result of a variety of abnormalities affecting each of these elements (alone or in combination), with consequent altered physiology of gut peristalsis and symptom generation.

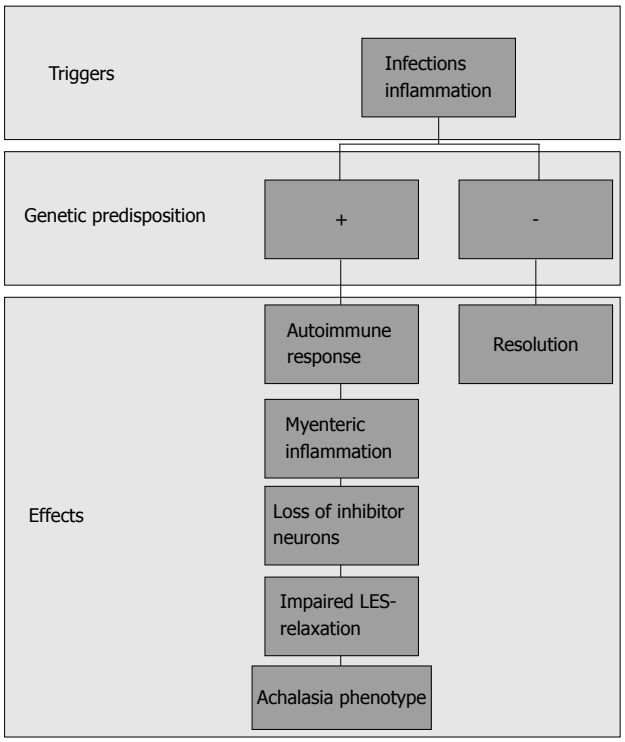

Figure 1 Supposed pathophysiology of achalasia: triggering external factors are able to induce a loss of inhibitor neurons in individuals genetically predisposed, causing an impaired lower esophageal sphincter relaxation.

The symptom complex is site specific and depends on the gastrointestinal tract involved. Thus, patients with disturbed esophageal motility may complain of dysphagia, regurgitation or chest pain, whereas patients with gastric dysmotility may report symptoms of nausea, vomiting, postprandial fullness, early satiety or epigastric pain. Esophageal achalasia and functional dyspepsia are the most representative motility disorders of the upper gastrointestinal (GI) tract, with the first rare and well characterized and the latter highly prevalent and less defined.

Like the majority of functional gastrointestinal disorders (FGIDs), they are characterized by the persistence of symptoms in the absence of reliable biological markers. As for other complex diseases, the pathophysiology of impaired upper GI tract motility diseases is supposed to be multifactorial, with triggering external factors that are able to generate the gastrointestinal dysfunction in individuals who are more or less genetically predisposed (Figure 1). The idea that an individual genotype may contribute to the development of FGIDs is suggested by clinical observations and prospective studies indicating that there is clustering of FGIDs within families[3,4] and an increased concordance in monozygotic twins[5-7]. The most commonly used approach in functional dyspepsia (FD) to date has been to search for correlations of a candidate gene polymorphism with the symptom based phenotype. More recently, a few studies have aimed to identify correlations between gene polymorphism and biological intermediate phenotypes, such as impaired motility.

In this review, we summarize the current knowledge on the genetic contribution to upper GI motor disorders. We evaluate the most recent literature on the genetic epidemiology of representative motility disorders of the upper GI tract (UGT). We describe the putative genetic contributions that have been addressed and the potential association with single mechanisms, such as receptors, transporters and translation, or transduction mechanisms involved in the disturbed motility of the esophagus, stomach and pylorus, respectively impaired in esophageal achalasia, functional dyspepsia and hypertrophic pyloric stenosis. Each of the diseases can be considered paradigmatic because of the following: (1) hypertrophic pyloric stenosis (HPS) is characteristic of infants and there is significant evidence about the role of genetic factors; (2) isolated idiopathic achalasia can be considered the prototype of defective esophageal motility in adults and the role of genetic factors is emerging as a major challenge to explain the disease; and (3) functional dyspepsia cannot be paradigmatically considered a primary gastric motor disorder, but is characterized by a significant association between impaired gastric motility and symptoms to which genetic factors contribute.

IMPAIRED MOTILITY OF THE ESOPHAGUS

Achalasia

Idiopathic achalasia is the best recognized primary motor disorder of the esophagus. It is characterized by incomplete relaxation of the lower esophageal sphincter (LOS) and absence of peristalsis that causes bolus impaction and generation of symptoms like dysphagia, regurgitation and chest pain[8]. Although the pathogenesis of achalasia is still largely unknown, it is now clear that a major issue is the loss of neurons in the esophageal myenteric plexus. We know that neurons gradually disappear in the lower part of the esophagus of achalasia patients. In most cases, this process is the result of a localized infiltration of immune cells, determining an inflammatory-based neurodegeneration[9,10]. Interestingly, this process is prevalent for inhibitory neurons containing nitric oxide (NO) and vasoactive intestinal polypeptides (VIP) and accounts for the loss of inhibitory inputs, with consequent abnormal esophageal function[11-13]. Notwithstanding this histological background, we do not know the precise pathogenic mechanism of achalasia; consequently, hereditary, degenerative, autoimmune and infectious factors have all been claimed to be possible causes of the disease.

A certain degree of genetic influence in achalasia is suggested by the familial occurrence and twin concordance. However, a systematic analysis of the literature revealed that twin concordance was significant but still inconclusive[6]. On the other hand, the only family study of achalasia conducted to date is biased by the small sample size and because the diagnosis of achalasia was based on a self-reported questionnaire[14]. In addition, achalasia may present as part of a genetic syndrome or in association with isolated abnormalities or diseases[15].

Table 1 Overview of genetic association studies in achalasia

Protein (gene)	Polymorphism	Finding	Ref.
PTPN22	C1858T	Risk factor	[28]
IL-10	GCC	Protective	[29]
IL-23R	G381A	Protective	[30]
iNOS	iNOS22*A/A	No association	[35]
eNOS	eNOS*4°4°	No association	[35]
iNOS	(CCTTT) n > 12	Risk factor	[36]
cKit	Rs6554199	Risk factor	[37]
VIPR type 1	Rs437876 and rs896	Risk factor	[38]

iNOS: Inducible nitric oxide synthase; IL: Interleukin; PTPN22: Polymorphism C1858T of phosphatase N22.

An achalasia phenotype is indeed present in well characterized genetic syndromes, like Down[16] and Allgrove syndromes[17], the familial visceral neuropathy[18] and the achalasia microcephaly syndrome[19]. In many of the above mentioned syndromes, achalasia does occur in the majority of the patients, but it generally presents in infancy.

Although these findings indicate that genetic factors are involved in the development of an achalasia phenotype, they do not provide insights into the pathogenesis of the sporadic form of achalasia, which affects the vast majority of patients and presents with adult-onset achalasia. As for other complex diseases, it is likely that the etiology of this form of achalasia is multifactorial, *i.e.,* a combination of the cumulative effect of variants in various risk genes and environmental factors leads to the disorder. The idea of an infectious origin of achalasia was first suggested by the evidence of viruses or signs of viral infection in the esophageal tissues of achalasia patients[20-22], but the possibility that the presence of viruses could be sufficient *per se* to explain the disease was ruled out by other observations[23]. More recently, the concept that achalasia could be the result of an immune mediated inflammatory disorder induced by a virus has been strongly repurposed[24]. Facco *et al*[24] demonstrated that HSV-1 or HSV-like antigens were responsible for a significant activation of $CD3^+$T cells infiltrating the LES in achalasia patients, likely resulting in an immune-mediated destruction of the myenteric neurons of the LOS. The reasons whereby this process occurs only in esophageal tissues of achalasia patients are unknown, but it is reasonable to assume that some genetic influence may affect the disease phenotype, making some individuals more susceptible to the disease. In addition and most interestingly, several evidences strongly support the idea that genes encoding for proteins involved in the immune response are likely candidates in achalasia.

A significant association between HLA DR or DQ, especially DQA1 *0103 and DQB1 *0603, and achalasia has been indeed described[25-27]. The increased risk for the development of achalasia in individuals with specific polymorphisms of genes involved in the immune response was also supported by the finding that the polymorphism C1858T of phosphatase N22 (PTPN22 gene, chromosome 1), which is a down-regulator of T-cell activation, is significantly associated with achalasia in Spanish women[28]. The same researchers have also demonstrated that the GCC haplotype of *IL-10* gene promoter is a protective factor for achalasia. This specific polymorphism enhances the release of IL-10, an anti-inflammatory cytokine, resulting in a downregulation of immune response[29]. In a similar manner, a single nucleotide polymorphism (SNP) of the IL 23 receptor (G381A), which regulates T cell differentiation, appears to be protective against achalasia. De León *et al*[30] reported that the coding variant 381Gln of *IL-23R* was significantly more common in patients with achalasia compared with healthy controls.

This evidence sustains the role of both genetic predisposition and immune alteration in the pathogenesis of idiopathic achalasia. All data are summarized in Table 1.

Contribution of genes with a dual effect on motility and immunity

A disturbed inhibitory neurotransmission is a trademark of achalasia[11]. In keeping with this, several studies have addressed the role of NO and VIP that are involved in both defense against infections and inhibitory neurotransmission and may represent ideal candidates to explain the spread of inflammation and inhibitory nerve degeneration[12,13,19,31-33].

Nitric oxide is produced by three different forms of NO synthases: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). As NO production is genetically regulated, Mearin *et al*[34] firstly investigated whether SNP in the different *NOS* genes were respectively involved in the susceptibility to suffer from achalasia. Although a trend toward a higher prevalence of genotypes iNOS22?*A/A and eNOS*4a4a in patients than in controls was observed, the authors failed to find a significant association between *NOS* gene polymorphisms and susceptibility to achalasia. Although the simplest conclusion was that NO is not involved in the pathogenesis of achalasia, this study is biased by the small sample size and by the fact that the SNPs analyzed do not play a major role in gene expression. The lack of any association between the same SNP in the iNOS was also confirmed by a Spanish group in a larger population of achalasia patients, further suggesting that these specific polymorphisms play a minor role in the functional expression of the iNOS gene[35]. More recently, our group showed a significant association between the pentanucleotide (CCTTT) polymorphism in the *iNOS* gene promoter and achalasia. Since *in vitro* data showed that the iNOS promoter activity increases in parallel with the repeat number of (CCTTT)n, we concluded that individuals carrying longer forms have an increased risk of achalasia by higher nitric oxide production.

Moreover, growing evidence suggests that esophageal interstitial cells of Cajal, a group of specialized cells that constitutively expresses c-kit and contribute to nitrergic neurotransmission, may be involved in the pathogenesis

of achalasia. Alahdab *et al*[36] have indeed shown that the rs6554199, but not rs2237025, c-kit polymorphism is significantly prevalent in achalasia patients in a Turkish population. Importantly, alterations in ICC have already been reported in other congenital diseases with abnormal peristalsis, emphasizing the key role of these cells in the regulation of GI motor function.

The implication of VIP in the pathogenesis of achalasia was also recently reported[37]. Two different SNPs, rs437876 and rs896, in the VIP-receptor type 1 gene were found to be significantly associated with the late onset achalasia. Interestingly, the authors suggest that this was probably related to a genetically based abnormal VIP-R1 signaling that may protect individuals carrying the specific genotype by delaying the immune-mediated neurodegeneration. Although these data need to be replicated, they are extremely interesting because they may suggest that early and late onset forms of idiopathic achalasia are genetically distinct disorders[38] (Table 1).

IMPAIRED MOTILITY OF THE STOMACH

FD

FD is defined by the presence of persistent symptoms in the upper part of the abdomen in the absence of organic or metabolic pathology[39]. On the basis of the Rome Ⅲ criteria, patients who suffer from functional dyspepsia in the absence of any organic disease are categorized as having postprandial distress syndrome or epigastric pain syndrome[40].

In spite of this, the pathogenesis of FD is still largely unknown and, although delayed gastric emptying, impaired gastric accommodation and visceral hypersensitivity have been all claimed as major underlying mechanisms, it is supposed to be a multifactorial disease[39].

The classic assumption in studies addressing the association between genetic factors and a single or a cluster of diseases postulates that a specific functional gene polymorphism that results in altered protein function may play a role in disease pathophysiology[41]. This paradigm that implies a clear correlation between impaired physiological function and symptom generation, however, cannot be applied to FD whose pathophysiology is complex and not necessarily associated with specific symptoms. Since functional dyspepsia is one of the most prevalent FGIDs, a certain genetic influence is suggested by both symptom familial clustering and twin studies reported for FD and IBS[3]. So far, most of the studies conducted were designed to search for correlations of a candidate gene polymorphism with the symptom-based phenotype of FD. The candidate genes that have been studied for possible associations with functional dyspepsia are summarized in Table 1.

In a subset of dyspepsia patients, *Helicobacter pylori* (*H. pylori*) infection, even in the absence of ulceration or erosion in gastroduodenal mucosa, has been proposed to play a role in generation of symptoms. A Japanese group found an association between TLR-2 -196 to -174 allele with a lower risk for developing FD in Helicobacter pylori positive subjects. Since the same group had previously demonstrated that the TLR-2 -196 to -174 increases the severity of *H. pylori*-induced inflammation, it is reasonable to hypothesize that the TLR-2 genotype influences the onset of dyspeptic symptoms modulating the degree of inflammatory response[42].

Serotonin (5-HT) is a key signaling molecule affecting upper GI motor and sensory functions[43] and thus genes of the serotonergic system are critical candidates in assessing the role of genetic determinants in FD. The action of 5-HT is terminated by the 5-HT transporter (serotonin transporter, SERT) mediated uptake, thereby determining 5-HT availability at the receptor level. A single nucleotide insertion/deletion in the SERT gene, by creating a long (L) and short (S) allelic variant, results in reduced SERT expression and 5-HT uptake[44]. Although the SS genotype of the SERT promoter polymorphism has been reported to be associated with diarrhea-predominant irritable bowel syndrome (IBS), two different studies from the United States[45] and Europe[46] failed to find any significant association between such polymorphisms and FD. Similarly, association studies between SNPs of the 5-HT3, 5-HT1A and 5-HT2A receptors in FD patients failed to yield significant association[45,46]. Although both studies were conducted on small populations of patients, the results suggest that the serotoninergic pathway plays a minor role in the pathophysiology or, at least in part, in the generation of dyspeptic symptoms.

Another genetic association reported in functional dyspepsia is the link to GNβ3[47]. G-proteins mediate the response to the release of 5-HT and several other neurotransmitters modulating gastroduodenal sensory and motor function.

A common polymorphism of *GNβ3* gene has been described and is associated with different genotypes that are predictive of an enhanced G-protein activation. A significant association between different GNβ3 polymorphisms and both uninvestigated and investigated dyspepsia has been reported in different studies from the United States[45], Europe[46,47] and Asia[48,49]. Although the sample sizes remain relatively small in all of these studies, which may account for some of the variability in the associations detected, it is of note that the different classifications of dyspepsia also contribute to the diverse distribution of polymorphisms. In fact, while homozygous GNB3 825T allele was found to influence the susceptibility to EPS-like dyspepsia in a Japanese population of dyspeptics[49], in a report from the United States, the same polymorphism was associated with postprandial dyspeptic symptoms and lower fasting gastric volumes[45]. Other studies failed to identify single genetic factors as a predominant factor in the pathogenesis of functional dyspepsia and symptom generation. As sympathetic adrenergic dysfunctions may affect both gastric sensitivity, Camilleri *et al*[45] analyzed the role of presynaptic inhibi-

Table 2 Overview of genetic association studies in functional dyspepsia

Protein (gene)	Polymorphism	Finding	Ref.
TLR-2	(192-174)del	Protective for *Helicobacter pylori*-infected patients	[43]
SERT	SS variant	No association	[45]
5-HT1A	-Pro16Leu	No association	[46,47]
5-HT2A	-1438 G/A	No association	[46,47]
HTR3A	C178T	No association	[46,47]
GNB3	C825T CT and TT carriers	Risk factor	[48-50]
GNB3	C825T CC and TT carriers	Risk factor for PDS	[48-50]
GNB3	C825T CC carriers	Risk factor	[48-50]
GNB3	C825T TT carriers	Risk factor for EPS	[50]
Presynaptic inhibitory α2A and a2C adrenoceptor	-1291 C>G (α2A) and -del 1322-325 (α2A)	No association	[46]
Fatty acid amide hydrolase	C385A	No association	[51]
TRPV1	G315C	Risk factor	[52]
Na (V) 1.8	SCN10A 3218CC	Protective	[53]

PDS: Post discharge surveillance; EPS: Encapsulating peritoneal sclerosis; TLR: Toll-like receptor; TRPV: Transient receptor potential vanilloid.

tory α2A and α2B adrenoceptors polymorphism, but they failed to find any significant association with dyspepsia symptoms. The same group also failed to find any significant association between the genetic variation in the endocannabinoid metabolism (*i.e.,* a SNP in the human fatty acid amide hydrolase gene-C385A-) and both impaired fundus accommodation and delayed gastric emptying in a small subgroup of dyspepsia patients[50]. Conversely, in a recent report, the involvement of the transient receptor potential vanilloid 1 (TRPV1) was investigated in a small population of Japanese dyspeptic patients. The authors found that in a population of 109 dyspeptics, individuals carrying the G315C polymorphism, known to affect the *TRPV1* gene and to alter its protein level, were at lower risk for both epigastric pain and postprandial distress syndrome. In addition, the authors showed that dyspeptics with that specific polymorphism had a baseline and cold water induced symptom severity[47]. Although this finding needs to be reproduced in different ethnic populations and validated on a large sample, it is of note that TRPV1 pathways may be ideal candidates as they are involved in nociception and in acid sensitivity, with the latter being claimed to have a role in the generation of dyspepsia symptoms. On the same line, the same group have found a significant association between a polymorphism of the tetrodotoxin-resistant (TTX-r) sodium channel Na (V) 1.8, a channel expressed by C-fibers and involved in nociception, and functional dyspepsia. Indeed, the SCN10A 3218 CC variant, which determines a lower activity of this Na (V)-channel, was significantly associated with a decreased risk for the development of FD[52] (all data are summarized in Table 2).

Post-infectious dyspepsia

By summarizing what we have described above, we can say that although it is unlikely that a single genetic factor causes FD, it is more likely that a genetic factor (or factors) modulates the risk of developing the abnormalities after exposure to one or more specific environmental factors[53]. This paradigm is well supported by the hypothesis of a post-infectious origin of dyspepsia. Indeed, it is now evident that in a subgroup of patients with functional dyspepsia, acute GI infections may precipitate symptoms. Data from the literature indicate that in presumed post-infectious dyspepsia patients there is an increased prevalence of impaired gastric accommodation to the meal that is likely dependent on inflammatory-induced impaired nitrergic innervation of the gastric wall[54]. However, only one study systematically addressed the genetic contribution to post-infectious dyspepsia and the data presented are quite controversial since a significant association between macrophage migration inhibitory factor-173C and IL17F-7488T polymorphisms was only observed in the subgroup of patients with symptoms suggestive of EPS-like dyspepsia[55]. Indirect evidence for a role of inflammatory products gene in the genesis of FD is also suggested by a Japanese study in which a significant association between a COX1 polymorphism and EPS-like symptoms was observed in a subgroup of dyspeptic female patients[56]. However, a very recent study investigating the role of a polymorphism in the receptor for neuropeptide S receptor gene (NPSR1) that is involved in inflammation, anxiety and nociception failed to reveal any significant association with FD[57] (Table 3).

Hypertrophic pyloric stenosis

Isolated HPS is a common condition characterized by the hypertrophy of the muscle surrounding the pylorus, with impaired sphincter relaxation and consequent gastric outlet obstruction which causes severe non-bilious vomiting. The etiology of this disease is still largely unknown; however, epidemiological studies indicate that genetic factors play an important role in the pathophysiology of this entity[58]. Krogh *et al*[59] have shown a 200-fold increased risk of HPS in monozygotic twins and a 20-fold increased risk among siblings of affected children. In particular, different studies have demonstrated a higher prevalence of HPS in offspring of an affected mother compared with offspring of an affected father, suggesting a major role of maternal factors; however, this evidence was not confirmed in further studies[60]. In addition, HPS is associated with several well-defined genetic syndromes, sustaining the hypothesis that genetic factors are largely involved in the pathogenesis of this disease[58].

A reduced expression of NOS1 was demonstrated at the mRNA level in pyloric tissue of patients with HPS, suggesting a pathogenic role of nNOS in this disease.

Table 3 Overview of genetic association studies in post-infectious dyspepsia

Protein (gene)	Polymorphism	Finding	Ref.
MIF	-173C	Risk factor for EPS	[56]
IL17	-7488T	Risk factor for EPS	[56]
COX-1	T1676C	Risk factor for EPS	[57]
NPS-R	rs2609234, rs6972158, and rs1379928	No association	[58]

IL: Interleukin; COX-1: Cyclooxygenase-1; EPS: Encapsulating peritoneal sclerosis; MIF: Macrophage migration inhibitory factor.

For this reason, several researchers have analyzed the gene of *NOS1*; however, only a Chinese group found a linkage between HPS and NOS1a on chromosome 12q[61], but this result was not confirmed by another study[62]. Moreover, the analysis of the complete coding region of NOS1 in patients and controls revealed no significant difference, confirming that the impaired nNOS function in the pylorus of these patients is not related to a direct mutation of the gene encoding for NOS1[63].

Several studies have demonstrated a linkage between HPS and different loci in affected patients of the same family, but larger studies on additional families were usually unsuccessful. A Chinese group described a linkage of a SNPs of chromosome 16 (16p12-p13) in 10 affected members of the same family, but this association was not observed in 10 additional families[64]. Moreover, the major candidate genes of this region encoding for MYH11 and GRIN2A, proteins involved in smooth muscle relaxation, have not shown mutations[64]. In members of the same family, Everett *et al*[65] found an association with SLC7A5 (16q24), a gene which influences NO activity; however, they failed to confirm these data on additional 14 families. In a genome-wide linkage study, the same group demonstrated an association between HPS and some loci on chromosome 11q14-q22 and Xq23, both encoding for protein involved in the functioning of ion channels (TRPC5 and TRPC6) in 81 families with 206 affected members[66,67]; however, a subsequent Chinese study failed to replicate the association with TRPC6[68].

Finally, a Danish group in a recent genome-wide association study (GWAS) on 1001 individuals affected by HPS and 2401 healthy controls demonstrated an association between HPS and three SNPs of MBNL1 and NKX 2-5, genes involved in the splicing and transcription processes[69], but it was the first GWAS and these results could explain only a very small proportion of HPS cases (all reviewed associations are summarized in Table 4).

In conclusion, hypertrophic pyloric stenosis is a multifactorial disease and both genetic and environmental factors could contribute to the pathophysiology of this condition. Especially for the syndromic form of HPS, different mutations in different genes involved in different functions have been associated with the onset of this disease, but we are still far from a single unifying pathogenetic factor. Conversely, in the sporadic form of the disease, the contribution of the genetic background is even more scanty and complex and no clear triggering factors have been described yet, further sustaining the great heterogeneity of this disease.

Table 4 Overview of genetic association studies in hypertrophic pyloric stenosis

Protein (gene)	Chromosome	Finding	Ref.
NOS1	12q	Association	[62,63]
MYH11-GRIN2A	16p12-p13	Association	[65]
SLC7A5	16q24	Association	[66]
TRPC5 and TRPC6	11q14-q22 and Xq23	Association	[67-69]

CONCLUSION

Several association studies have established a genetic component in the genesis of motility disorders of the upper gastrointestinal tract, like esophageal achalasia, functional dyspepsia and hypertrophic pyloric stenosis. Although candidate gene studies have identified a few gene polymorphisms that may be correlated with these syndromes, small sample size, lack of reproducibility in large data sets, and the unreliability of the clinical phenotype represent a major limit to identify a unifying factor in the pathophysiology of these syndromes in any of the reported polymorphisms.

Whether the genetic contribution plays a crucial role in the generation of upper GI tract symptoms therefore deserves further studies. More specifically, the recruitment of large case-control samples appears to be mandatory in order to provide a powerful tool for the identification of risk genes, especially for diseases like achalasia and functional dyspepsia, in which a multifactorial inheritance is assumed. In this direction, genome-wide association studies will allow for the unbiased and systematic identification of risk genes and may represent the greatest challenge for all future studies of upper GI tract motility disorders.

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REVIEW

Pancreatic cancer diagnosis by free and exosomal miRNA

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Abstract

Patients with pancreatic adenocarcinoma (PaCa) have a dismal prognosis. This is in part due to late diagnosis prohibiting surgical intervention, which provides the only curative option as PaCa are mostly chemo- and radiation resistance. Hope is raised on a reliable non-invasive/minimally invasive diagnosis that is still missing. Recently two diagnostic options are discussed, serum MicroRNA (miRNA) and serum exosomes. Serum miRNA can be free or vesicle-, particularly, exosomes-enclosed. This review will provide an overview on the current state of the diagnostic trials on free serum miRNA and proceed with an introduction of exosomes that use as a diagnostic tool in serum and other body fluids has not received sufficient attention, although serum exosome miRNA in combination with protein marker expression likely will increase the diagnostic and prognostic power. By their crosstalk with host cells, which includes binding-initiated signal transduction, as well as reprogramming target cells *via* the transfer of proteins, mRNA and miRNA exosomes are suggested to become a most powerful therapeutics. I will discuss which hurdles have still to be taken as well as the different modalities, which can be envisaged to make therapeutic use of exosomes. PaCa are known to most intensely crosstalk with the host as apparent by desmoplasia and frequent paraneoplastic syndromes. Thus, there is hope that the therapeutic application of exosomes brings about a major breakthrough.

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Key words: Pancreatic cancer; Exosomes; MicroRNA; Diagnosis; Serum

Core tip: Patients with pancreatic adenocarcinoma have a dismal prognosis due to late diagnosis prohibiting surgical intervention, which is further burdened by chemo- and radiation resistance. Hope is raised on a minimally invasive diagnosis that is still missing. Recently two diagnostic options are discussed, serum microRNA (miRNA) and serum exosomes. Serum miRNA can be free or vesicle-, particularly, exosomes-enclosed. This review presents an overview on the current state on miRNA as a cancer diagnostics and discusses arguments in favor of tumor exosomes as a diagnostic tool that additionally could provide a powerful therapeutic option in the near future.

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INTRODUCTION

Pancreatic adenocarcinoma (PaCa) ranks fourth in mortality among cancer-related deaths. With an overall 5-year survival rate of below 1% and a mean survival time of 4-6 mo it is the deadliest cancer[1,2]. There has been considerable progress in the treatment of patients with early stage PaCa. But late initial diagnosis that prohibits resection, chemotherapy and radiation resistance and the early metastatic spread of PaCa account for the non-satisfactory progress in therapy[3,4]. Thus, research has focused on defining a reliable non-invasive or minimally invasive diagnosis. So far, serum markers allowing for a non-invasive diagnosis and follow up studies are rare.

CA19-9 is still the most reliable diagnostic serum marker, but should be used in conjunction with other diagnostic tools. Additional markers are carcino-embryonic antigen (CEA), CA125 and CA242, their specificity and particularly sensitivity being below that of CA19-9[5-7]. However, recently, two non-invasive diagnostic tools have come into focus. First, serum microRNA (miRNA) was repeatedly described to allow for differential diagnosis of cancer, where PaCa patients' serum miRNA might allow differentiating between benign and malignant tumors as well as inflammation[8,9]. Second, tumor-derived exosomes are readily detected in body fluids. Their protein, mRNA and miRNA profiles might well serve as diagnostic tools[10]. In addition, exosomes are hotly debated as potent therapeutics[11-13].

TUMOR DIAGNOSIS AND miRNA

Recovery of non-coding RNA in body fluids

A new class of small noncoding RNA known as miRNA endogenously regulates gene expression at the posttranscriptional level[14]. miRNA range in size from 19 to 25 nucleotides. They regulate translation and degradation of mRNA through base pairing to complementary sites mostly in the untranslated region[15]. MiR constitute only 1%-3% of the human genome, but control about 30% of the coding genes[16], most miR controlling multiple mRNA[17]. miR biogenesis is a multistep process, where a long primary transcript (pri-miR) is processed into a 70-100 nt hairpin precursor pre-miR. The pre-miR is translocated to the cytoplasm, where it is cleaved by the ribonuclease Dicer into a mature miR duplex, which is incorporated into the RNA-induced silencing complex (RISC) resulting in degradation of the duplex and binding to target mRNA by complementary base pairing at the 3'-untranslated region[14]. Seed sequence complementarity of about 7 base pairs enables miRNA to bind the target mRNA, which results in inhibition of translation or a reduction in mRNA stability[18]. miRNA in the serum may derive from necrosis, apoptosis[19] or be actively released in microvesicles[20]. Free extracellular miRNA is associated with argonaute proteins (Ago) The Ago2-miRNA complex accounts for the stability of the free miRNA[21,22].

In advance of discussing serum miRNA as a potential diagnostic tool, it should be stated that data normalization is an important factor and that due to any fluctuation, epigenetic factors or others, like age, gender, diurnal changes and many more, cohort sizes should be large. Also due to these variabilities, it is very unlikely that a set of reference housekeeping miRNA with universal applicability can be identified[23,24]. Furthermore, it has to be kept in mind that most miRNA regulate more than one mRNA. Thus, in turn, a given miRNA may be deregulated in multiple diseases, including different types of cancer[25,26].

miRNA and cancer

The increased knowledge on miRNA greatly fostered progress in oncology, where miRNA could be linked to prognosis, disease progression, local recurrence and metastasis[24,27-29]. As summarized in a recent review[30] miRNA plays an important role in epithelial-mesenchymal transition (EMT), maintenance of cancer stem cells as well as tumor invasion and migration. EMT is regulated by the mir-200 family, miR-141, miR-429 and miR-205. The expression level of miR-200 negatively correlates with znc finger E-box-binding homeobox (ZEB)1 and 2, which inhibit E-cadherin expression[31]. In PaCa, down-regulation of miR-30 correlates with EMT, targets being vimentin and snail-1[32]. Examples for the involvement of miR in cancer stem cell (CSC) control, including pancreatic cancer, are the tumor suppressor miR-34 that regulates Notch and Bcl2[33,34] and miR-21 that correlates with chemoresistance[35]. Instead, miR-9, regulating E-cadherin expression, is suggested to be of major importance for metastasis-associated mobility and invasiveness[36,37]. miR-34a overexpression can inhibit metastasis by regulating CD44[38] and miR-340 suppresses invasion and metastasis by regulating c-Met and *via* c-Met MMP2 and MMP9[39,40].

For PaCa Jamieson *et al*[41] performed microarray analysis on resected PaCa tissue on a cohort of 48 and 24 patients. They describe associations with lymph node involvement, tumor grading and overall survival, where high expression of miR-21 and low expression of miR-34a significantly correlated with poor survival. Additional studies on PaCa tissue, non-transformed pancreatic ductal cells, CP samples and on PaCa culture lines by array or RT-PCR[42-46] have been summarized by Li *et al*[47], which also provides an overview on their function as tumor suppressors (miR-15a, miR-34a, miR-96, miR-375) or oncogenes (miR-27a, miR-132, miR-155, miR-194, miR-200b, miR-220c, miR-429, miR-212, miR-214, miR-301a, miR-421, miR-483-3p) and potential molecular targets, which include besides others WNT3A, p53, K-Ras, Akt, 14-3-3zeta and Smad4[43,48-56].

Taken together, there is increasing evidence that miRNA plays a central role in carcinogenesis and tumor progression, where the recovery of miRNA in body fluids may, additionally, provide a minimally invasive diagnostic tool. This has created hope particularly for most deadly PaCa, late diagnosis considerably contributing to the poor prognosis.

Serum miRNA as a diagnostic tool in pancreatic cancer

The stability of free miRNA in serum and other body fluids has fostered the hope for a minimally invasive diagnostic tool that may also be of prognostic value[57-59], which meanwhile has been experimentally supported for different types of cancer[60-62] including PaCa, where it will be particularly important as late diagnosis prohibits a curative intervention.

In an earlier study 4 miRNA, miR-21, miR-210, miR-155 and miR-196a have been found to differentiate PaCa patients' serum from that of healthy controls, where miR-155 is a biomarker of early PaCa and miR-196a cor-

relates with progression[63]. Evaluating a combination of CA19-9 with plasma miRNA in PaCa revealed 4 miRNA, miR-155, miR-181a, miR-181b and miR-196a, to differ significantly from healthy donors' miRNA, where only miR-16 and miR-196a allowed for discrimination from chronic pancreatitis (CP). Including CA19-9 increased sensitivity and specificity of the analysis, 85.2% of PaCa samples being positive even at stage 1[64]. An elegant recent study on serum miRNA in PaCa based on sequencing of pooled samples, a selection phase based on quantitative reverse transcriptase PCR (qRT-PCR) followed by a testing phase revealed upregulation of miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 and miR-191 in the serum of PaCa patients as compared to healthy controls. The authors also confirmed that these 7 miRNA allowed for differentiation towards CP, where expression in CP did not differ significantly from that of healthy donors[9]. Additional studies mostly confirmed abundance of miR-21, miR-155, miR-196a, miR-210 and miR-16 in PaCa patients' sera[65-69]. A statistical meta-analysis, which includes 9 studies, from which 5 were performed with tissue and 4 with serum or plasma[9,63,64] suggests a potential role for miRNA assays in screening for and confirming PaCa diagnosis[70]. However, the authors also point out that none of these miRNA is selective for PaCa. An additional concern should be mentioned. A differential analysis of free versus vesicular, particularly, exosomal miRNA in the serum of PaCa patients appears to be missing. An exosomal miRNA analysis may well be advantageous as exosomal miRNA derives from living cells, whereas free miRNA may mostly derive from dead cells and thus could significantly change particularly during therapy or in late stage PaCa[19,71,72]. Serum exosome screening could have an additional advantage. Membrane integrated PaCa markers will be recovered on exosomes, thus allowing for a concomitant screening of miRNA and proteins.

EXOSOMES AS A DIAGNOSTIC TOOL

Exosomes are small 40-100 nm vesicles, which derive from the fusion of the intraluminal vesicles of multivesicular bodies (MVB) with the plasma membrane[10,73]. Their homogeneous size is one of the major criteria to differentiate exosomes from apoptotic blebs, microparticles and microvesicles, which vary in size[74]. Exosomes are composed of a lipid bilayer; they contain selected proteins, mRNA and miRNA[75]. Exosomes are secreted by many cells and abundantly by tumor cells[76] and are found in all body fluids[77]. Due to their presence in all body fluids and the expression of selected markers, exosomes are suggested to be optimal candidates for non-invasive diagnosis[78,79]. Exosomal proteins, mRNA and miRNA being functionally active[80,81] and transferred into target cells[13,81-85], exosomes are the most important intercellular communicators[75] and are suggested to become a very powerful therapeutic tool[12,86,87]. To reach the goals of exosomes as diagnostics and therapeutics great efforts are taken to elaborate the prerequisites, such as exosome assembly and exosomal message transfer.

Exosome assembly and secretion

It is well known that the relative abundance of proteins, mRNA and miRNAs differs between exosomes and donor cells, which implies active sorting into MVB. Indeed, the sorting of proteins into exosomes is a highly regulated process, where monoubiquitinylation as well as the endosomal sorting complex required for transport (ESCRT) play a role, some components of ESCRT, like Tsg101 and Alix being recovered in exosomes. The ESCRT machinery consists of 3 complexes, ESCRT Ⅰ, Ⅱ and Ⅲ, where Tsg1 in the ESCRT complex I binds ubiquitinated protein and recruits ESCRT Ⅱ. ESCRT Ⅲ becomes recruited *via* ESCRT Ⅱ or Alix. ESCRT Ⅲ recruits a deubiquitinating enzyme that removes the ubiquitin tag from the cargo proteins prior to sorting into MVB[88,89]. However, not all proteins require the ESCRT complex for incorporation into exosomes. Alternatively, proteins in detergent resistant membrane complexes can become incorporated into MVB like MHC Ⅱ molecules in dendritic cells[90]. Lipid affinity also can account for MVB incorporation[91]. Tetraspanins and other proteins with high affinity for cholesterol and sphingolipids are partitioned into membrane domains which according to their physical properties are prone for internalization[92-95]. Proteins also may become recruited by associated proteins such as integrins associated with tetraspanins or the transferin receptor (TfR), which associates with heat shock proteins (HSP)[94]. In particular for tetraspanin-associated molecules it has been described that protein complexes rather than singular molecules are recruited into exosomes. This complex binding severely influences exosome targeting and the crosstalk with target structures[96-98]. Besides members of the tetraspanin family (CD9, CD63, CD81, CD82, CD151, Tspan8), where tetraspanins are constitutive components of exosomes[91,99] and are frequently used to differentiate exosomes from other extracellular vesicles[75,91], additional molecules most abundantly recovered in exosomes are HSP[100,101], proteases[102,103], MHC molecules, cytoskeletal proteins and signal transduction molecules[104], where engulfment of cytosolic proteins involves proteins located close to the outer membrane of MVB by automicrophagy[105].

Interest in exosomes has steeply increased, when it was reported that exosomes contain mRNA and microRNA that will be transferred into target cells[106]. Exosomal mRNA and miRNA also differs from that in the donor cell. mRNA recruitment can be guided by a zip code in the 3'-UTR[107]. Exosomal mRNA is less abundant than exosomal miRNA. Exosomal mRNA are mostly involved in cell cycle progression, angiogenesis, migration, or histone modification[98,108,109]. Exosomes also contain selected miRNA. miRNA recruitment is facilitated by coupling of RISCs (RNA-induced silencing complexes) to components of the sorting complex[110,111], the release

of miRNA being controlled through ceramide-dependent machinery associated with exosome secretion[112]. Exosomes contain > 120 miRNA from a selected number of genes. Network based analysis of exosomal miRNA points towards an involvement in stem cell differentiation (let-7), organogenesis (miR-1), hematopoiesis (miR-181) tumorigenesis (miR-17, miR-18, miR-19a, miR-20, miR-19b-1, miR-93-1)[13,14] and metastasis[105].

As exosomes are found in all body fluids[77], the selective enrichment of "marker" proteins as well as of miRNA makes exosomes a very attractive means for non-invasive diagnosis[104,113].

Tumor diagnosis by serum exosomes

Exosomes are separated by sequential centrifugation steps followed or preceded by 0.2 μm filtering. For pre-evaluation exosomes should be further purified by sucrose density gradient centrifugation[114,115]. This, however may not be possible for large sample number evaluation and also may not be feasible with the amount of available serum. According to our experience and in line with literature reports, 1 mL of serum will be sufficient for screening of a limited number of proteins and miRNA. Particularly for miRNA screening, recently a thorough comparative evaluation of mRNA preparation has been published[116], which should be taken into account as in dependence of the exosome source minor differences may lead to a pronounced loss of miRNA. Besides these "home made" exosomes, several commercially available exosome purification kits are available that were described to reveal comparable results. In addition, there are special diagnostic kits on the market, which will be helpful, if a clearly defined question is to be answered, *e.g.*, searching for one marker or a few selected miRNA. As far as one is interested to find out the protein marker or miRNA profile of exosomes of a tumor entity that has not yet already been analyzed, it may be preferable to start open minded without any preselection. In concern of the readout system, I strongly recommend for miRNA the protocol of Liu *et al*[9] described above for free serum miRNA, starting with a microarray of pooled serum exosomes from patients and control donors. According to our unpublished experience the ten most abundant miRNA are with high likeliness recovered in exosome pools of different patients. As the serum contains much more exosomes that are not tumor-derived, taking into account that only platelets account for roughly 50% of serum exosomes[117] , the comparison to healthy donors' exosomes provides already a good means to select out non-tumor exosomal miRNA. As an additional control, I would recommend exosomes from culture supernatant of tumor lines from the same cancer type.

It also should be remembered that exosome collect a limited number of mRNA and miRNA that does not correlate to the mRNA or miRNA profile of the cell, which we confirmed for a rat pancreatic cancer line and exosomes derived thereof[109]. Our unpublished study on human PaCa serum exosomes confirms this inasmuch as the miRNA profile of serum exosomes and of culture supernatant exosomes show abundance of the same miRNA. In addition, the absence of a miRNA that is recovered in serum exosomes from healthy donors and PaCa patients provides a strong hint towards this miRNA being not derived from tumor exosomes. Having selected for miRNA abundant in pools of PaCa patients serum exosomes, one can proceed with verification by qRT-PCR.

In concern of serum exosome marker profiles one should also start with pooled healthy donors serum exosomes and select for markers that are undetectable on healthy donors' exosomes. Antibodies against constitutive exosome markers may serve as controls. After this screening one can either proceed with enzyme-linked immunosorbent assay (ELISA)[118] or flow cytometry, where latex beads can be coated with antibody in advance or latex beads are loaded with exosomes and marker expression is evaluated by incubation with antibodies after blocking free binding sites of the latex beads[114,119,120]. Both procedures have advantages and disadvantages and it depends on the individual question, which to prefer. For diagnostic purposes several kits are commercially available.

So far, at least to my knowledge studies being concerned about serum diagnosis or diagnosis in other body fluids, like the urine, by miRNA have rarely taken into account the particular profile of exosomes. To give a few examples. In glioblastoma serum exosomes miR-21 was 40-fold increased[108]. In serum exosomes from ovarian cancer patients, 8 miRNA were significantly increased[121] and in prostate cancer urine exosomal miR-107 and miR-574-3p are upregulated[122].

In concern of the comparably rare trials on serum or other body fluids exosomes as diagnostic tool, I want to stress again that only exosomal miRNA is delivered by live cells[19,71,72]. Thus, this miRNA can be expected to be particularly selected for functional relevance. In addition, CSC/migrating tumor cells are suggested to be enriched in the serum[123,124] and could well contribute to the serum exosome pool and to its diagnostic validity, cancer progression relying on the small population of CSC, which account for drug resistance, metastasis and late recurrence[125-127]. Finally, exosomes being delivered by live tumor cells, the amount of exosomal miRNA may change with the size of the tumor, but the miRNA profile most likely will be stable.

Serum exosomes as a diagnostic tool have an additional advantage, as besides tumor miRNA, membrane bound tumor markers can be searched for. Thus, in ovarian cancer, $CD24^+$ and $EpCAM^+$ exosomes were recovered in ascites of tumor patients and in serum $CD24^+$ exosomes were detected, the absence of $EpCAM^+$ exosomes in serum being due to cleavage by exosomal ADAM10[128,129]. Also in ovarian cancer claudin4 was upregulated in 32 of 63 patients' serum exosomes, but only in 1 of 50 control serum exosomes[130]. In plasma exo-

somes of prostate cancer patients' survivin is upregulated compared to controls and benign prostate hyperplasia[131]. In urine exosomes of prostate cancer also PCA3 and TMPRSS2: ERG, deriving from a chromosomal rearrangement were detected, verifying body fluid exosomes as diagnostic marker[132], though in another study on prostate cancer urinary exosomes PSA and PSMA were detected, but exosomes in urine showed great variability[133]. Also in plasma exosomes from melanoma patients caveolin-1 and CD63 were consistently elevated[134] and tumor exosomes could be efficiently isolated with anti-HER2/neu from ascites of cancer patients[135]. Last, not least, the tumor-specific epidermal growth factor receptor VIII (EGFRVIII) was detected in 7 out of 25 glioblastoma patients serum exosomes[108] and our ongoing study on pancreatic cancer serum exosomes confirms recovery of exosomes carrying PaCa stem cell markers[124].

Taken together, comparably few studies on cancer patients serum/plasma or urinary exosomes confirmed the suggestion that exosomes in body fluids can serve as a diagnostic tool. Unfortunately, at least according to my stage of knowledge, PaCa serum exosomes have not yet been evaluated, where I strongly recommend to take into account that exosomes offer the possibility to evaluate both miRNA and protein markers. Our ongoing studies strongly suggest that combining the analysis of these two parameters most likely will bring about a considerable improvement in early PaCa diagnosis.

EXOSOMES AS A THERAPEUTIC TOOL

Exosomes are hotly debated as the most potent gen therapeutic option of the futur4[12]. In advance of discussing this option, I should briefly introduce what is known so far about the interaction between exosomes and target cells. I will first discuss exosome binding and uptake and proceed giving a brief overview on exosome binding and uptake-induced target modulation.

Exosome binding and uptake

In advance of considering options for the therapeutic use of exosomes, it is a conditio sine qua non to be aware, which cells in the body are potentially targeted by exosomes. Though it is well appreciated that exosomes only interact with selected targets[97,98,136], the mode of selection requires further clarification. Several options, which are mutually not exclusive are discussed, receptor-ligand interactions, attachment, fusion with the target cell membrane, or internalization[136-138].

Due to inward budding of endosomes into MVB, the outer membrane of exosomes is characterized by phosphatidylserine (PS), which can trigger exosome uptake by binding to scavenger receptors, integrins, complement receptors and PS receptors (TIM), particularly TIM-4[139,140]. In line with this, macrophages (Mϕ) very rapidly bind exosomes, binding being efficiently blocked by anti-CD11b[141]. However, *in vivo* studies did not provide evidence that exosome uptake is dictated by scavenger receptors. Furthermore, the selectivity of exosome uptake argues for PS facilitating binding, but not for being involved in exosome uptake[97,141,142].

Instead, already in 2004 evidence was presented that exosome uptake by dendritic cells (DC), Kupffer cells and some macrophages (Mϕ) involves, besides PS, milk fat globulin-E8, CD11a, CD54, CD9 and CD81 on exosomes and requires αvβ3, CD11a and CD54 as ligands on DC[143] suggesting exosome binding and uptake to involve receptor-ligand interactions that may vary depending on the protein pattern on exosomes and target cells[144]. Notably, this early study also pointed towards a later on confirmed contribution of tetraspanins[97,145,146]. We additionally unraveled that target cell ligands are also located in internalization prone protein clusters, which include annexins, chaperons, molecules involved in vesicular transport, tetraspanins and tetraspanin-associated molecules[97]. Thus, internalization by donor cells and the exosome uptake by target cells use similar fusion/fission machineries, maintenance of internalization complexes and re-use of these complexes for exosome uptake apparently being a common theme[146-148]. Furthermore, antibody blocking of CD91, a common receptor for several HSP interferes with exosome activity[149]. Of note, exosomes also bind with high avidity several matrix proteins[102], where matrix protein binding is selective and requires defined tetraspanin-adhesion molecule complexes[103]. Less is known about the discussed mechanism allowing for fusion of exosomes with their target cell. However, it has been shown that exosome fusion is facilitated or requires an acid pH[150].

Thus, exosomes display target cell selectivity, which at least partly builds on the engagement of protein complexes in internalization prone membrane domains.

Target modulation by exosomes

First to note, exosomal proteins, mRNA and miRNA are function competent[112,145]. Accordingly, there are several modes, whereby exosomes can modulate their targets. Binding-induced target modulation mostly relies on activation of exosome ligands and protein cleavage by exosomal proteases. Exosome uptake-initiated changes can be brought about by transferred proteins, mRNA and miRNA. These distinct activities of exosomes are far from being comprehensively understood, but all have exemplary been confirmed. I will mention some examples, as I feel it is important to be aware of this ongoing research to understand the potential power of an exosome based therapy.

Exosome-binding induced target modulation

Exosomes are rich in proteases[102], which modulate the exosomes protein profile as well the ECM and target cells.

A tumor creates its own matrix, but also influences the host matrix to generate surroundings promoting tumor cell migration and survival. The phenomenon is poorly understood and the impact of tumor exosomes is largely unexplored. First to note, exosome proteases

modulate the exosome protein profile, described for L1 and CD44 shedding by ADAM10 and for EpCAM, CD46, TNFR1 by unknown metalloproteinases[151-153]. Exosomal proteases also modulate the ECM, where exosomal tetraspanins due to their association with proteases and integrins become important[152,154-156]. The collagenolytic and laminin-degrading activity of exosomes facilitates angiogenesis and metastasis[142,157-162], degradation of aggrecan increases invasiveness[163,164] and exosomal MMP2, MMP9, MMP14 and cathepsinB correlate with invasiveness[160,165]. Focalizing exosomal matrix degrading enzymes allows for paving the path of metastasizing CSC towards the premetastatic niche, which we confirmed for a rat metastasizing pancreatic adenocarcinoma[103,166]. As the ECM also is a storage of bioactive compounds[167], modulation of the ECM by exosomal proteases[168] can account for cytokine/chemokine and protease liberation and generation of cleavage products that promote motility, angiogenesis and stroma cell activation[102]. Thus, the modulation of the ECM by exosomal proteases creates a path for migrating cells, favors a tumor growth promoting microenvironment, angiogenesis and premetastatic niche establishment.

Exosome-initiated signal transduction: Exosome-initiated signal transduction can be promoted by exosome binding and exosome uptake, which in most instances is experimentally difficult to decipher. Nonetheless, the impact of tumor exosome binding-initiated signal transduction on tumor immunity, angiogenesis, tumor growth/metastasis has been convincingly demonstrated.

DC-exosomes are one of the best explored examples for exosome binding-initiated signal transduction. DC-exosomes can replace DC in immune response induction and exosome-based therapy was first explored using DC-exosomes as a cancer vaccine. DC also take up exosomes secreted by other cells, including tumor cells, which they internalize and process for presentation. Thus, DC use exosomes as a source of antigen and produce exosomes that suffice for T cell activation, both features expanding the operational range of DC[143,169-171].

Tumor exosomes also affect the immune system[172]. Tumor exosomes inhibit $CD4^+$ T cell proliferation, which is accompanied by up-regulation and stronger suppressive activity of regulatory T cells (Treg) due to exosome-associated transforming growth factor beta 1 (TGF-β1)[168]. NK activity also becomes impaired *via* tumor exosome inhibiting activation of Stat5, Jak3, cyclinD3 expression and perforin release[173] or due to blocking NK cells *via* NKG2D binding[174]. Induction of myeloid-derived suppressor cell (MDSC) is promoted by exosomal TGFβ and PGE2[175]. *Via* stimulating TGFβ1 secretion by Mϕ, tumor exosomes suppress anti-tumor immune responses allowing for tumor growth and metastasis formation in allogeneic mice[176] and by high ICAM1 expression, tumor exosomes block the interaction between T cells and endothelial cells, thereby decreasing T cell recruitment[177]. On the other hand. high level HSP expression on tumor exosomes-HSP functioning as an endogenous danger signal-promotes NK activation and tumor cell lysis[178,179] and supports T cell activation and effector functions[180] as well as induction of costimulatory molecule expression in DC[181,182]. Tumor exosomal chemokines attract and activate DC and T cells, such that intratumoral injection efficiently inhibits tumor growth[183]. Tumor exosomes also can be an efficient antigen source, which induce a potent Th, CTL and B cell response, even where lysates of the same tumor are non-immunogenic[141,184].

Taken together, there is an intense crosstalk between tumor exosomes and the immune system that may be due predominantly to exosome binding-initiated signal transduction. Depending on the individual tumor's exosome composition, immune responses are suppressed, but also can be strengthened and in combination with DC tumor exosomes could well contribute to cancer immunotherapy.

Angiogenesis induction being one of the hallmarks of cancer, intense efforts have been taken to elaborate the contribution of tumor exosomes. Tumor exosomes containing tumor necrosis factor alpha (TNF-α), IL1β, TGFβ and TNFR1 recruit endothelial cell (EC) progenitors, promote angiogenesis[107] and stimulate EC by paracrine signaling[185]. Delta-like4 bearing tumor exosomes confer a tip cell phenotype to EC with filipodia formation, enhanced vessel density and branching[186], which involves activation of PPARα and NFκB activation[187]. In a feedback, prostate cancer exosomes lead to activation of fibroblasts, which then shed exosomes that increase tumor cell migration *via* CX3C-CX3CR1[188].

Another elegant examples of tumor exosome-mediated signal transduction describes overexpression of CD9 or CD82 promoting formation and secretion of exosomes that contain β-catenin, thereby reducing its cellular content and impairing Wnt signaling, which proceeds *via* tetraspanin-associated E-cadherin[189]. Besides indicating that the cargo of exosomes differs depending on ESCRT- or tetraspanin-initiated internalization, this study demonstrates that by depletion of inhibitors or stimulators tumor exosomes can opposingly affect signal transduction[190]. Also, tumor exosome-promoted tumor growth may vary for individual tumors. Thus, a deficit in Rab27a leading to reduced exosome production affected growth of a tumor line that required recruitment of neutrophils, but not of another neutrophil-independent line[191].

Briefly, binding of tumor exosomes to hematopoietic cells, EC and stroma cells can severely affect the target cell, which may become activated or suppressed. Additionally, the export of proteins into tumor exosomes affects the tumor cell itself. It also has to be kept in mind that tumor exosome-initiated signaling varies with the origin and composition of tumor exosomes. Last and importantly, the strength of tumor exosome initiated signaling relies on their accessibility throughout the body.

Exosome uptake promoted target cell modulation: Early reports on the information transfer *via* exosomes

showed that embryonic stem cell exosomes transfer messages into hematopoietic progenitor cells that promoted survival and expression of early pluripotency markers[20]. Adult tissue exosomes, too, had the capacity to alter the phenotype of their target such that upon coculture bone marrow cells (BMC) express markers found on the exosome donor cell[192], where uptake of exosome proteins, mRNA and miRNA are contributing. These findings also account for tumor exosomes, which transfer receptor and oncoproteins or miRNA[20,193].

One of the first evidences to support tumor exosome-uptake plays a critical role in autocrine stimulation of tumor growth revealed that the intercellular transfer of the oncogenic receptor EGFRVIII *via* tumor exosomes to glioma cells, lacking this receptor, causes transformation of indolent glioma cells[194] and reprograms growth factor pathways in EC[126]. Other oncogenes, like Ras, Myc, SV40T also induce signaling and gene expression[195-197], where *e.g.*, exosomal amphiregulin, an EGFR ligand, increased tumor invasiveness 5-fold compared to the recombinant protein[198].

Tumor exosome uptake-induced changes in recipient non-tumor cells can be transient, but also suffice to drive tumor growth as described for tissue transglutaminase and fibronectin[199] or high level c-Met uptake by BMC, which leads to their re-education to support premetastatic niche formation for melanoma cells, where in melanoma patients, too, circulating BM-derived cells express Met[200]. Tumor exosomes also transport apoptosis inhibitory proteins[201] and present TGFβ. This drives differentiation of fibroblasts towards myofibroblasts, which support tumor growth[202]. Adipose-tissue derived mesenchymal stem cells (MSC) also can be driven into myofibroblasts by tumor exosomes[203]. Lung cancer tumor exosome uptake stimulates IL8, VEGF, LIF, oncostatin and MMP secretion, which promotes tumor growth[204]. Instead, uptake of tumor suppressor genes from non-transformed cells can mitigate cancer cell aggressiveness[12,205].

An involvement of exosomes in metastasis was first described for platelet-derived exosomes, which transferred the αIIb integrin chain to lung cancer cells, stimulated the MAPK pathway and increased expression of MT1-MMP, cyclin D2 and angiogenic factors and enhanced adhesion to fibrinogen and human umbilical vein EC[206]. We explored that exosomes from a PaCa together with a soluble tumor matrix facilitated recruitment of hematopoietic progenitors from the BM as well as activation of stroma cells and leukocytes in premetastatic lymph nodes such that a non-metastatic tumor line settled and formed metastases[207]. The recruitment of tumor cells also becomes facilitated by exosomal HSP90, a complex of exosomal HSP90 with MMP2 and tissue plasminogen activator promoting together with exosomal annexin II plasmin activation tumor cell motility[208]. As already mentioned, the transfer of c-Met contributes to premetastatic niche formation mostly *via* bone marrow cell modulation[200]. Thus, tumor exosomes enhance migration and homing of tumor cells in sentinel lymph nodes due to stroma and hematopoietic cell as well as matrix modulation[77,108,200,207]. Finally, uptake of exosomes from non-transformed cells in the tumor surrounding can affect tumor cells such that fibroblast-exosomes promote breast cancer motility *via* Wnt planar polarity signaling[209].

Tumor exosome uptake also accounts for EC modulation. Colorectal cancer exosomes, enriched in cell cycle-related mRNA, promote EC proliferation[210]. Glioblastoma-exosome-induced angiogenesis relies on the transfer of exosomal proteins and mRNA[108]. Uptake of EGFR-positive tumor exosomes by EC elicit EGFR-dependent responses including activation of the MAPK and Akt pathway and VEGFR2 expression[211]. Transfer of exosomal Notch-ligand-delta-like-4 increases angiogenesis[183] and tumor exosomes expressing a complex of Tspan8 with CD49d preferentially are taken up by EC and EC progenitors, which initiates progenitor maturation and EC activation including VEGFR transcription[60]. Chronic myeloid leukemia (CML)-exosomes induce angiogenic activity in EC, where a Src inhibitor affects exosome production as well as vascular differentiation[212].

As mentioned tumor exosome uptake-induced target cell modulation frequently represent the net result of protein transfer-initiated signal transduction, transferred mRNA translation and mRNA silencing by miRNA. Though a separation between these activities appears somewhat artificial, a few reports describing preferential activities of mRNA and miRNA should be mentioned.

By the transfer of miR-150 in AML-exosomes to hematopoietic progenitors CXCR4 expression becomes reduced and HSC migration is impaired[213]. $CD105^+$ renal cell CSC exosomes carry proangiogenic mRNA and miRNA, which trigger the angiogenic switch[158]. mRNA and miRNA of exosomes from a metastasizing PaCa are recovered in lymph node stroma and lung fibroblasts, and transferred miRNA significantly affects mRNA translation, which was exemplified for abundant exosomal miR-494 and miR-542-3p, which target cadherin17. Concomitantly, MMP transcription, accompanying cadherin17 downregulation, was up-regulated in lymph node stroma cells transfected with miR-494 or miR-542-3p or co-cultured with tumor exosomes. Thus, tumor exosome miRNA uptake affected premetastatic organ stroma cells towards supporting tumor cell hosting[109]. Exosomes from virus transfected cells transfer viral miRNA[214,215]. Leukemia cell exosomes contain miR-92a that is transferred into EC, downregulates CD49e and increases migration and tube formation[216]. In lung cancer exosomes miR-21 and miR-29a act as a ligand of mouse TLR7 or human TLR8, functioning as agonist and leading to NFκB activation and IL6 and TNFα secretion, which promotes metastasis[217]. Hepatocellular carcinoma exosomes abundantly contain miR-584, miR-517c, one of the potential targets, TGFβ activated kinase 1, activates JNK and MAPK pathway and NFκB, where transfer of exosomal miRNA in coculture promoted anchorage-

independent growth and apoptosis resistance[218].

Stroma cells also release exosomes, whose miRNA can influence tumor cells. BM stroma cell exosomes inhibit the growth of multiple myeloma, but those derived from patients with multiple myeloma force multiple myeloma progression, the latter exosomes showing a lower content of tumor suppressor miR-15a, but high levels of oncogenic proteins, cytokines and adhesion molecules[219]. Tumor-associated Mϕ secret exosomes with high miR-223, that binds Mef2c, causing nuclear accumulation of β-catenin[220]. Monocyte exosomal miR-150, when transferred to EC, promotes migration[221].

Taken together, transferred exosomal miRNA can reprogram target cells, the linkage between exosomal miRNA and the targeted mRNA remaining to be elaborated in detail in many instances. In concern of the described impact of transferred proteins and mRNA, the question on long-lasting *in vivo* efficacy awaits clarification. Exosomes being a most powerful means of intercellular communication that function across long distance, it is utmost important to answer these open questions. Nonetheless, therapeutic exploitation of exosomes appears promising.

EXOSOMES AS THERAPEUTICS

Exosomes are discussed as most potent gene delivery system, as they are easy to manipulate and efficiently transfer proteins and genes. This could offer a means to interfere with tumor exosome promoted angiogenesis and metastasis, two major targets in cancer therapy[91,222]. In addition, exosomes are discussed as cancer vaccine[172]. Nonetheless, in advance of discussing the possibilities to interfere with tumor growth and progression *via* exosomes, I want to stress three points. First, uptake by selective target cells needs to be most thoroughly controlled. Second, the pathway whereby exosomes affect a selected target cells has to be well defined. Besides the still open question, whether transferred proteins, mRNA and miRNA or a combination account for observed effects, the multiple targets of individual miRNA could create problems such that side effects at the present state of knowledge can not be excluded. Third, it should be mentioned that the indispensability of exosome transfer in human cancer remains questionable. In A431 PS blocking inhibits uptake of exosomes by EC, but the antiangiogenic effect was only transient[194]. Also a blockade of cellular vesiculation (TSAP6, acidic sphingomyelinase) did not prevent tumorigenesis[223,224]. Furthermore, blocking of Rab27a involved in exosome biogenesis exerts distinct effects on primary versus metastatic tumor growth and also differs between tumors[200,225]. These findings should not be taken to discourage attempts to translate experimental studies on the power of exosomes into therapeutic settings, but should foster the point that clinical translation in many instances essentially awaits progress in elaborating the mode of exosome activities. These clauses account particularly for active interference with tumor exosomes. Instead, DC exosomes are already used as a vaccine[226,227].

Exosomes to substitute or support dendritic cells

Exosome research became highly stimulated, when it was noted that antigen presenting cells release exosomes derived from MVB of the MHC class Ⅱ compartment, which can stimulate T cells *in vitro* and *in vivo*[228]. Several studies report that DC-exosomes were well tolerated, induced an antigen-specific response and or NK recovery and that the disease-free survival time was mostly prolonged. For the therapeutic translation it is also beneficial that exosomes can be stored at -80 ℃ and that recovery is high. Limitation were mostly restricted to the requirement of large amounts of DC-exosomes[229-231].

Though tumor exosomes can be immunosuppressive, this does not affect their use for loading DC. Several groups report that exosomes delivered from DC after coculture with tumor exosomes might be superior to exosomes derived from peptide-pulsed DC. DC pulsed with exosomes of an AML line provoked a strong anti-leukemia response[232]. In line with this, directing tumor-associated, non-mutated antigens like CEA and HER2 to exosomes by coupling to lactadherin increased their immunogenicity[233]. Targeting prostate-specific antigen or prostatic acid phosphatase *via* lactadherin to exosomes also induced a superior immune response[234]. Furthermore, anticancer drug force the release of HSP-bearing exosomes, which efficiently activate NK cells[235]. Taking this into account, tumor exosomes should be particularly helpful as antigen source, when immunogenic entities of a tumor are unknown.

Competing with tumor exosomes

Even taking into account that an individual tumor may not essentially depend on exosomes for survival and progression, tumor exosomes doubtless support the tumor by modulating the host. Thus, competing with tumor exosomes might be a means to retard metastasis formation.

Blocking of exosome uptake could be performed at the exosome or the target cell level[229], where PS blocking of tumor exosomes only transiently inhibited angiogenesis[194]. Instead, in a rat PaCa, where exosomes expressing the tetraspanin Tspan8 induced a lethal systemic consumption coagulopathy, blocking exosomes by a Tspan8-specific antibody completely prevented undue angiogenesis, although primary tumor growth was not impaired[236,237]. Based on this finding and our ongoing studies that exosomes bind *via* tetraspanin-complexes to ligands also located in internalization prone membrane domains[59], we speculate that a scrutinized analysis of an individual tumors' exosome-binding complex should provide the information for hampering undue tumor exosome-initiated angiogenesis and premetastatic niche formation, where exosomes from non-transformed cells modulated to express the tumor exosome-binding complex will be most promising[59]. As an alternative

approach, tumor exosomes can be removed by affinity plasmapheresis known as Aethlon ADAPT™[238]. Blocking of tumor exosomes also can affect drug and radiation resistance due to enhanced release of export transporter MRP2, ATP7A and ATP7B or Annexin A3[239,240].

Tailored exosomes for drug delivery

Greatest hope in exosome therapy is based on the discovery of horizontal transfer of mRNA and miRNA[106,241], which can be translated or mediate RNA silencing[20,73,242].

As exosomes are natural products, are small and flexible, which allows them to cross biological membranes and to protect their cargo from degradation by a lipid bilayer[138], they are discussed as ideal and possibly the most potent gene delivery system[73,86,138,241,243]. Notably, exosome electroporation efficiently transfers siRNA into exosomes[112]. Furthermore, special devices can be developed, *e.g.*, to cross the blood-brain barrier, which was explored for the delivery of BACE1 siRNA, where mast cell exosomes were equipped with a brain penetrating peptide fused to the vesicular membrane protein Lamp2[244,245]. Also, curcumin or Stat3 inhibitor delivery confirmed exosomes to be well suited for drug delivery[246,247], where chemotherapeutic drug efficacy was increased by lowering the pH of exosomes[150,248]. Adenoviral vectors associated with exosomes displayed higher transduction efficacy than purified AAV vectors[249]. As exosomes from non-tumor cells contain tumor-suppressive miRNA, it was suggested to use exosomes loaded with those miRNA, which was exemplified for miR-143 as a therapeutic strategy in cancer[213]. In a mouse hepatoma, systemic administration of miR-26a, inducing cell cycle arrest, exerted a dramatic protective effect without toxicity[250]. Additional approaches like miRNA inhibitors (miRNA sponges), antagomirs, locked-nucleic-acid-modified oligonucleotides are reviewed in[23].

At the present state of knowledge miRNA based therapies have to be considered as double-edged sword as most miRNA have a multitude of targets. However, as soon as the above mentioned hurdles are solved, rapid progress in clinical translation can be expected[251,252].

CONCLUSION

The recovery of tumor-associated miRNA and of tumor exosomes in serum and other body fluids has created hope for non/minimally invasive diagnostics, where our own, unpublished data indicate that an exosome-based screening may be advantageous as it offers the possibility to search concomitantly for tumor-related protein markers as well as tumor-associated miRNA. Taking into account that the poor prognosis of PaCa patients despite considerable progress in surgical treatment is mostly due to late diagnosis, a reliable serum-based diagnosis at early stages could already significantly contribute improving the rate of curative treatment.

Beyond diagnosis, the discovery of exosomes as intercellular communicators throughout the body fostered reconsideration of many aspects of tumor biology and is hoped to bring a major breakthrough in therapy. The power of exosomes is due to their ubiquitous presence, their particular protein profile and their equipment with mRNA and miRNA as well as their most efficient transfer in target cells. Together with the ease of transfecting exosomes, there should be hardly any limits in the use of exosomes as therapeutics. The therapeutic use of exosomes from non-transformed cells to compete, to induce an immune response or to silence immunosuppression should not become a danger for the patient's organism. Instead, therapeutic approaches based on tailored tumor exosomes still awaits answers to the targeting receptors and their ligands, which most likely will offer modalities to further restrict the panel of potential targets of natural tumor exosomes and a precise knowledge on miRNA targets and consequences on release from repression. Answering these questions will take time, but is not an insurmountable hurdle.

PaCa are burdened by desmoplasia and early metastatic spread. Both features essentially depend on the crosstalk with the host, which has been convincingly demonstrated to be to a considerably degree mediated by tumor exosomes. Thus, it is my personal opinion that PaCa treatment/rognosis will particularly profit from unraveling the option of exosome-based therapy.

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MINIREVIEWS

Prospects and challenges for intestinal microbiome therapy in pediatric gastrointestinal disorders

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Abstract

Fecal microbiome (microbiota) transplantation is an emerging treatment not only for refractory/recurrent *Clostridium difficile* infections and chronic gastrointestinal diseases, but also for metabolic syndrome, and even possibly for neurological disorders. This non-conventional therapy has been perhaps more appropriately designated as fecal bacteriotherapy (FB) as well. The employment of FB is spreading into pediatric gastroenterology. This focused review highlights the pediatric applications of FB and discusses hypotheses for its mechanism of action. We propose that intestinal microbiome therapy may be a more appropriate term for FB, which integrates its potential future applications.

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Key words: Microbiome; Fecal transplant; Children; Inflammatory bowel disease; Ulcerative colitis; *Clostridium difficile*

Core tip: This review provides a focused overview of fecal bacteriotherapy and discusses possible mechanisms of action for this unconventional treatment. It also highlights the challenges, which this therapy faces.

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INTRODUCTION

The alarming increase in recurrent *Clostridium difficile* (*C. difficile*) infections (CDI) and associated deaths[1] geared the attention of gastroenterologists around the world towards fecal microbiome (or microbiota) transplantation (FMT)[2]. This non-conventional therapeutic approach has also been designated as fecal bacteriotherapy (FB)[3].

Human fecal preparations have been used for centuries in traditional Chinese medicine to treat various disorders[4]. However, it was not until 1958 when fecal preparations from healthy donors were employed by bold surgeons as enemas to treat critically ill patients with pseudomembranous colitis (PC)[5]. In spite of the surgeons' dramatic success, fecal bacteriotherapy has received less attention up to the 1980's perhaps secondary to the recognition that *C. difficile* is the pathogen for PC and that it can be effectively treated with antibiotics. Indeed, the short term efficacy of current antimicrobials is around 90% against CDI[6]. However, the infection may recur in 13%-24% of cases within 4 wk[6]. In such instances FB has been utilized with a cure approaching 90% irrespective of the mode of delivery (*i.e.*, upper gastrointestinal, colonoscopic, or large volume retention enema)[7]. The first randomized control trial comparing FB with vancomycin therapy for recurrent CDI showed the overwhelming superiority of the fecal preparation[8]. In-spite of this finding we do not understand clearly

how FB works. It appears that live bacteria are required for FB to be efficacious based on mouse model studies[9]. Many researchers argue that it is true engraftment of the donor microbiota that occurs in the recipients through FB, hence is designation as "transplantation"[10]. Only limited high-throughput metagenomic studies have addressed this question, especially over a prolonged time course after the treatments. Work with an artificial fecal bacterial preparation of 33 species found that there was a steady decline in the transplanted strains within the stool of the 2 recipients studied[11]. More specifically, only about 25%-30% of the species received remained in the recipient community by 6 mo after the "transplant". This result shows that some donor bacteria truly populate the recipient microbiome at least for several months. However, I propose that FB works by shock therapy or "enslavement" of the recipient microbiota, rather than just engrafting absent bacterial species into the recipient population. More specifically, a brief shock from a healthy donor bacterial community may restructure the recipient microbiota, which acts as a dynamic organ. The short-term to long-term engraftment of a few bacterial species from the donor stool into the restructuring recipient microbiota may aid/participate in this process. I use the example of crystallization induced by a bit of crystal placed into an over-saturated solution, such as in the case of sodium acetate (http://www.youtube.com/watch?v=HnSg2cl09PI) to demonstrate my hypothesis. In the case of FB, the stool donor is the bit of crystal and the dysbiotic recipient microbiota is the over-saturated solution. Upon the induction from the healthy donor stool, the recipient microbiota reverts back ("crystallizes") to a healthier state of the microbial community supporting its ability to overcome CDI. The arguments for the shock therapy are: (1) a single FB enema works as effectively as any other mode of delivery. Enema volumes are about 5%-10% of the colonic volume. Those reach only the hepatic flexure at best, and are evacuated within a few hours after delivery. It is physiologically rather difficult to imagine that such a preparation could truly transplant the whole intestine of the recipient with microbes; and (2) a simple cultured mixture of 10 bacterial species worked as effectively in treating CDI as a retention enema preparation[12]. It is unlikely that the treated recipients harbored only the 10 "transplanted" species following the resolution of CDI.

Based on the above, FB appears to be a more appropriate designation of this treatment modality than fecal transplantation. Even more, "intestinal microbiome therapy" (IMT) may be the most proper term for FB, since the future will likely bring the development of restricted microbial communities for the treatment of human diseases. In fact, FB has been used with benefit in inflammatory bowel diseases (IBD)[13], chronic constipation and irritable bowel syndrome[14], metabolic syndrome[15], and even in isolated cases of neurological diseases[2]. As for IBD, it was an academic physician, Justin Bennet, who innovatively treated his own ulcerative colitis (UC) with serial large volume retention enemas of stool preparation from a healthy donor[16]. Thereafter, Thomas Borody and colleagues treated 6 UC patients with 5 consecutive daily enemas resulting in over 1 year remission off all medications in all[17]. One of these patients has been in remission for over 11 years implicating the potential curative nature of FB for UC. However, in a recent review Borody *et al*[18] states that more than 5 enemas are needed for most patients, but does not define how many. This statement leads to valid concerns raised about FB in the medical community[19]. The absence of consensus in regards to volume, route, donor screening, safety measures, and the potential lack of medical supervision has been discussed. Consequently, a fecal microbiota FMT workgroup has formed, and established guidelines for donor screening and recipient selection primarily for CDI[20]. To further standardization, "universal" frozen stool preparations to treat CDI were employed with success[21]. The establishment and adherence to stringent guidelines and methods should aid the safety and future utilization of this unconventional treatment option for various human diseases.

FB has been less investigated in children, most likely secondary to safety concerns. In the meantime, parents of children suffering from UC, for example, are eager for this treatment option to become available[22]. There are only two case reports in children supporting the safety and efficacy of FB for pediatric recurrent CDI[23,24]. Additionally, a very recent publication demonstrated that serial retention enemas from healthy donors can be of benefit for pediatric and young-adult patients with mild to moderately active UC[25]. The study participants experienced only mild to moderate, self-resolving side effects from FB. These publications clearly indicate the potential utility of FB in pediatric gastrointestinal disorders as well.

At present, FB appears to hold great prospects, but also significant challenges for the treatment of human diseases. For chronic disorders, such as IBD, the end point of therapy may be difficult to define. Long-term potential side effects such as modified metabolism, changes in mood and affect, altered susceptibility to malignancies, *etc*. have not been examined. The most optimal route of delivery may vary between diseases, and between differing phenotypes of a single disease. The need for donor-recipient matching is also of question. The importance of age, gender, race, and microbiome composition (among others) are also unknown in this respect. The potential significance of fungi and viruses during IMT has not even been addressed to date. Perhaps the greatest challenge for the future will be to define restricted microbial communities for specific diseases. Only dedicated academic scientists will be able to meet these challenges and optimize metagenomic medicine for current and future generations to come.

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Intestinal barrier: Molecular pathways and modifiers

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Abstract

The gastrointestinal tract is frequently challenged by pathogens/antigens contained in food and water and the intestinal epithelium must be capable of rapid regeneration in the event of tissue damage. Disruption of the intestinal barrier leads to a number of immune-mediated diseases, including inflammatory bowel disease, food allergy, and celiac disease. The intestinal mucosa is composed of different types of epithelial cells in specific barrier functions. Epithelial cells control surface-associated bacterial populations without disrupting the intestinal microflora that is crucial for host health. They are also capable of modulating mucosal immune system, and are thus essential in maintaining homeostasis in the gut. Thus, the regulation of intestinal epithelial homeostasis is crucial for the maintenance of the structure of the mucosa and the defensive barrier functions. Recent studies have demonstrated that multiple molecular pathways are involved in the regulation of intestinal epithelial cell polarity. These include the Wnt, Notch, Hippo, transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) and Hedgehog pathways, most of which were identified in lower organisms where they play important roles during embryogenesis. These pathways are also used in adult organisms to regulate multiple self-renewing organs. Understanding the interactions between these molecular mechanisms and intestinal barrier function will therefore provide important insight into the pathogenesis of intestinal-based immune-mediated diseases.

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Key words: Intestinal epithelium; Mucosal barrier; Homeostasis; Molecular pathways; Immune-mediated disease

Core tip: The pathogenesis of gastrointestinal diseases is associated with important molecular pathways such as Wnt, Notch, Hippo, transforming growth factor-β/bone morphogenetic protein or Hedgehog in controlling cell-fate determination. Here, we discuss how they contribute to homeostasis of intestinal epithelium.

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INTRODUCTION

The intestinal epithelium is a single-cell layer that serves as a protective barrier against the external environment. It supports nutrients and water transport, while maintaining a defense against intraluminal toxins, bacteria, and antigens. The epithelial barrier is governed by the expression of adherens junctions (AJs) and tight junctions (TJs), including cadherins, claudins, occludin, and junctional adhesion molecules (JAM) proteins, which seal adjacent cells together[1]. Expression of AJ and TJ proteins is regulated by phosphorylation and it can either promote or destabilize TJ formation[2]. The AJ and TJ complexes also play a crucial role in the regulation of cellular polarization, proliferation, and differentiation[3].

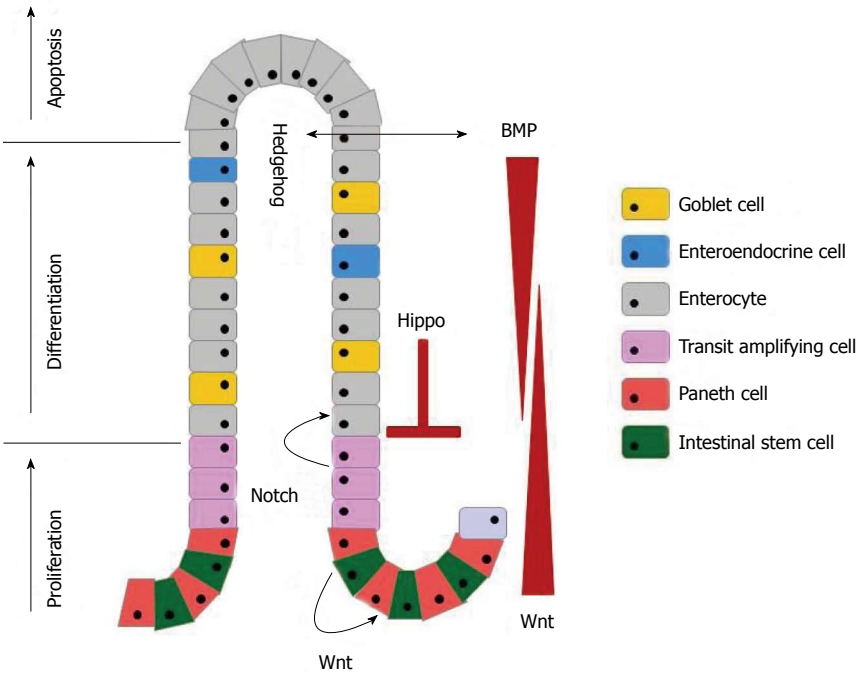

Figure 1 The molecular regulatory pathways in intestinal epithelial homeostasis. Wnt signaling promotes the proliferation of TA/stem cells and drives Paneth cell maturation. Notch signaling cooperates with Wnt to drive proliferation of intestinal stem cells and regulates maintenance of undifferentiated TA/stem cells. Hippo and bone morphogenetic protein (BMP) signaling inhibits proliferation and induces maturation of all secretory cell types. Hedgehog signaling activates the expression of BMP in the mesenchyme.

The intestinal epithelium is populated by distinct types of cells derived from stem cells, such as the absorptive cells (enterocytes) and the secretory cells (mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, Tuft cells, and antimicrobial peptides-secreting Paneth cells). All but the Paneth cells differentiate into mature forms from the crypts to replace cells extruded from the tips of the villi[4,5]. This continuous replenishment of intestinal epithelium takes generally 4-7 d and it is important for the maintenance of epithelial integrity. Several conserved signaling cascades, *i.e.*, the Wnt, transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP), Notch, Hippo, and Hedgehog pathway are associated with the maintenance of the morphological and functional features of diverse epithelial cell types (Figure 1).

Disruption of this delicate balance causes a variety of intestinal-related inflammatory and autoimmune syndromes[6-8]. For example, healthy first-degree relatives of patients with inflammatory bowel disease (IBD) and celiac disease have increased intestinal permeability and epithelial apoptosis[9,10]. Intestinal barrier dysfunction also contributes to the severity of food allergen-induced clinical symptoms[11]. In this review, we summarize current studies for major molecular signaling pathways in intestinal homeostasis.

SIGNALING PATHWAYS REGULATING INTESTINAL HOMEOSTASIS

WNT PATHWAY

Wnt signaling plays multiple roles during intestinal homeostasis. It contains the canonical and non-canonical pathway. The central player in the canonical Wnt signaling cascade is β-catenin, a cytoplasmic protein the stability of which is regulated by the APC tumor suppressor. Complexes of frizzled seven transmembrane molecules and low-density lipoprotein receptor related proteins (LRP-5/-6) serve as receptors for secreted Wnts[12]. When Wnt receptor complexes are not engaged, caseine kinase-1 and GSK3-β, both residing in the APC complex, sequentially phosphorylate β-catenin at a series of highly conserved Ser/Thr residues near its N-terminus and are thereby tagged for ubiquitination and proteasomal degradation. In contrast, Wnt stimulation blocks the intrinsic kinase activity of the APC complex, leading to β-catenin accumulation. Unphosphorylated β-catenin translocates into the nucleus where it engages transcription factors of the TCF/LEF family, thereby translating the Wnt signal into the transcription of a TCF target gene[13].

The canonical Wnt pathway is essential for epithelial proliferation and crypt maintenance[14,15]. Mutations in the *APC* gene, a negative regulator of Wnt signaling, also results in hyperproliferation of the epithelium followed by the development of adenomas[16]. Moreover, β-catenin plays a role as an AJ component, thereby linking it to the cytoskeleton of epithelial cells. Wnt signaling is necessary for positioning and maturation of Paneth cells in the crypts, and for separating proliferating and differentiated cells. These processes are controlled by the Wnt-dependent expression of specific ephrin receptors in the intestine[17]. Non-canonical (β-catenin

independent) Wnt signaling is termed the planar cell polarity pathway, which is activated by the GTPases Rho and Rac. These induce cytoskeletal rearrangements and help to form new crypts[18].

NOTCH PATHWAY

Notch signaling is active in intestinal crypt compartments and assists the Wnt pathway in promoting stem cell proliferation and negatively regulates differentiation into the secretory lineages[19]. Interaction of the Notch receptor (NOTCH 1-4) and ligand (Delta-like 1, 3, and 4; Jagged 1 and 2) between two adjacent cells results in proteolytic cleavages of the receptor by the γ-secretase enzyme complex, leading to the translocation of the Notch intracellular domain (NICD) into the nucleus. There, NICD binds to the transcription factor CSL (CBF1 in human, RBPjk in mice, Su (H) in Drosophila, Lag-1 in C. elegans) and activates target genes such as *HES-1* (Hairy and enhancer of split 1), *HES-3,* and *HES-5*, which are important for the differentiation of absorptive cells. The transcription factor MATH-1 is a downstream target of HES-1 repression in the intestine and its activity leads to generation of the secretory cell lineages[20,21]. These results suggest that the absorptive versus secretory epithelial cell type fate decision is established through the HES/MATH1 axis. In addition, Gfi-1 and neurogenin-3 (Ngn-3), other transcription factors, compete for selection of enteroendocrine versus goblet or Paneth cell fates[22].

The dysregulation of Notch activity is related to the pathogenesis of IBDs, such as ulcerative colitis (UC) and Crohn's disease (CD). A histological study in UC revealed that the depletion of goblet cells with loss of ATOH1 expression and CD is caused by dysregulation of secretory cell differentiation[23-25]. Additional evidence supporting such an important role for Notch in the intestine is derived from studies of γ-secretase inhibitors (GSIs). Treatment of mice with GSIs induced colitis due to inhibition of Notch signaling[26]. Notch activity may thus contribute to the regenerating epithelium and enhance the barrier function of the intestinal epithelium.

HIPPO PATHWAY

The Hippo pathway plays a crucial role in controlling organ size by inhibiting cell proliferation and apoptosis in response to cell-cell contact. This tumor suppressor pathway regulates intestinal regeneration and tumorigenesis[27,28]. When the Hippo pathway is active, the downstream effector of this pathway Yes-associated protein (YAP) is phosphorylated at S127 by the LAT1/2 kinases. Phosphorylated YAP remains in the cytoplasm and inhibits its proliferative and anti-apoptotic function in the nucleus, which is mediated by its binding to TEAD1-4 transcription factors. Cytoplasmic YAP has the Wnt antagonizing effects, thereby contributing to the prevention of proliferation and intestinal stem cell expansion[29]. In contrast, the deletion of Hippo pathway component Mst1/2 in mouse intestinal epithelial cells results in an expansion of undifferentiated stem cells and an absence of all secretory lineages[30].

In the small intestinal and the colonic epithelium of the normal mouse, YAP protein is found in the crypts and under normal conditions YAP makes no contribution to intestinal epithelial proliferation. However, it is required for tissue regeneration caused by injury. YAP protein is overexpressed in the crypts of the recovery phase from the DSS-treated mice[31]. Treatment of GSIs suppressed the intestinal dysplasia caused by YAP and this result suggests that YAP stimulates Notch signaling. YAP is commonly overexpressed in colorectal cancers[32]. Therefore, the activation of YAP for regulating intestinal stem cells regeneration indicates that a deficiency of Hippo signaling may contribute to tumorigenesis in the intestine.

HEDGEHOG PATHWAY

The Hedgehog signaling is initiated through the binding of Hedgehog ligands to the patched homolog 1 (Ptch1) receptor. In the absence of ligands, Ptch1 inhibits the activity of smoothened (Smo). Hedgehog binding inactivates Ptch1 and in contrast, Smo inhibition is released and these mechanisms lead to the translocation of members of the GLI family (GLI-1, GLI-2, and GLI-3 in mammals) of Zn-finger transcription factors from the cytoplasm to the nucleus. Upon nuclear translocation, they activate the transcription of target genes, such as Ptch1, Gli-1, Bmp4, Hhip1[33]. Three Hedgehog homologs, such as sonic hedgehog (Shh), indian hedgehog (Ihh), and desert hedgehog (Dhh) are known to be highly conserved in mammals and of these, only Shh and Ihh are expressed in epithelium during development, but are redistributed after villus formation to be localized to the intervillus region[34]. Thus, Hedgehog signaling is required for the formation of villi. Shh has been localized to the region of the crypts and Shh plays a role in the regulation of epithelial proliferation[35]. Furthermore, Hedgehog ligands have been reported to be anti-inflammatory epithelial modulators in the intestine[36]. Hedgehog signaling pathway inhibitors induced hypoplasia of Paneth cells, thus this signaling pathway may influence intestinal epithelium repair partly through the regulation of Paneth cells[37]. Hedgehog signaling decreases during the injury phase and increases during the repair phase[38]. It confirms an important role of Hedgehog signaling in the repair of intestinal epithelium after injury.

TGF-β/BMP PATHWAY

TGF-β signaling regulates embryonic development, wound healing, proliferation, and cell differentiation[39,40]. The TGF-β family consists of cytokines including TGF-β isoform, BMPs, and activins. Signaling is induced by ligand binding to type Ⅱ serine/threonine kinase receptors, which results in the phosphorylation of

the type I receptor. Then, signaling of these activated receptors is transduced through three classes of SMAD proteins: receptor-regulated SMADs (R-SMADs: SMAD -1, -2, -3, -5, and -8), common SMAD (SMAD-4), and inhibitory SMADs (I-SMADs: SMAD-6 and -7). R-SMADs become phosphorylated by activated type I receptors and subsequent translocate to the nucleus. The SMAD complex interacts with transcription factors, thereby inducing target gene expression. TGF-β signaling components are expressed in the differentiated compartment of the intestine[41]. Inactivation of TGF-β signaling components has also been identified at the adenoma-to-carcinoma transition[42].

BMP signaling mediates the action of hedgehog, blocking the formation of ectopic crypts, and the expression of BMP antagonist noggin in the crypts prevents the activity of BMP, thereby enabling proliferation to continue[43]. TGF-β ligands signal through SMAD-2 and -3, whereas BMP signaling is mediated through SMAD-1, -5 and -8. Phosphorylated SMAD-1, -5, and -8 are observed in the villus epithelium and this modification prevents the villus epithelium from adopting a crypt-like proliferative character[44]. BMP signaling antagonizes the Wnt pathway within the differentiated compartment, thereby positioning transient amplifying cells in the crypts[45]. In addition, humans with germline mutations in SMAD-4 or BMP receptor type 1A (BMPR1A) are associated with up to 50% of JPS (Juvenile polyposis syndrome) cases[46,47].

CONCLUSION

Intestinal epithelial barrier dysfunction has been implicated as a critical role in the predisposition to a number of gastrointestinal diseases such as IBD, food allergy, and celiac disease. It could lead to defects in multiple aspects of microbial, epithelial, and immune interactions, and thus injury to the epithelial lining thought to be an important factor in the pathogenesis of intestinal-based immune-mediated diseases[48-50].

The homeostasis of the intestinal epithelium is maintained by a complex interplay of multiple regulatory mechanisms. Together, the Wnt, Hedgehog and TFG-β/BMP pathways maintain the crypt-villus architecture. The Wnt, Notch, and Hippo signaling pathways combine to control the cell fate choices from stem cells. Thus and understanding of critical signaling pathways for maintaining intestinal homeostasis is essential.

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MINIREVIEWS

Fibrogenesis and fibrosis in inflammatory bowel diseases: Good and bad side of same coin?

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Abstract

Fibrogenesis in inflammatory bowel diseases is a complex phenomenon aimed at mucosal repair. However, it may provoke intestinal fibrosis with the development of strictures which require surgery. Therefore, fibrogenesis may be considered as a "two-faced" process when related to chronic intestinal inflammation. Many types of cells may be converted into the fibrogenic phenotype at different levels of the intestinal wall. A complex interaction of cytokines, adhesion molecules and growth factors is involved in the process. We report an overview of recent advances in molecular mechanisms of stricturizing Crohn's disease (CD) including the potential role of trasforming growth factor beta, protein kinase C and Ras, Raf and ERK proteins. Fibrotic growth factors such as vascular endothelial growth factor and platelet-derived growth factor, as well as the Endothelial-to-Mesenchymal Transition induced by transforming growth factor-β, are considered. Finally, our experience, focused on tumor necrosis factor α (the main cytokine of inflammatory bowel diseases) and the link between syndecan 1 (a heparan sulphate adhesion molecule) and basic fibroblast growth factor (a strong stimulator of collagen synthesis) is described. We hypothesize a possible molecular pattern for mucosal healing as well as how its deregulation could be involved in fibrotic complications of CD. A final clinical point is the importance of performing an accurate evaluation of the presence of fibrotic strictures before starting anti-tumor necrosis α treatment, which could worsen the lesions.

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Key words: Fibrogenesis; Fibrosis; Tumor necrosis factor -α; Syndecan 1; Basic fibroblast growth factor; Cellular fibrogenic phenotype; Inflammatory bowel diseases

Core tip: The present minireview reports an outline of the mechanisms of fibrogenesis in inflammatory bowel diseases. Potential fibrogenetic cells and their characterization are detailed. Recent advances in possible molecular mechanisms are highlighted. Our experience, suggesting the hypothesis of a possible molecular mechanism of mucosal healing, is described. The modalities whereby a deregulation of this molecular pattern may lead to fibrotic strictures in Crohn's disease are also illustrated. Finally, possible clinical implications are outlined.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are immunologically mediated disorders of the gastrointestinal tract in which inflammation and damage are the main findings. Fibrosis may be a complication of both processes, occurring more frequently in CD and often requiring surgical treatment due to the development of stenosis and hence bowel occlusion. However, fibrogenesis is a phenomenon that is intimately involved in mucosal repair[1] and, therefore, fibrotic complications of the disorders are paradoxically closely linked to a physiological phenomenon aimed at restoring damaged mucosa.

On these bases, a better understanding of the modalities of the evolution of fibrogenesis into fibrosis is essential, and the issues of how fibrosis differs from normal tissue repair, as well as the identification of cellular mediators for testing possible therapies, are intriguing emerging research concepts.

CELLULAR BASIS OF FIBROSIS

Fibrosis in CD can be viewed as an extreme healing response to injury. This model predicts that injury causes an initial activation of normal intestinal mesenchymal cells, with a shift to a "fibrogenic" phenotype[2,3]. These cells are characterized by an enhanced ability to trigger extracellular matrix (ECM) synthesis. Following acute injury, however, the normal intestinal architecture is restored because post-transcriptional and post-translational mechanisms prevent the accumulation of ECM, while fibrogenic cells are eliminated. By contrast, in fibrosis the mechanisms serving to degrade ECM are not operative at appropriate levels and fibrogenic cells are not only maintained but increase in number. The mechanisms regulating these effects are unknown but may include factors associated with CD, such as cytokines or transmural inflammation.

The normal intestine has a large, heterogeneous population of mesenchymal cells, some of which synthesize significant amounts of collagen. These cells could be considered to have a fibrogenic phenotype and are mainly constituted by fibroblasts and smooth muscle cells or myofibroblasts, as shown by their immunostaining properties with antibodies to vimentin (V) and α-smooth muscle actin (α-SMA)[2,3].

Fibroblasts are V+/A-, while smooth muscle cells are V-/A+ and predominate in the normal muscularis mucosa and muscularis propria. Subepithelial myofibroblasts (SEMF) with a V+/A+ phenotype are found adjacent to epithelial cells. However, some of these, that share common features with V+/A+ myofibroblasts, do not express α but γ-SMA[2,3].

Interstitial cells of Cajal (ICC) are a myofibroblast-related subtype specific to the intestine. They are located between enteric smooth muscle layers and serve to regulate gut motility. The c-kit receptor, which binds the protooncogene stem cell or steel factor, is a marker of cells of Cajal. Recent studies suggest that in normal human intestine these elements also express vimentin, but not α-SMA. It has been suggested that ICC could transform into a collagen-expressing fibroblast or myofibroblast phenotype. Another possibility is that ICC are destroyed during fibrosis and replaced by cells with a fibroblast phenotype[2,3].

Inflammatory cells that infiltrate the gut in UC and CD include macrophages, lymphocytes, and plasma cells. These may have important interactions with mesenchymal cells and thereby impact fibrosis[4,5].

In normal intestine, SEMF and fibroblasts found in submucosa, serosa, and intermuscular connective tissue are the primary sites of expression of collagen mRNA and protein. In UC, collagen mRNA expression is upregulated in SEMF, suggesting that chronic inflammation further increases the activity of fibrogenic cells.

Recent studies in fibrotic intestine from CD patients indicate that V+/A- or V+/A+ fibroblasts and myofibroblasts are the major sites of increased collagen mRNA expression and collagen deposition[6]. An overgrowth of the muscularis mucosa and muscularis propria occurs in CD but not UC, and this contributes to the development of stenosis, strictures, and obstruction[7]. Muscularis overgrowth also occurs in some animal models of chronic intestinal inflammation and these data support the concept that in muscularis overgrowth in CD, but not in UC, a change in enteric smooth muscle cells towards a fibroblast or myofibroblast phenotype[8] is implicated.

MOLECULAR MECHANISMS OF FIBROGENESIS

Cytokines are a heterogeneous class of secretory proteins produced by several types of cells. For some of them, they act as growth factors, for others as regulators of cellular division and finally, cytokines may paradoxically trigger mechanisms which mediate cell death. All these effects occur *via* regulation of the immune system and inflammation, so cytokines are currently subdivided into pro and anti-inflammatory types. The main cytokine involved in the pathogenesis of both UC and CD is tumor necrosis factor-α (TNF-α)[9,10]. The sites of TNF-α production are the mononuclear phagocytes, antigen activated T-lymphocytes, activated mast cells and natural killer cells. Conventional stimulators of TNF-α production are the lipopolysaccharides of the Gram negative bacteria cellular wall, since they are the main mediators of the host response to these organisms. However, TNF-α may be seen as two-faced, since it is able to trigger a closed circle in which, starting from tissue damage, it generates an inflammatory response that exacerbates the damage itself. Moreover, increasing doses of TNF-α may have a lethal effect. Nevertheless, TNF-α plays a key role in the maintenance of tuberculous granuloma, allowing Koch's bacilli to "be walled alive" and thus pre-

venting their spread in the body of an infected person (miliary tuberculosis)[11].

Cell adhesion molecules (CAMs) are proteins located on the cell surface involved in binding with other cells or with the ECM. Essentially, cell adhesion molecules help cells to join together and to their surroundings. These proteins are typically transmembrane receptors and are composed of three domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain, and an extracellular domain that interacts either with other CAMs of the same kind (homophilic binding) or with other CAMs or the extracellular matrix (heterophilic binding)[12]. A subtype of adhesion molecules containing heparan sulphate (syndecan family) is chemically a proteo-glycan and plays a significant role in tissue repair[13]. At intestinal level, syndecan 1 is located in the basolateral region of the columnar epithelium[14] and is a relevant factor in the reversal of inflammatory bowel disease (IBD) damage[15,16]. Indeed, in inflamed mucosa, these molecules are mainly located in the cells of the stroma and apical epithelium, where the repair of ulcerative lesions will presumably occur.

Basic fibroblast growth factor (bFGF) is a member of the fibroblast growth factor family[17], comprising at least 22 factors with pleiotropic functions[18]. This peptide is able to repair ulcerative lesions because of its capacity to bind epithelial and stromal cells. In normal tissue, basic fibroblast growth factor is present in basement membranes and in the subendothelial extracellular matrix of blood vessels. It stays membrane-bound as long as there is no signal peptide, when it acts as a potent angiogenic factor in patho-physiological processes that include wound healing and tissue repair[19-22]. The bFGF has been shown to promote proliferation of endothelial cells, to increase the number of fibroblasts and myofibroblasts and to activate these fibroblasts. The induction of collagen secretion from CD and UC fibroblasts by bFGF may be one of the mechanisms inherent to the stromal processes of the disease, including transmural fibrosis and stricture formation, as well as tissue repair and healing.

Klagsbrun *et al*[23] suggested that heparan sulphate proteoglycans (and, therefore, syndecan 1) change the bFGF morphology and modulate the structure of its receptors, allowing it to bind to the cells dedicated to the repair process, such as those located at the margins of an ulcerative lesion[24,25]. bFGF, when not activated by syndecan 1, is destroyed by luminal and circulating proteases, which may be activated by TNF-α, thus impeding the tissue restoration process.

Molecular mechanisms: recent advances

A relevant actor in the pathogenesis of fibrotic complications of CD is the cytokine transforming growth factor beta (TGF-β)[26]. TGF-β is secreted by many cell types, including macrophages, and has a controlling role in cellular proliferation, differentiation and apoptosis, immunoregulation, supervision of the inflammatory response, as well as fibrosis and other functions including tissue healing. It is known that TGF-β promotes collagen expression by intestinal fibroblasts and smooth muscle cells[27,28]. This process is mediated by an intracellular signaling pathway in which a cascade constituted by protein kinase C and Ras, Raf and ERK proteins plays a key role[26,29,30]. Moreover, it has been hypothesized that TGF-β promotes the overexpression of adhesion molecules (*e.g.*, intracellular adhesion molecule-1)[31] by fibroblasts and other pro-fibrotic growth factors such as vascular endothelial growth factor[32] and platelet-derived growth factor[33]. Finally, a recently revealed mechanism of fibrogenesis in CD, is the Endothelial-to-Mesenchymal Transition induced by TGF-β[34]. This cytokine is able to induce a protein expression pattern in endothelium that leads to a de-differentiation of these cells and to a transformation to a fibrogenetic phenotype, similar to fibroblasts.

In conclusion, the overexpression of TGF-β and its receptors in both the intestinal wall, and in fibroblast cultures taken from sites of intestinal stricture in patients with CD, suggests a potential regulatory role for this cytokine in intestinal fibrogenesis[35].

Adipokines are cell-to-cell signaling proteins produced by adipose tissue. The best known adipokines are leptin, adiponectin and resistin. They play a key role in regulating energy intake and expenditure, including appetite and hunger, metabolism, and behavior: indeed, their role has beenwidely studied in diseases like metabolic syndrome and type 2 diabetes. Recently, however, further functions of these molecules have been discovered, as mediators of systemic inflammation. It has been shown that obesity per se, and in particular visceral adiposity, is associated with systemic microinflammation, and disturbed circulating adipokines levels[36,37]. This is why numerous studies have been focused on the role of adipokines in the pathogenesis of IBD[38].

The outer intestinal wall in patients affected by IBD is enveloped by fat deposits called "wrapping" or "creeping" fat that seem to play an important role in the pathogenesis of IBD. An overexpression of leptin mRNA in mesenteric visceral adipose tissue (mWAT) has also been found in IBD subjects[39], and is correlated with local macrophages infiltration, which drives a high expression of interleukin (IL)-10, IL-6 and TNF-α[40]. On the other hand, adiponectin seems to have a protective function against inflammation in IBD[41,42]. Rodrigues *et al*[43] evaluated serum adiponectin and leptin by enzyme-linked immunosorbent assay in patients with active CD (ACD group), CD in remission (RCD group) and in six healthy controls, and found that serum adiponectin was lower in the ACD group as compared to controls, whereas there were no differences between the ACD and RCD groups.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is involved in the control of the expression of DNA sequences that affect basic cell functions, like cell growth, differentiation and death[44]. This pathway is triggered by the binding

of a ligand (usually a pro-inflammatory cytokine such as IL-6[45], IL-12, IL-1, TNF-α or interferon gamma[46]) to a tyrosine-kinase membrane receptor. Wu *et al*[47] evaluated whether intestinal myofibroblasts could produce nitric oxide (NO) in response to the IBD-associated cytokines IL-1b, TNF-α, and interferon gamma in a JAK-STAT dependant pathway, using intestinal myofibroblasts isolated from mice and cultured. The result was an increasing expression of inducible nitric oxide synthase (iNOS) mRNA (evaluated by real time polymerase chain reaction, RT-PCR), but not endothelial NOS or neuronal NOS. This mechanism was shown to be enhanced by a protein cascade constituted by JAK-STAT, Akt and NF-κB. The importance of NO in the pathogenesis of IBD has long been known and is widely discussed in literature[48,49]. Furthermore, genetic studies investigating polymorphisms in the *JAK* gene[50] revealed that same genetic variants (the G allele of rs744166 and the C allele of rs3816769) increase not only the risk of onset of CD, but even the risk of strictures requiring surgery, because of the interaction with the *CARD15* gene[51]. Considering the JAK2 rs10758669 polymorphism, the homozygous C/C or heterozygous A/C genotypes had a higher risk of CD as compared with the homozygous A/A (OR = 1.76, 95%CI: 1.26-2.45 and OR= 2.36, 95%CI: 1.44-3.86, respectively). On these bases, future therapy with JAK inhibitors for their anti-inflammatory effects appears promising[52].

A novel field of genetics which is attracting the attention of researchers is epigenetics, *i.e.*, the study of all the heritable modifications that vary gene expression while not altering the DNA sequence. Micro RNAs (miRNA)[53] are just one example. miRNAs are small non-coding RNA oligonucleotides that can regulate the expression of a large number of genes and have been implicated in different human diseases like cancer[54] and inflammatory diseases[55,56] including IBD[57]. A very recent study[58] performed on NCM460 human colonocytes incubated with interleukin-6 and on colon biopsies from pediatric and adult patients with UC revealed that a deregulation (low levels) of miRNA-124 can cause a hyper-phosphorylation of STAT3 (and consequently, hyper-activation), *via* a mechanism induced by IL-6, very likely resulting in a pathogenic system leading to IBD. miRNA-124 is only a second lead on the crowded stage of miRNAs: miRNA-192, which is normally expressed in colonic epithelial cells, is significantly reduced in tissues of patients with active UC[59]; miR-150 is up-regulated in mice with dextran sulfate sodium-induced colitis and in colon tissues from patients with UC[60]; an overexpression of miRNA-21, which promotes inflammation, has been reported in several studies of patients with active UC and CD colitis[61].

The process of DNA methylation is another form of epigenetic regulation of gene expression. It consists in the binding of a methyl group to cytosines that are part of cytosine-guanine dinucleotides (CpG), and has a gene silencing function. The main genes whose methylation is involved in the pathogenesis of IBD are the CDH1[62], BCL3, STAT3, OSM, STAT5[63] proteins involved in the IL-12 and IL-23 pathways.

Finally, histone modifications are an epigenetic process that may modify genomic expression. Histones are alkaline proteins that package and order the DNA into structural units called nucleosomes and chromatine[64]. They have N-terminal amino acid tails that protrude and can be modified by acetylation, methylation, ubiquitination, and phosphorylation. Acetylation, however, is the most closely studied phenomenon, because it improves gene transcription and recruitment of transcriptional factors. In the course of IBD, the main genes targeted by histone acetylation are *p53*, *STAT3*, and *HIF1a*[65]. Furthermore, an innate immune response to microbiota has been proposed to link histone modifications with inflammation: butyrate, an endogenous metabolite formed during fermentation of dietary fibers by the intestinal microbiota, is a histone deacetylase inhibitor. Butyrate increases the expression of NOD2 by increasing histone acetylation in its promoter region[66].

The importance of all these epigenetic mechanisms lies in possible future therapeutic applications: inhibitors of deacetylation, demethylating agents and miRNA produced by genetic engineering could be potential targets in IBD[67].

Molecular mechanisms in mucosal healing and strictures: our experience

Our previous investigations[68] demonstrated that a decrease of TNF-α induced by anti-TNF-α (infliximab) treatment is accompanied by a decrease in both syndecan 1 and bFGF when mucosal healing occurs. A possible explanation is that infliximab therapy may downregulate, *via* a marked reduction of TNF-α mucosal levels, the bFGF/syndecan 1 link. This molecular profile could represent a pathway of mucosal healing. However, the parallel trend of TNF-α, syndecan 1 and bFGF could be just a simultaneous consequence of the control of inflammation. To dispel this doubt, we analyzed the "timing" of the TNF-α decrease and bFGF/syndecan 1 reversal to normal levels and sites in cultured biopsy samples taken from patients with both CD and UC and incubated in a medium containing comparable amounts of infliximab similar to those reached in the serum of treated patients. After 24 h we assayed TNF-α, syndecan 1 and bFGF in tissue homogenates. TNF-α was found to be decreased, while syndecan 1 and bFGF levels were still high when evaluated by both a molecular method (reverse transcriptase real time polymerase chain reaction) and immunohistochemistry[69]. This last finding supports our primary hypothesis that a mucosal TNF-α reset, induced by biological drugs, is followed by a mucosal restoration in which syndecan 1 modulates the strong reparative bFGF aptitudes. Finally, in healed mucosa, cytokines, adhesion molecules and growth factors resume their normal pattern.

A report by Bousvaros *et al*[70] showed that in chil-

dren with CD there was a strong correlation between the bFGF level and disease activity. The relationship of bFGF with disease activity persisted even after adjusting for other covariates (including age, sex, hematocrit, albumin, and sedimentation rate) in a multivariate linear regression model. There was also a statistically significant, although less strong, correlation between the bFGF level and disease activity in UC. Although bFGF is not a specific marker for CD, its serum levels reflect disease activity. Therefore, bFGF release may be important in mediating the angiogenesis and wound healing seen in CD. This report, as well as our experience both *in vivo* and *in vitro*[68,69], suggest a similar molecular mechanism for mucosal healing both in CD and UC.

In a further study, we investigated the pattern of TNF-α, syndecan 1 and bFGF in patients with CD complicated by fibrotic stenosis undergoing surgical resection[71]. We observed that TNF-α mucosal levels were not significantly increased. A possible explanation for this finding may be that an overgrowth of fibrotic tissue may be a successive stage after inflammation, where the increase in TNF-α is the peculiar aspect. Therefore, at the stage of fibrotic stenosis requiring surgery, inflammatory mucosal changes may be an irrelevant phenomenon in most patients. Syndecan 1 levels were increased, showing a pattern similar to the one observed in damaged tissues. It may be presumed that the molecule location, albeit limited to the mucosal layer, reflects an attempt at bFGF modulation. However, this function cannot be effectively carried out due to the bFGF overexpression and location all along the intestinal wall, *i.e.*, outside the district where syndecan 1 could operate[14]. Indeed, bFGF overexpression affects all intestinal wall layers, being expressed in epithelium, stroma, muscularis propria and endothelium. It is possible that: (1) the low levels of TNF-α may provoke a failure in cytokine induced bFGF proteolysis; (2) the presence of syndecan 1 is limited to the mucosal layer with a consequent very partial regulation of bFGF binding to specific receptors dedicated to tissue repair; and (3) an irreversible transformation of different type of cells to the fibrogenetic phenotype occurs, thus provoking the prevalence of fibrotic over inflammatory stenotic lesions[72].

In strictures, it is possible that the excess extracellular matrix cannot be inhibited by the regulatory mechanisms of the phenomenon, in accordance with the hypothesis proposed by Pucilowska *et al*[72]. On these bases, we may conclude that the different molecular patterns in repair dedicated fibrogenesis and stricturizing fibrosis in CD could be the consequence of different mucosal levels of TNF-α. These are very high in the active disorder, but undergo a progressive depletion in the long term, and this event may trigger a polymorphic regulation of the syndecan 1/bFGF system.

FINAL REMARKS

Fibrogenesis in inflammatory bowel diseases is a phenomenon aimed at tissue repair. Many cellular types are involved in this process and cytokines, adhesion molecules and growth factors interact to achieve mucosal repair. However, a deregulation of the healing molecular pathway can progress towards fibrosis and stenotic complications often requiring surgical therapy[73,74]. Therefore, fibrogenesis and fibrosis may represent the good and bad sides of the same coin, the former allowing lesion healing but the latter leading to severe complications. The main tool for discriminating between them is, in our experience, the presence/absence of inflammation (and, consequently, the level of TNF-α expression).

A final clinical consideration is the importance of making an accurate evaluation[75] in cases of stenosis in the course of CD using all available diagnostic tools (histology, ultrasonography[76] with Doppler evaluation of the resistance index, magnetic resonance[77], computed tomo-enterography[78], biochemical indices of inflammation[79,80]) in order to distinguish inflammatory from fibrotic stenosis. This could orient anti-TNF-α therapy[81], that should be limited to the first case, avoiding the risk that the cytokine decrease could support fibrotic complications rather than mucosal healing.

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Effects of occupational stress on the gastrointestinal tract

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Abstract

The aim of this review is to provide a general overview of the relationship between occupational stress and gastrointestinal alterations. The International Labour Organization suggests occupational health includes psychological aspects to achieve mental well-being. However, the definition of health risks for an occupation includes biological, chemical, physical and ergonomic factors but does not address psychological stress or other affective disorders. Nevertheless, multiple investigations have studied occupational stress and its physiological consequences, focusing on specific risk groups and occupations considered stressful. Among the physiological effects of stress, gastrointestinal tract (GIT) alterations are highly prevalent. The relationship between occupational stress and GIT diseases is evident in everyday clinical practice; however, the usual strategy is to attack the effects but not the root of the problem. That is, in clinics, occupational stress is recognized as a source of GIT problems, but employers do not ascribe it enough importance as a risk factor, in general, and for gastrointestinal health, in particular. The identification, stratification, measurement and evaluation of stress and its associated corrective strategies, particularly for occupational stress, are important topics to address in the near future to establish the basis for considering stress as an important risk factor in occupational health.

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Key words: Stress; Occupation; Gastric alterations; Gastrointestinal tract diseases; Health risks

Core tip: In workers, the combination of personality patterns (anxiety/depression), stress and negative emotions contribute to gastrointestinal tract (GIT) alterations. In particular, jobs that produce privation, fatigue, chronic mental anxiety and a long past history of tension, frustration, resentment, psychological disturbance or emotional conflict have been shown to produce gastric ulcers. Irritable bowel syndrome and functional dyspepsia also have significant co-morbidity with mood alterations. Workers with unipolar depression have been shown to be more prone to present irritable bowel syndrome-like symptoms. Moreover, three systems are known to participate in the GIT alterations of workers: sympathetic autonomic nervous system, the hypothalamic-pituitary-adrenal axis and genetic factors.

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INTRODUCTION

Stress is a term that is often used by the global population. This term was first described as a "syndrome produced by diverse nocuous agents" in the 1930s by Selye[1] and was later called General Adaptation Syndrome. Stress refers to the consequences of the failure of a living organism (*i.e.*, human or animal) to respond appropriately to emotional or physical threats, whether actual or imagined[2]. Stress can be defined as any threat to an organism's homeostasis[2,3]. The function of the stress response is to maintain homeostasis and may involve both physiological and behavioral adaptations[2]. Currently, stress is a condition that affects people daily. Environmental factors, such as work pressures, financial conditions, family situations and social issues, contribute to stress. Factors related to job stress include the need for counseling, lack of leisure time, daily shift work, dissatisfaction with the workplace, work absenteeism due to health problems and insufficient work incentives. All of these situations produce psychological stress that may affect different physiological functions in the gastrointestinal tract (GIT)[3], including gastric secretion, gut motility, mucosal permeability, mucosal barrier function, visceral sensitivity and mucosal blood flow[4]. There have been several studies on the effects of psychological stress on the GIT that debate if these effects constitute a physiological response of the body or if they can be considered a pathology[2-5]. In relation to occupations, it is difficult to define a psychological stress classification for determining stress levels, exposure duration, exposure limits to stressors, the sensitivity of the worker, *etc.*; therefore, as evidenced in most of the literature, the delimitation of the problem to be addressed is almost forced. In this review article, we consider general occupational stress as assessed using multiple approaches. Additionally, we focus on occupational stress associated with specific GIT problems in any worker group, but particularly in those groups working stressful jobs.

EFFECT OF PSYCHOLOGICAL STRESS AND EMOTIONS ON THE GASTROINTESTINAL TRACT

The relationship between psychological stress and disease has already been recognized by the ancient Greeks, who hypothesized that moods affect the body. Hippocrates described how psychosomatic disorders produce abnormal physical reactions due to stressful emotions, and Galen supported the idea that emotions and pain are diseases of the soul. However, in 1637, Descartes changed the paradigm by proposing the separation of the thinking mind from the material body[5]. Currently, there are an increasing number of reports regarding cognitive and psychological declines related to stress, including occupational stress, in subjects without psychiatric premorbidities or major life trauma[6].

The relationship between emotions and gastric motility has been documented since the nineteenth century and the beginning of the twentieth century by Charles Cabanis and William Beaumont and thereafter by Ivan Pavlov, Walter Canon and Stewart Wolf, who were the pioneers in determining the gastric response after an emotional stimulus in animal models[7,8]. Based on these antecedents, researchers and clinicians were curious about the relationship between stress and gastric motility. For example, Muth *et al*[9] reported the case of a fistulated patient who displayed increased gastric motility when he was angry but decreased gastric motility when he was fearful. Nevertheless, in general, the role of occupational stress in gastric motility has not been closely examined. The main limitation, as far as we perceive, occurs because most of the techniques used for GIT evaluations are invasive[10].

The combination of personality patterns and emotional stress also has an important contribution to GIT alterations. In a review by Alp *et al*[11], studies were mentioned that suggested privation, fatigue and mental anxiety frequently coincided with the presence of gastric ulceration[12] and that a psychological disturbance or emotional conflict might be transformed into an organic disease, *e.g.*, a peptic ulcer[13,14]. The same paper also mentioned that a significant correlation exists between the onset of peptic ulcer symptoms and domestic upset, financial stress or an extensive past history of tension[15]. Furthermore, anxiety, frustration, resentment and fatigue were suggested to be important aggravating factors in the symptomology of peptic ulceration[16]. Many of the emotional states previously described by these researchers are related to psychological stress.

In relation to other GIT alterations, such as irritable bowel syndrome (IBS) and functional dyspepsia, a significant co-morbidity reportedly exists between mood alterations (*e.g.*, anxiety and depression) and functional gastrointestinal syndromes[17]. However, the exact pathophysiological link between emotions and the gut is not yet well established (see below). A model of an emotional motor system (EMS) that reacts to interoceptive and exteroceptive stress was proposed by Karling *et al*[18]. These investigators found that recurrent unipolar depression patients who were experiencing remission did not have a greater number of IBS-like symptoms than the controls, indicating that GIT dysfunctions may resolve when depression is treated to remission. Apparently, there is a relationship between mood alterations (*e.g.*, anxiety and depression) and IBS-like symptoms in patients with unipolar depression, in patients with IBS and in a sample of the normal population. In addition, the investigators suggested that, during the regulation of the emotional-motor system, there is a participation of the following three systems (the interrelationships of which are presented schematically in Figure 1): (1) the sympathetic autonomic nervous system (ANS), which explains symptoms that occur when patients change their body position; (2) the hypothalamic-pituitary-adrenal (HPA) axis, which explains the symptoms of diarrhea and early satiety by the stimulation of

Corticotrophin-releasing hormone (CRH) receptors and (3) the *val158met* COMT polymorphism (single nucleotide polymorphism in the *COMT* gene that encodes Catechol-O-methyl-transferase), which is associated with IBS-like symptoms. IBS patients tend to have a lower frequency of the heterozygous val/met genotype, and this genotype may be protective against IBS/IBS-like symptoms. Moreover, a higher frequency of the val/val genotype is associated with diarrhea symptoms[19].

JOB, OCCUPATION AND PSYCHOLOGICAL STRESS

A job is defined in the OECD Glossary of statistical terms[20] as a set of tasks and duties executed, or meant to be executed, by one person. Based on this description, an occupation is defined as a set of jobs whose main tasks and duties are characterized by a high degree of similarity. An occupational classification is a tool for organizing all jobs in an establishment, an industry or a country into a clearly defined set of groups according to the tasks and duties undertaken in the job. The occupation classification is generally not based on health risk factors. However, the concept of occupational health has been well-defined since 1950, when the International Labour Organization (ILO) and the World Health Organization (WHO) created a common definition through the ILO/WHO Committee on Occupational Health. The definition reads as follows: "Occupational health should aim at the promotion and maintenance of the highest degree of physical, mental and social well-being of workers in all occupations…the placing and maintenance of the worker in an occupational environment adapted to his (her) physiological and psychological capabilities..."[21]. In this definition, the mental well-being and the psychological capabilities are mentioned; moreover, the main focus of occupational health describes the promotion of a "positive social climate". The term health, in relation to work, indicates not merely the absence of disease or infirmity but also includes the physical and mental elements affecting health, which are directly related to safety and hygiene at work[22].

In 2001, the ILO published the ILO-OSH 2001 document titled "Guidelines on Occupational Safety and Health Management Systems" to assist organizations introducing OSH management systems[23]. Usually, occupational health hazards are considered as physical, chemical, biological and/or ergonomic factors; almost everywhere, psychological factors, such as stress[24], are not even mentioned. We consider that one of the main reasons for minimizing psycho-social factors is because the principles of occupational hygiene are the "recognition and/or identification" of occupational health hazards, the "measurement" of the level or concentration of such factors, the "evaluation" of the likelihood and severity of harm and the "control strategies" available to reduce or eliminate risks. Usually, recognition and identification implies the obvious correlation between cause and effect, with the effect being associated with physical illness or injury. In our case, psychological stress has been recognized and correlated with physical effects in many studies[25]. Furthermore, the control strategies (although a subjective topic) appear to be well known by professionals and therefore are well-established[26]. The measurement of levels or concentrations and the evaluation of the likelihood and severity of the stress factors are the most complicated factors to be studied quantitatively, and we consider these factors to be poorly established aspects of the problem.

Despite the discussion above, stress, particularly occupational stress, has become one of the most serious health issues in the modern world[27]. The concept of occupational stress can be observed as a natural extension of the classical concept of stress introduced by Selye[1] to a specific form of human activity, namely work. Steers[28] indicated that occupational stress has become an important topic study of organizational behavior for several reasons: (1) stress has harmful psychological and physiological effects on employees; (2) stress is a major cause of employee turnover and absenteeism; (3) stress experienced by one employee can affect the safety of other employees and (4) by controlling dysfunctional stress, individuals and organizations can be managed more effectively. More recently, Beheshtifar and Modaber[29] described five types of sources of occupational stress: (1) causes intrinsic to the job, including factors such as poor physical working conditions, work overload or time pressures; (2) the role in the organization, including role ambiguity and role conflict; (3) career development, including lack of job security and under/over promotion; (4) relationships at work, including poor relationships with the boss or colleagues, an extreme component of which is mobbing in the workplace; and (5) the organizational structure and climate, including the experience of having little involvement in decision-making and office politics.

The large diversity of stress factors, the complexity of labour activities and the large number of worker health-status levels make it very difficult to complete an exhaustive review of the relationships among GIT alterations, stress and occupational activity. Nevertheless, many delimited investigations have been performed around these topics, mainly for specific activities, delimited work places and/or specific groups of people. For example, in a controlled study of lorry drivers, de Croon *et al*[30] investigated the result of specific job demands on job stress (fatigue and job dissatisfaction), thereby identifying the risk factors associated with the psychosocial work environment to begin building an effective stress-reducing strategy. Another study of telemarketers directly addressed occupational stress by reporting the prevalence of stressors affecting job performance[31]. In a non-specific occupational study of migrants in Spain, Ronda *et al*[32] reported the occupational health-risk differences between local and foreign-born workers. These investigators listed what they called psychosocial factors, of which most were identified with stressful work condi-

tions. Based on the self-reported exposure, this study revealed a larger difference in females in non-service jobs; although no specific attention was paid to psychosocial factors, the prevalence of exposure to occupational risk factors appeared to be, on average, higher for migrants. The same research group in Spain searched for risk factors during pregnancy using self-reports, which are the predominant method to report psychosocial risks. The results of these investigations revealed that the prevalence of the psychosocial risks was, on average, higher than any other chemical, physical or biological factors[33].

Several researchers studied the psychological stress in a specific pathology, for example, in diabetes. Golmohammadi *et al*[34] investigated the occupational stress in diabetic workers from Iran and found that the type of occupation was not an important factor in psychological stress, although a difference was evident in the patients compared with the control group. The researchers concluded that occupational stress may be a risk factor in the development of diabetes. Specific studies addressing jobs identified as stressful have been conducted. Examples of this type of work are the study of stress-factor risks in nurses from England[35] or in psychiatric nurses (*i.e.*, a job with more exposure to psychological risks) in Japan[36]. Other stressful jobs are those related to the army and security services. Martins *et al*[37] studied the military hierarchy in peace times in the Brazilian Army, finding correlations with common mental disorders. Berg *et al*[38] studied security service workers, focusing on personality, anxiety and depression. Another type of occupation considered stressful is the mental health profession[39,40]. These professionals, similar to other workers exposed to long-term occupational stress, often experience the stage known as burnout. According to Selye's definition, if stress is associated with adaptation, then occupational stress should be identified conceptually with a temporary adaptation to work that is associated with psychological and physical symptoms. The long-term process of adaptation to certain jobs yields chronic physical and psychological symptoms. The final stage in a breakdown during this adaptation is known as burnout and is caused by prolonged occupational stress[39,40]. In 1982, Belcastro[41] stated that several somatic complaints have been suggested to be associated with burnout, including gastrointestinal disturbances, nausea and loss of weight, which are some of the most common symptoms. The author suggested that specific illnesses appear to be associated with burnout, including colitis, gastrointestinal problems and drug and alcohol addiction, among others. Developed countries face different challenges than do non-developed countries, specifically, economic[42] and cultural differences, which have been considered in stress research[43]. Nonetheless, it is difficult to describe the occupational psychological-stress classifications, levels, exposure durations, exposure limits, sensitivity, *etc*. Therefore, in most of the literature, the delimitation of the problem to be addressed is almost forced. In this review article, we focus on occupational stress in general, assessed in any manner. Additionally, we focus on specific GIT problems in any worker group, but mainly in those groups dedicated to some jobs well-identified as stressful.

TYPE OF JOB, STRESS AND GIT ALTERATIONS

Currently, gastric ulcers are identified as an extremely common chronic disease in working-age adults. The first description of an association between stress and peptic ulcer disease was in men with supervisory jobs; these individuals had a higher ulcer prevalence than executives or artisans. Cobb and Rose[44] found that air traffic controllers, particularly those individuals with higher stress levels in their workplace, were almost twice as likely to have ulcers than the civilian copilots. Hui *et al*[45] noted that the numbers of positive and negative life events were similar in both subjects with dyspepsia and control subjects, but the former had a higher negative perception of major life events and daily stresses. Police officers' work-stress reactions have been classified as physiological, emotional and behavioral reactions[46]. Physiological reactions have been termed as having a higher-than-normal probability of death from certain illnesses; after cardiovascular problems, stomach problems are the most frequent. Changing work shifts has also been associated with changes in the digestive system, circadian rhythm and other bodily reactions. Angolla[46] studied 229 police officers (163 males and 66 females) who answered a questionnaire consisting of five parts: demographics, external and internal work environments, coping mechanisms and symptoms. This investigator found that police work is highly stressful, and the highest rated symptoms were as follows: feeling a lack of energy, loss of personal enjoyment, increased appetite, feeling depressed, trouble concentrating, feeling restless, nervousness and indigestion. Satija *et al*[47] evaluated 150 professional workers (100 males and 50 females) who self-completed the Emotional Intelligence and Occupational Stress Scale. The authors' findings demonstrated a negative correlation between emotional intelligence and occupational stress; those professionals with a high score in overall emotional intelligence suffered less stress. Shigemi *et al*[48] evaluated 585 employees (296 males and 289 females), all of whom were working at a middle-sized company in Japan. A self-administered questionnaire about smoking habits and perceived job stress was administered, and the patients were followed for two years. In addition, previous or current gastric or duodenal ulcers were evaluated. The researchers found 32 incidences of peptic ulcers over the two years, and the risk ratio (RR) was 2.13 (95%CI: 1.09-4.16) between job stress and peptic ulcers. Susheela *et al*[49] evaluated 462 smelter workers, 60 supervisors working in the smelter unit and 62 non-smelter workers (control group). The participants' state of health and gastrointestinal complaints were recorded and included the following symptoms: nausea/loss of ap-

petite, gas formation, pain in the stomach, constipation, diarrhea (intermittent) and headaches. The researchers found that the total number of complaints reported by the study groups was significantly higher than in the control group. The prevalence of gastrointestinal complaints in the smelter workers was significantly higher ($P < 0.001$) than in the non-smelter workers (control group). In 2006, Nakadaira *et al*[50] investigated the effects of tanshin funin (*i.e.*, working far from one's hometown and therefore far from one's family) on the health of married male workers. A prospective study using the pair-matched method was performed in 129 married male tanshin funin workers who were 40-50 years of age. Matched workers living with their families also participated. These researchers demonstrated that fewer tanshin funin workers ate breakfast every day. Moreover, these workers more frequently suffered from stress due to daily chores and from stress-related health problems, namely, headache and gastric/duodenal ulcers (21% and 2.4%, respectively). The levels of gamma-glutamyl-transpeptidase in workers reluctant to work under tanshin funin conditions and in workers who spent less than two years in tanshin funin conditions increased significantly, although the corresponding levels in the matched regular workers did not exhibit significant changes. The investigators concluded that abrupt changes in lifestyle and elevated mental stress were thus important effects of tanshin funin.

Although jobs and occupations are considered a risk factor for stress and morbidity for gastric and duodenal ulcers[9,11,48], there are studies that report discrepancies. For example, Westerling *et al*[51] evaluated the socioeconomic differences in avoidable mortality in a Swedish population from 1986 to 1990. Using the population of 21- to 64-year-old individuals, the researchers performed analyses for different socioeconomic groups [blue-collar workers (BCWs), white-collar workers (WCWs) and the self-employed] and for individuals outside the labor market. The researchers demonstrated that the largest differences were found in ulcers of the stomach and duodenum, in addition to other symptoms. For these causes of death, the risk of dying was between 3.1 and 7.5 times greater in the non-working population than in the workforce. The differences in avoidable mortality between BCWs and WCWs and the self-employed were much smaller. However, the death rate for ulcers of the stomach and duodenum in BCWs was 2.8 times higher than for other work categories. The GIT and mortality problems reported by Wesrterling *et al*[51] used standardized mortality ratios for the occupied population. The ratios for malignant neoplasms of the large intestine, except for the rectum, were 95 in BCWs and 104 in WCWs and for malignant neoplasms in the rectum and rectum-sigmoid junction were 103 and 100 for BCWs and WCWs, respectively. However, for gastric and duodenal ulcers, the investigators reported ratios of 163 and 59 for these BCWs and WCWs, respectively. Moreover, the causes for mortality reported for abdominal hernia, cholelithiasis and cholecystitis were 127 and 86 for BCWs and WCWs, respectively. However, the researchers suggested that, as in most other studies, the follow-up period was short and that the exposure data from earlier censuses would be advantageous.

In Table 1, we summarize the international literature regarding the GIT disorders most frequently reported by workers experiencing job-related psychological stress and other alterations in their affective states. In a cross-sectional study with a population of 2237 subjects from San Marino, Italy, Gasbarrini *et al*[52] demonstrated that the prevalence of *Helicobacter pylori* (*H. pylori*) was 51%; this prevalence increased with age from 23% (20-29 years) to 68% (> 70 years) and was higher among manual workers. In San Marino, there was a higher incidence of clinically relevant gastroduodenal diseases, such as peptic ulcer and gastric cancer (25 of 10000 and 8 of 10000 in 1990, respectively). With respect to Italy and other European countries, Gasbarrini *et al*[52] demonstrated that *H. pylori* infections tended to be more frequent among BCWs ($P < 0.001$), especially those doing manual work (miners 78%, road sweepers 65%, plumbers/painters 61%, housekeepers 60% and cooks 58%) compared with WCWs (physicians 15%, clerks 21%, secretaries 37%, nurses 38%, general managers and lawyers 38%, teachers 39% and shop workers 50%). A high prevalence of infection was also noted among social workers (74%). The researchers concluded that social workers with a high educational standard had a higher rate of seropositivity to *H. pylori* (74%) than did subjects with a similar socioeconomic status but a different type of job, thereby emphasizing the relevance of direct person-to-person spread of the infection. This result was also recently demonstrated in nurses and in cohabiting children. These findings suggest that poor hygienic standards and a low socioeconomic status (which frequently reflects the former) are important factors for acquiring *H. pylori* during the first years of life, thereby confirming previous findings regarding the differences between developed and developing countries and the importance of overcrowding and close person-to-person contact during childhood.

In 2009, Lin *et al*[53] investigated 289 call-center workers (mean age of 33.6 years) to investigate how these workers perceived their job stress and health status and the relationships among inbound (incoming calls) versus outbound (outgoing calls) workers in a Taiwanese bank. Data were obtained on individual factors, health complaints, perceived job stress levels and major job stressors (using the 22-item Job Content Questionnaire, C-JCQ). For inbound services, operators handled approximately 120 to 150 calls during each 8 h per day. Outbound operators were primarily responsible for sales and handled approximately 120 calls daily. The subjects completed the self-administered questionnaires during their leisure time (taking between 15 and 20 min). The results demonstrated that 33.5% of outbound service-call center workers and 27.1% of inbound service-call

Table 1 Summary of the international literature on the gastrointestinal tract disorders most frequently reported by workers experiencing job-related psychological stress and other alterations in their affective states

GIT alteration	Ref.	Study/procedure	Conclusions
Generalized GIT disturbances/IBS	Konturek *et al*[4]	Impact of stress on the GIT. The study addresses the role of stress in the pathophysiology of the most common GIT diseases.	The exposure to stress is the major risk factor in the pathogenesis of various GIT diseases.
	Bhatia *et al*[5]	Association between stress and various GIT pathologies.	The mind directly influences the gut. The enteric nervous system is connected bidirectionally to the brain by the parasympathetic and sympathetic pathways, forming the brain-gut axis.
	Karling *et al*[18]	Pre- and post-dexamethasone morning serum cortisol levels were analyzed in 124 subjects with symptoms of IBS.	There is a relationship between mood alterations (anxiety/depression) and IBS-like symptoms in patients with unipolar depression, in patients with IBS and in a sample of the normal population.
	Karling *et al*[19]	In total, 867 subjects representative of the general population and 70 patients with IBS were genotyped for the val158met polymorphism. The IBS patients completed the Hospital Anxiety and Depression Scale questionnaire.	There is an association between the val/val genotype of the val158met *COMT* gene and IBS and with the specific IBS-related bowel pattern in IBS patients.
	Belcastro *et al*[41]	Estimation of the relationship between teachers' somatic complaints and illnesses and their self-reported job-related stresses. Stress group: teachers	Several somatic complaints have been suggested to be associated with burnout.
	Hui *et al*[45]	Perception of life events and the role of daily "hassles" (stressful events) in 33 dyspeptic patients *vs* 33 controls of comparable sex, age and social class.	Patients with non-ulcer dyspepsia have a higher negative perception of major life events than controls. Psychological factors may play a role in the pathogenesis of non-ulcer dyspepsia.
	Angolla *et al*[46]	Empirical study of police-work stress, symptoms and coping strategies among police service workers in Botswana measured by a questionnaire. Sample size n = 229 (163 males and 66 females). Stress group: the Botswana Police Service.	Police duties are highly stressful. The highest rated symptoms were as follows: feeling a lack of energy, loss of personal enjoyment, increased appetite, feeling depressed, trouble concentrating, feeling restless, nervousness and indigestion.
Ulcers	Alp *et al*[11]	Comparative study using a neuroticism-scale questionnaire administered to 181 patients with previously diagnosed gastric ulcers and 181 controls without any previous history of gastric ulcers.	People with a past history of chronic gastric ulcers have an increased incidence of domestic and financial stress compared with age- and sex-matched individuals with no previous history of gastric ulcers.
	Cobb *et al*[44]	Review of aeromedical certification examinations of 4325 traffic controllers and 8435 second-class aviators. Stress group: air traffic controllers.	Air traffic controllers were almost twice as likely to have stomach ulcers as civilian copilots .
	Shigemi *et al*[48]	Two-year study to examine the role of perceived job stress on the relationship between smoking and peptic ulcers.	These results suggest that specific and perceived job stress is an effect modifier in the relationship between the history of peptic ulcers and smoking.
	Nakadaira *et al*[50]	Effects of working far from family on the health of 129 married male workers (40-50 yr of age) compared with the control group.	The tanshin funin workers had higher rates of missing breakfast, stress due to daily chores and stress-related health problems (*e.g.*, headache, gastric/duodenal ulcers and common colds/bronchitis).
	Westerling *et al*[51]	1985 study of the Swedish population (21-64 yr of age). Analyses of standardized mortality ratios (avoidable mortality) of blue-collar workers, white-collar workers, self-employed workers, and individuals outside the labor market. Stress group: Unemployed individuals	The death rates for the non-workers were higher than for the workers. The largest differences were found for stomach and duodenal ulcers.
	Lin *et al*[53]	289 call center workers in Taiwan, 19 to 54 yr of age. Health complaints, perceived level of job stress and major job stressors were considered. Stress group: call center workers.	Workers who perceived higher job stress had significantly increased risks of multiple health problems, including hoarse or sore throat, irritable stomach and peptic ulcers.
Gastric motility alterations	Mawdsley *et al*[2]	Review of recent advances in the understanding of the pathogenic role of psychological stress in IBD, with an emphasis on the necessity of investigating the therapeutic potential of stress reduction.	The living-organism (human or animal) responds to emotional or physical threats. Psychological stress contributes to the risk of IBD relapse.
	Huerta-Franco *et al*[3]	Bioimpedance technique. In this study, 57 healthy women (40-60 yr of age) were analyzed.	Assessment of the changes in gastric motility induced by acute psychological stress.
	Mai *et al*[7]	Description or tracking of 238 experiments conducted over more than 10 yr on a young man (Beaumont) with digestive disorders.	Emotions can cause bile reflux into the stomach and may delay gastric emptying.
	Wolf *et al*[8]	Description of the work of the French physiologist Cabanis.	Inhibitory and excitatory effects of gastric secretory and motor function were described.

Muth *et al*[9]	Electrogastrograms were recorded, and the inter-beat intervals were obtained from electrocardiographic recordings from 20 subjects during baseline and in response to a shock avoidance task (shock stimulus) and forehead cooling (dive stimulus).	Acute stress can evoke arousal and dysrhythmic gastric myoelectrical activity. These acute changes, which occur in healthy individuals, may provide insight into functional gastrointestinal disorders.

GIT: Gastrointestinal tract; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; COMT: catechol-O-methyltransferase.

center workers were classified as suffering from high stress, which was considerably higher than figures from a wider survey of the working population in Taiwan (7.6%). The researchers demonstrated a relationship between the perceived job stress and health complaints, indicating that workers who perceived a higher job stress had a significantly increased risk of multiple health problems (OR ranging from 2.13 to 8.24), including an irritable stomach and peptic ulcers [42% and 57% for inbound and outbound operators, respectively ($P < 0.05$)]. For example, the OR of irritable stomach and peptic ulcers when the stress was moderate (*i.e.*, sometimes feeling extremely stressed at work) was 3.03 (95%CI: 1.40-6.55) and when the stress was high (*i.e.*, often or always feeling extremely stressed at work) was 8.24 (95%CI: 3.56-19.09). The researchers concluded that there is an association between the perceived job stress and health complaints, as workers who perceived a high level of job stress had significantly increased risks of irritable stomach and peptic ulcers.

In 2011, Nabavizadeh[54] demonstrated that physical and psychological stress increase gastric acid and pepsin secretions possibly by raising the gastric tissue nitric oxide level. In return, the increased gastric acid and pepsin secretions cause necrotic and inflammatory changes in the gastric and duodenal tissue.

STRESS IN THE PATHOPHYSIOLOGY OF GASTROINTESTINAL ALTERATIONS

Currently, many adults die from diseases caused by the relationship between stress, moods and vital organs; among these diseases, GIT has become a major clinical problem. Stress is an acute threat to the homeostasis of an organism, either physically or psychologically. A number of studies have shown that stress can delay gastric emptying, impair gastro-duodenal motility[3], modify gastric secretions[55] and pancreatic output and alter intestinal transit and colonic motility. Owing to its considerable effects on physiological and pathophysiological processes of gastrointestinal (GI) motility, stress is thought to play an important role in the development, maintenance and exacerbation of symptoms related to functional GI disorders. To analyze the effects of occupational stress on GIT function, it is important to have an understanding of the physiology of GIT motility and emptying. Similar to almost every other system, the physiological processes that occur in the GIT are wide-ranging; the major function of the GIT includes swallowing, motility, emptying (of every section), assimilation and elimination. Motility enables swallowing, transit, emptying and elimination, and all these functions are essential for proper assimilation[56]. Beginning with swallowing and ending with elimination, motility is required for GIT function[57,58]. Two variables related to GIT motility are particularly important: (1) peristalsis, which is a function of the frequency and magnitude of the gastric contractions that are generated by the pacemaker area and (2) gastric emptying, which is a measure of the average time the stomach takes to empty half of its luminal content. The ANS regulates GIT motility, controlling peristaltic activity through the myenteric system[59,60] (Figure 1). In fact, alterations in GIT motility are frequently viewed as signs of neuropathy of the myenteric plexus or other pathologies of neuropathic origin. Abnormal gastric emptying is considered a clinical marker for a gastric or intestinal motility disorder[61,62]. Quigley and other researchers have found a relationship between stress and delayed gastric emptying or other motor disturbances[63-65]. This factor is understood by analyzing how the human body reacts defensively when threatened by the environment and when attempting to achieve both physical and psychological balance. However, activation of these adaptive or allostatic systems can become maladaptive because of frequent, chronic or excessive stress and can cause a predisposition to disease[5]. This explanation leads to the concept of brain-gut interaction described by Mawdsley and Rampton[2]. These authors mentioned that, to maintain homeostasis, a living organism must constantly adapt to environmental alterations at a molecular, cellular, physiological and behavioral level. As presented in Figure 1, these investigators hypothesized that exposure to psychological stress causes alterations of the brain-gut interactions (brain-gut axis), ultimately leading to the development of a broad array of GIT disorders, including inflammatory bowel disease, irritable bowel syndrome, other functional GIT diseases, food antigen-related adverse responses, peptic ulcers and gastro-esophageal reflux disease (GERD)[16,19]. For instance, IBS is presumed to be a disorder of the brain-gut link associated with an exaggerated response to stress[66].

More recently, relative importance has been ascribed to the hypothesis that emotional and environmental states in females play an important role in the genesis of IBS[2]. This hypothesis has been proved by demonstrating that, worldwide, women present the highest prevalence of physical and psychological symptoms compared with males[67]. Men may be more apt to experience stress due to unfamiliar house chores than women, and women are more likely to experience occupational stress than

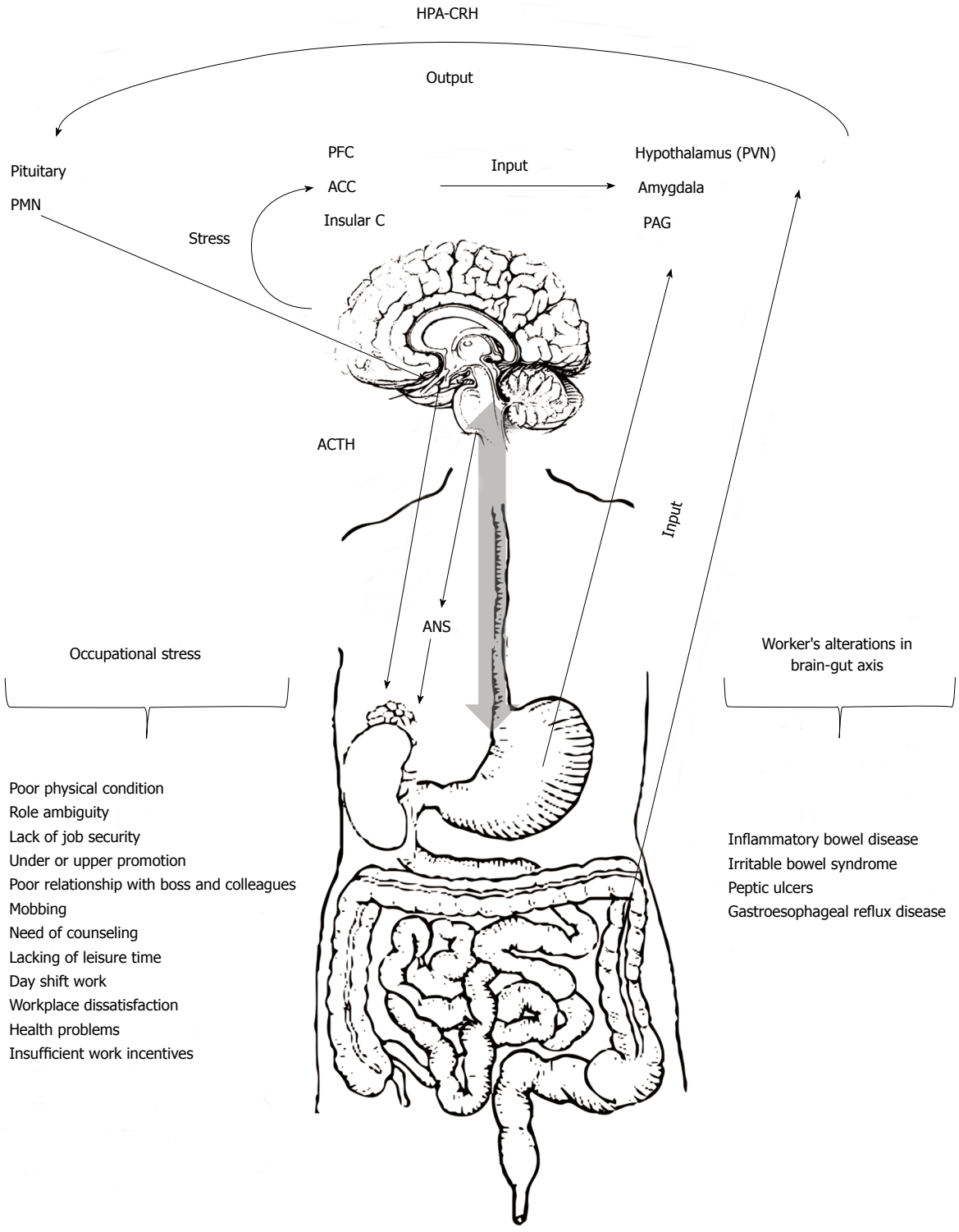

Figure 1 Representation of the hypothetical mechanism by which occupational stress produces gastrointestinal tract alterations in workers. Stress during job development (see occupational stress) generates a response of the network integrated by the hypothalamus (paraventricular nucleus), amygdala and periaqueductal grey. These brain regions receive input from visceral and somatic afferents and from the medial prefrontal cortex (PFC) and the anterior cingulated (ACC) and insular cortices (Insular C). In turn, output from this integrated network to the pituitary and ponto-medullary nuclei (PMN) mediates the neuroendocrine and autonomic responses in the body. The final output of this central stress circuitry is called the emotional motor system and includes the autonomic neurotransmitters norepinephrine and epinephrine and neuroendocrine (hypothalamus-pituitary-adrenal axis, HPA) and pain modulatory systems. PVN: Paraventricular nucleus; PAG: Periaqueductal grey; GIT: Gastrointestinal tract; ACTH: Adrenocorticotropic hormone; CRH: Corticotrophin-releasing hormone; ANS: Autonomic nervous system.

men[50]. The emotions and stress experienced by workers (Figure 1) play an important role in exaggerated gut responses. Stress affects the relationship between the brain and gut, leading these systems to act defensively to real or imaginary threats.

In Figure 1, we present the pathophysiological mech-

anism by which stress has been proposed to produce GIT alterations in workers. The workers' responses to stress are generated by a network comprising integrated brain structures, particularly sub-regions of the hypothalamus (paraventricular nucleus), amygdala and periaqueductal grey. These brain regions receive input from visceral and somatic afferents and from cortical regions, including the medial prefrontal cortex and sub-regions of the anterior cingulated and insular cortices. In turn, output from this integrated network to the pituitary and ponto-medullary nuclei mediates the neuroendocrine and autonomic responses in the body[4,18,19]. The final output of this central stress circuitry is called the emotionalmotorsystem and includes the autonomic neurotransmitters norepinephrine and epinephrine, the neuroendocrine HPA axis and the pain modulatory systems. This circuit is under feedback control by serotonergic neurons from the raphe nuclei and noradrenergic neurons from the locus coeruleus[5].

The neuroendocrine response to stress is mediated by corticotropin-releasing hormone (CRH). In the brain-gut-axis, CRH is considered a major mediator of the stress response. Particularly, the stress-related activation of CRH receptors has been reported to produce alterations in GIT function. Physical and psychological stress delays gastric emptying, accelerates colonic transit and evokes colonic motility in rats. Accelerated colonic motor function can be produced by the central or peripheral administration of CRH and is blocked by treatment with a variety of CRH antagonists. In a clinical trial, Sagami *et al*[68] administrated a non-selective CRH antagonist (10 ug/kg of αhCRH) to 10 IBS patients and 10 healthy controls. The researchers demonstrated that the peripheral administration of αhCRH improved GIT motility, visceral perception and negative moods in response to gut stimulation, without affecting the HPA axis in IBS patients. This response was significantly suppressed in IBS patients but not in controls after the administration of αhCRH[68]. IBS is considered a disorder of the brain-gut-link. Psychological stress induces colonic segmental contractions, which are exaggerated in IBS patients. Similarly, the peripheral administration of CRH affects colonic motility, induces abdominal symptoms and stimulates adrenocorticotropic hormone (ACTH) secretion, all of which are also exaggerated in IBS patients[69]. Two CRH receptor subtypes, R1 and R2, have been suggested to mediate increased colonic motor activity and slowed gastric emptying, respectively, in response to stress[5].

The genesis of gastric ulcers by stress was demonstrated in the study of Saxena *et al*[70], who investigated the gastro-protective effect of citalopram (an antidepressant drug) both as a single dose pre-treatment and 14-d repeated pre-treatment for animals exposed to cold restraint stress (CRS). The results revealed that the plasma corticosterone level significantly increased in the stress group compared with the control group. Furthermore, mucosal ulceration, epithelial cell loss and a ruptured gastric mucosal layer at the ulcer site were observed in the gastric mucosa of rats exposed to CRS. Repeated citalopram pretreatment decreased the CRS-induced enhancement in the corticosterone level. The researchers also demonstrated that citalopram at doses of 5, 10 and 20 mg/kg significantly attenuated the CRS-induced gastric mucosal lesions.

In summary, gastric ulcers are identified as an extremely common chronic disease in working-age adults. In workers, the combination of personality patterns (*e.g.*, anxiety and depression), stress and negative emotions significantly contribute to GIT alterations. Particular jobs that produce privation, fatigue or chronic mental anxiety and a long past history of tension, frustration, resentment, psychological disturbance or emotional conflict lead to gastric ulcers (*e.g.*, in traffic controllers, police officers, smelter workers, tanshin funin workers, health professionals and manual workers). Irritable bowel syndrome and functional dyspepsia also exhibit significant co-morbidities between mood alterations in workers (*i.e.*, anxiety and depression). Workers with unipolar depression were shown to be more prone to present IBS-like symptoms. Moreover, three systems are known to participate in the mechanism of GIT alterations in workers: (1) the sympathetic ANS, (2) the HPA axis and (3) genetic factors.

Subjective evaluations of stress (mainly self-reported) are extremely common in the clinic and in research. However, much work must first be done to quantitatively identify the psychological stress (*i.e.*, the occupational stress level in this case), considering the particularity of each worker (*i.e.*, the general health, the social and physical adaptation capacity and the physical and psychological vulnerability). The unique demands of each occupation require unique profiles of each worker. It is essential to train workers not only in specific skills but also in human and social aspects that include stress control strategies. Although the word "stress" has been included in our everyday language (even in research), the term continues to be a vague concept, even with the clear definition coined in 1936 by Selye.

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MINIREVIEWS

Usefulness of percutaneous endoscopic gastrostomy for supportive therapy of advanced aerodigestive cancer

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Abstract

Aerodigestive cancer, like esophageal cancer or head and neck cancer, is well known to have a poor prognosis. It is often diagnosed in the late stages, with dysphagia being the major symptom. Insufficient nutrition and lack of stimulation of the intestinal mucosa may worsen immune compromise due to toxic side effects. A poor nutritional status is a significant prognostic factor for increased mortality. Therefore, it is most important to optimize enteral nutrition in patients with aerodigestive cancer before and during treatment, as well as during palliative treatment. Percutaneous endoscopic gastrostomy (PEG) may be useful for nutritional support. However, PEG tube placement is limited by digestive tract stenosis and is an invasive endoscopic procedure with a risk of complications. There are three PEG techniques. The pull/push and introducer methods have been established as standard techniques for PEG tube placement. The modified introducer method, namely the direct method, allows for direct placement of a larger button-bumper-type catheter device. PEG tube placement using the introducer method or the direct method may be a much safer alternative than the pull/push method. PEG may be recommended in patients with aerodigestive cancer because of the improved complication rate.

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Key words: Aerodigestive cancer; Percutaneous endoscopic gastrostomy; Direct method; Introducer method; Pull/push method; Complications

Core tip: Aerodigestive cancer is well known to have a poor prognosis and is often diagnosed in the late stages with dysphagia. Insufficient nutrition and lack of stimulation of the intestinal mucosa may worsen immune compromise. Therefore, it is most important to optimize enteral nutrition before and during treatment, as well as during palliative treatment. Percutaneous endoscopic gastrostomy (PEG) may be useful for nutritional support. PEG tube placement using the introducer method or the direct method may be a much safer alternative than the pull/push method. PEG may be recommended in patients with aerodigestive cancer because of the improved complication rate.

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INTRODUCTION

Tumors of the esophagus and gastroesophageal junction or head and neck are some of the most malignant cancers with high mortality rates because many patients are diagnosed in the advanced stages[1]. Dysphagia, or difficulty swallowing, is one of the most distressing and debilitating symptoms. Dysphagia leads to nutritional compromise and deterioration of quality of life[2,3]. When the esophageal lumen becomes stenotic to less

than 14 mm in diameter, dysphagia generally develops. It first becomes difficult to swallow solid food. Next, it becomes difficult to swallow semisolid food. Finally, fluids and even saliva are difficult to swallow[4]. Patients develop anorexia and significant weight loss secondary to the tumor effects and may present with varying degrees of malnutrition. A poor nutritional status is a significant prognostic factor for increased mortality[5].

Selection of therapy for aerodigestive cancer is dependent upon the tumor stage, location and histological type, and the physician's experience and preference. Therapeutic options include surgical resection of the primary tumor, chemotherapy and radiotherapy. Therapies are sometimes combined, such as chemotherapy plus surgery or chemotherapy and radiotherapy plus surgery. Many of these patients find that their initial dysphagia worsens during this treatment because of side effects such as esophagitis and oral mucositis. Moreover, insufficient nutrition and lack of stimulation of the intestinal mucosa may worsen immune compromise due to toxic side effects[6]. During these periods, it is most important to optimize enteral nutrition. Early enteral nutrition reduces the incidence of life-threatening surgical complications in patients who undergo esophagectomy or esophagogastrectomy for esophageal carcinoma[7-11]. Nutrition is administered through a transnasal feeding tube for short-term feeding when oral intake is not possible. When chemotherapy and/or radiotherapy are intended to be curative, they frequently compromise oral intake for a long period of time. Nasogastric tubes are easy to place but they are poorly tolerated for prolonged periods of feeding. Percutaneous endoscopic gastrostomy (PEG) may be one of the best options for nutritional support.

A majority of patients are destined to receive palliation only, which is associated with a severely impaired health-related quality of life. These patients require palliative treatment, including brachytherapy, chemotherapy and endoscopic palliation techniques, such as esophageal dilatation, intraluminal stents and laser therapy, to relieve progressive dysphagia[12,13]. The two most commonly used strategies for improving swallowing are stent insertion and radiation, including intraluminal brachytherapy. They allow for an almost normal oral intake. Unfortunately, some patients develop restenosis symptoms after palliative therapy and some develop severe treatment-related side effects such as mucositis from radiation therapy. Stent insertion is difficult in some patients with proximal esophageal cancers or head and neck cancers. For these patients, PEG or nasal tubes may be the best options for nutritional support.

PEG PROCEDURE

There are three PEG tube insertion methods. The pull/push and introducer methods have been established as standard techniques for PEG tube placement. In the pull/push method, the feeding tube is introduced through the mouth. In the introducer method, balloon-type catheter feeding tubes can be inserted directly into the stomach through the abdominal wall. The third method is the modified introducer method (*i.e.*, direct method). The direct method allows for direct placement of a larger button-bumper-type catheter device. Use of the direct method is spreading in Japan, but it is not yet used worldwide[14]. Each method has advantages and disadvantages.

Pull/push method

The pull/push PEG technique is based on the standard Ponsky technique in which a guidewire is inserted through the abdominal wall under endoscopic guidance, grasped by a snare through a port on the endoscope, and subsequently advanced in a retrograde manner through the patient's mouth. The remaining end exits the patient through the anterior abdominal wall. A 20-French Ross Flexiflo Inverta-PEG tube (Abbott Laboratories, Columbus, OH) is then secured to the transoral end of the patient's mouth and abdominal wall by pulling the extra-abdominal end of the wire to advance the gastrostomy tube[15].

Introducer method

The introducer PEG technique is based on the Russell introducer method of PEG placement. After the endoscope is inserted and the PEG site is marked, four T-fasteners are placed before gastrostomy tube insertion to secure the stomach to the anterior abdominal wall. This prevents gastric wall displacement while inserting the gastrostomy tube. Using the Seldinger technique, a short guidewire is then passed transabdominally under endoscope visualization. Serial dilators are passed over the guidewire to create a stoma tract; the endoscope remains in place for visualization and verification of gastrostomy tube placement. An 18-French Ross Flexiflo gastrostomy tube (Abbott Laboratories) is then inserted or pushed over the guidewire, directly through the anterior abdominal wall[16].

Direct method

The direct method is a modified version of the introducer method (Direct Ideal PEG kit; Olympus Corp., Tokyo, Japan). After the stomach is secured to the anterior abdominal wall, the skin incision is dilated by passing a dilator percutaneously into the stomach over the guidewire as the same as introducer method. After the dilator is removed, a 24-French PEG tube is inserted using an obturator[14] (Figure 1).

OUTCOMES OF PEG

PEG in patients with aerodigestive cancer

PEG tube feeding is the preferred method with which to provide long-term tube feeding and its use is currently widespread. Many studies have examined the usefulness of PEG for aerodigestive cancer. A PEG tube was inserted in patients with oral intake difficulties for the

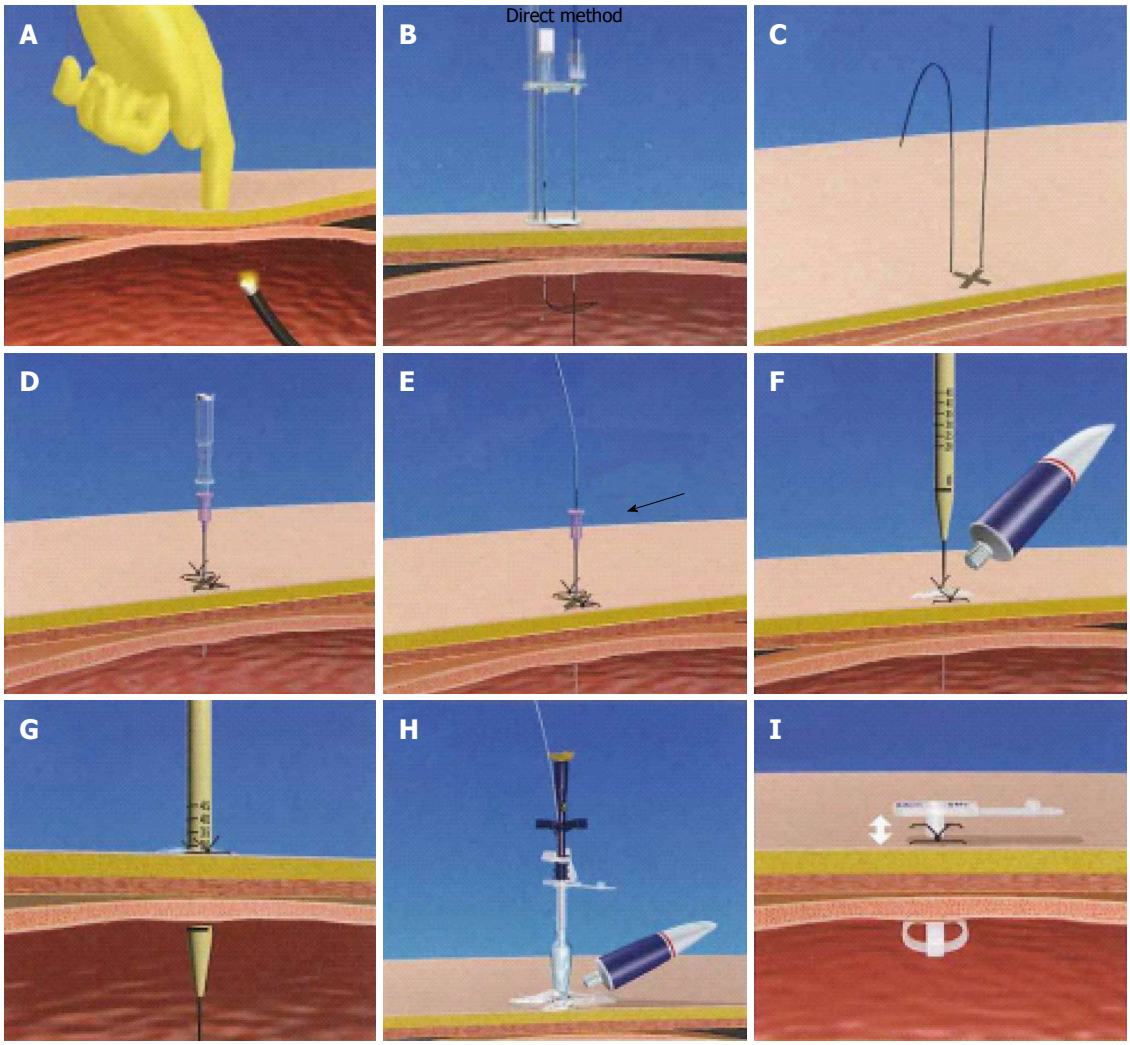

Figure 1 Direct method. A: The transilluminated area on the abdominal wall was pushed with a finger; B, C: The stomach was punctured using a double-lumen gastropexy device; D: A needle with an outer plastic sheath (18-French) was introduced into the stomach under endoscopic control; E: The needle was removed and the guidewire was replaced; F, G: The skin incision was dilated by passing a dilator percutaneously into the stomach over the guidewire under endoscopic visualization; H: After the dilator was removed, a 24-French percutaneous endoscopic gastrostomy tube using an obturator was inserted over the guidewire; I: The tube was fixed to the abdominal wall.

purpose of nutrition support in all stages and locations, including patients who had undergone chemotherapy and chemoradiation therapy with curative intent[17-22]. Chemotherapy or chemoradiation therapy is frequently associated with mucositis, dysphagia, loss of taste and anorexia. Chemotherapy, chemoradiation therapy and hyperfractionated radiation therapy are usually associated with even more severe treatment-related side effects and greater impairment of swallowing function. These treatments are long-term. Therefore, during these periods, PEG tube insertion may be one of the best options for nutritional support if the complication and mortality rates of PEG are low. Nasogastric tubes are easy to place but they are poorly tolerated for prolonged periods of feeding because they are associated with frequent ulceration, esophageal reflux and general discomfort. PEG tubes are better tolerated but they must be used selectively in patients who can be predicted to have a long-term need for nutritional support[23].

There are more reports of patients with head and neck cancer than patients with esophageal cancer. One of the reasons is that in the operation planned in esophageal cancer patients, PEG may limit the reconstruction of the stomach after esophagectomy because of the adhesion of the stomach and the abdominal wall, or the possibility of the injury for the right gastroepiploic artery which is needed in the reconstruction of the stomach[17]. Another reason for this is that stent insertion and brachytherapy are the first-choice palliative treatments in patients with middle and low esophageal cancers in many institutions. In terms of nutritional support, the most important factor is maintenance of oral food intake, which should stabilize or even improve quality of life. Dysphagia improves more rapidly after stent placement[12,13] and long-term relief of dysphagia is better after brachytherapy[24,25]. Therefore, stent placement may be reserved for esophageal cancer patients with severe dysphagia in combination with a short life expectancy who need more rapid relief of dysphagia and for patients with persistent or recurrent tumor growth after

Table 1 Comparison of the advantages and disadvantages of the pull, introducer and direct percutaneous endoscopic gastrostomy placement methods

	Advantages	Disadvantages
Pull method	Bumper type device inside stomach prevents misplacement of catheter Large-bore catheters can be used immediately after placement	Catheter may be contaminated during passage through mouth/esophagus → Increased risk of wound infection and tumor implantation Endoscope must be inserted twice to confirm correct placement
Introducer method	Adherence to aseptic technique guarantees low risk of wound infection Endoscope must be inserted only once	Risk of bleeding and incorrect puncture with large trocar Only small-lumen catheters can be used immediately after placement Catheter size must be increased step by step High probability of catheter misplacement (if using balloon type)
Direct PEG Kit	Adherence to aseptic technique guarantees low risk of wound infection Endoscope must be inserted only once Small puncture needle and blunt dilator → small wound One-step insertion of bumper type device Large-bore catheters can be used immediately after placement	Risk of bleeding

PEG: Percutaneous endoscopic gastrostomy.

brachytherapy[12,13]. When these modalities are technically not possible, nutritional support with a nasoenteric feeding tube or PEG tube should be considered to maintain adequate calorie intake. Grilo *et al*[22] suggest that PEG should be considered as a nutritional support method in patients with upper esophageal cancer that is unsuitable for esophageal stenting. For patients who suffer from restenosis symptoms after palliative therapy or who have proximal esophageal cancers or head and neck cancer, PEG may be one of the best options for nutritional support.

Thus, depending on the treatment, disease and the degree of stenosis, the following situations are indications for PEG. First, in aerodigestive cancer patients undergoing chemotherapy or chemoradiation therapy who are suffering from dysphagia, PEG is the first choice. Stenosis, even if not severe, and if lesions are located in the upper esophagus or head and neck, is an indication for PEG because difficult long-term oral intake is expected due to mucositis and esophagitis during the treatment. Next, in the operation planned for head and neck cancer patients, PEG is indicated because the stomach is not used for reconstruction. Lastly, in palliative treatment, patients with lesions of the upper esophagus or head and neck with the difficulty of a stent are indications for PEG. In addition, PEG will be indicated in patients in whom stenosis is severe even after palliative radiation therapy or a stent (Figure 2).

However, studies on this topic have weaknesses typical to retrospective studies. Nugent *et al*[26] and Locher *et al*[27] reported that there is insufficient evidence to determine the optimal method of enteral feeding for patients with head and neck cancer receiving radiotherapy and/or chemoradiotherapy. Larger studies of enteral feeding in patients with esophageal cancer are needed.

COMPLICATIONS

PEG tube placement is an invasive endoscopic procedure with a risk of complications. Minor complications resulting from PEG tube placement include cellulitis, ileus, peristomal leakage, extrusion, tube obstruction and gastric wall hematoma formation. Major complications include peritonitis, hemorrhage, airway aspiration, peristomal wound infection, buried bumper syndrome, tumor implantation and gastrocolic fistula[28,29] (Table 1).

The major complications of the standard pull/push method, which requires an esophageal lumen sufficient to pass a standard endoscope[30], include peristomal wound infections, presumably resulting from contamination of the gastrostomy catheter as it passes through the oral cavity[14,31], and tumor implantation at the PEG site[28,32] which are specific for pull/push method in the aerodigestive cancer patients. In the literature on patients with cancer, the overall complication and mortality rates of the pull/push method in patients with head and neck cancer are 10.9%-42.0% and 0%-5%, respectively[15,17,18,20-22,33-36].

An overall complication rate of 0%-11% and mortality rate of 0% have been reported with the introducer method[15,16,37,38] compared with the pull/push method in patients with aerodigestive cancer. In the pull/push method, one reason for the high complication rate may be that it is necessary to dilate the lumen before treatment when the stenosis caused by the tumor is severe. In many aerodigestive cancer patients, PEG tube placement by pull/push method can be limited by digestive tract stenosis. PEG tube placement using an introducer is the safest alternative in this group of patients but use of the available devices is difficult to implement.

In the past, the introducer technique was technically more demanding and associated with a lower success rate. This problem was solved by the use of T-fasteners to secure the anterior stomach to the abdominal wall[39,40]. Therefore, recent data on the introducer method using T-fasteners show low complication rates of less than 11% and no mortality[38,41-45]. However, Dyck's study shows that severe short-term complications may occur in patients with esophageal or head and neck tumors after place-

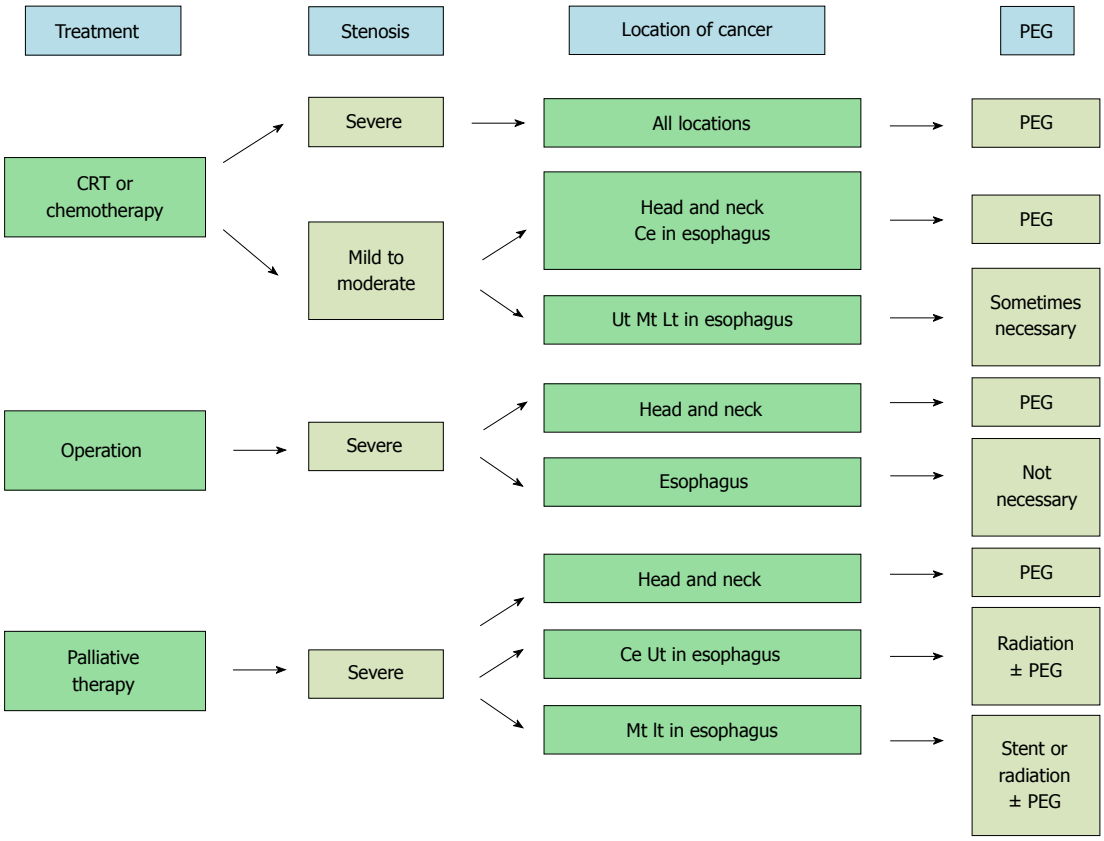

Figure 2 Algorithm of percutaneous endoscopic gastrostomy for aerodigestive cancer. CRT: Chemoradiation therapy; Ce: Cervical esophagus; Ut: Upper thoracic esophagus; Mt: Middle thoracic esophagus; Lt: Lower thoracic esophagus; PEG: Percutaneous endoscopic gastrostomy.

ment of the introducer PEG tube with T-fasteners, leading to urgent surgical intervention and even death in a substantial number of patients[20]. Why the complication and mortality rates were high in Dyck's study is unclear. Selection bias may be one reason. Van Dyck *et al*[20] reported that better follow-up of PEG tube daily care might be necessary. In almost all studies, the complication and mortality rates were low. Larger studies on the introducer method in patients with esophageal cancer are needed.

One disadvantage of the introducer method is that only small diameter balloon-type catheters are available and the requirement for frequent catheter changes when long-term tube feeding is needed[42,43]. The modification of the PEG device using the introducer technique is improved in this respect. It allows for the use of a larger-caliber tube with low complication rates and no procedure-related mortality. The direct method reduces the incidence of catheter changes compared with the 20-French catheter in the standard pull/push method. It is also feasible, safe and efficient in outpatients with obstructive head and neck cancer. However, procedure-related severe bleeding associated with the direct method has been reported[46].

TIMING OF PEG TUBE PLACEMENT

Cady[47] reported that patients who require therapeutic PEG tube placement in response to significant weight loss during treatment suffer greater morbidity than patients who receive PEG tubes prophylactically. Patients who have a PEG tube at treatment initiation experience less overall weight loss and fewer hospitalizations and toxicity-related treatment interruptions. However, Locher *et al*[27] reported that systematic evidence assessing both the benefits and harm associated with prophylactic PEG tube placement in patients undergoing treatment for head and neck cancer is weak and the benefits and potential for harm have not been established.

CONCLUSION

An optimal supportive treatment for aerodigestive carcinoma is not yet available. PEG has many advantages for aerodigestive cancer, although there is insufficient evidence to determine the optimal method of enteral feeding. Enteral nutrition by the introducer method or the direct method must be studied with an emphasis on the long-term effectiveness and safety of supportive therapy of the aerodigestive cancer.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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