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Mini Review

Achalasia Treatment: A Review of Per-oral Endoscopic Myotomy and Laparoscopic Heller Myotomy

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Abstract

Achalasia is a motility disorder of the esophagus, characterized by failure of relaxation of the lower esophageal sphincter and disordered peristalsis. Although it is a rare condition, its incidence is rising, likely due to advances in diagnostic techniques and the adoption of standardized definitions. Achalasia is associated with significant morbidity, and currently, there is no cure. Pharmacologic, endoscopic, and surgical interventions are aimed at symptom control. Laparoscopic Heller myotomy (LHM) has been the standard of care for achalasia since the 1990s. Over the past two decades, per-oral endoscopic myotomy (POEM) has emerged as a viable treatment option. Today, LHM and POEM represent the two most effective treatment modalities available for achalasia. This review aims to compare outcomes following LHM and POEM for achalasia and to explore patient characteristics and technical factors that guide optimal treatment selection. We examine the evidence regarding dysphagia relief, reflux, complications, and reintervention rates for both procedures, taking into account factors such as prior surgical history, achalasia subtype, and patient comorbidities.

Introduction

Achalasia is a motility disorder of the esophagus characterized by failure of relaxation of the lower esophageal sphincter (LES) and abnormal peristalsis of the esophageal body.[1] North American population studies estimate the incidence of achalasia to be 1.63 per 100,000 patients, suggesting that achalasia is an uncommon diagnosis but one associated with decreased survival compared to healthy controls.[2] More recent studies have found the incidence rate to be two to three times higher than previously thought, which may reflect the increasing utilization of modern diagnostic techniques such as high-resolution esophageal manometry (HREM).[3] The Chicago Classification utilizes HREM results to categorize esophageal motility disorders and has led to more standardized definitions of achalasia and its subtypes, improving diagnosis, management, and study of the disorder.[4] HREM is considered essential for the diagnosis of achalasia and can be supplemented with upper endoscopy and barium swallow studies.

The primary presenting symptom of achalasia is dysphagia; however, regurgitation, reflux, chest pain, aspiration, and weight loss are also commonly reported.[5] Given the non-specific symptoms associated with achalasia, there is often a delay in diagnosis and subsequent treatment.[6] The Eckardt score is a validated clinical tool used to assess achalasia severity and monitor treatment response based on patient-reported symptoms, including weight loss, dysphagia, chest pain, and regurgitation.[7] The pathophysiology of achalasia is poorly understood. It involves an inflammatory process causing degeneration of the inhibitory neurons of Auerbach's plexus in the esophagus. Genetic, autoimmune, infectious, and post-viral etiologies have been implicated.[8–10] In Chagas' disease, parasitic infection with *Trypanosoma cruzi* leads to an immune reaction and ultimately destruction of the esophageal myenteric plexus, causing secondary achalasia that cannot be distinguished clinically from the idiopathic form.[11] Achalasia is considered a chronic inflammatory disease, and to date, there is no cure.[12]

The treatment of achalasia aims to reduce symptoms and improve quality of life. This is primarily accomplished by decreasing LES pressure, which can be achieved through surgical and non-surgical modalities.[1,9] Pharmacologic treatment mainly consists of nitrates, calcium channel blockers, and botulinum toxin injections.[1] Nitrates, such as isosorbide dinitrate, act by inhibiting smooth muscle contraction via a cyclic GMP-mediated pathway and have been proposed as a treatment for achalasia since the 1940s.[1,13] Calcium channel blockers, such as nifedipine, block calcium action necessary for smooth muscle contraction, thereby reducing LES tone.[14,15] These medications are typically administered sublingually before meals; unfortunately, their therapeutic effect and long-term clinical response are limited.[1,9,13–15] Targeted

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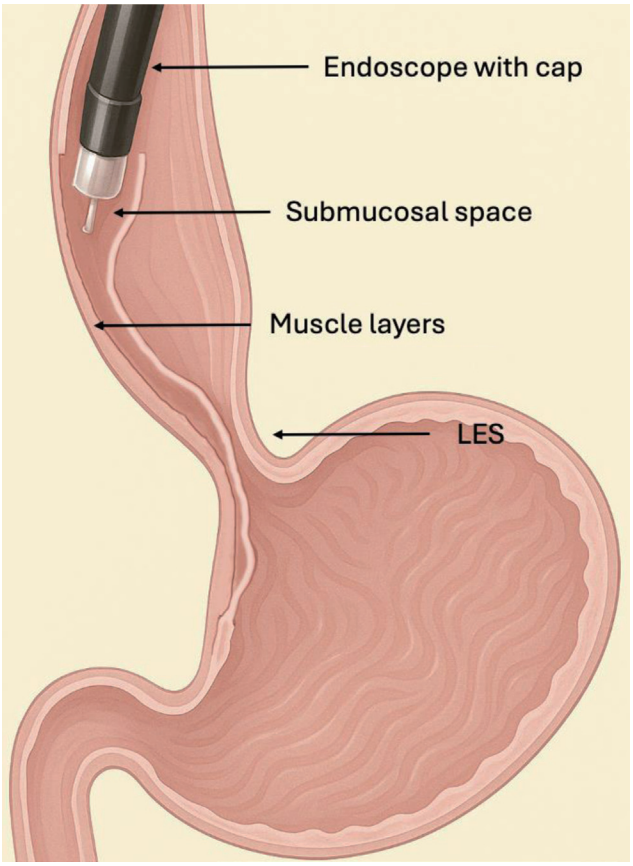

Fig. 1. POEM procedure: Cross-sectional schematic of the distal esophagus and gastric cardia after submucosal tunnel formation and just before myotomy creation. An endoscope with a transparent cap enters through a 2 cm mucosal incision into the submucosal "third" space, approximately 5 cm proximal to the lower esophageal sphincter. Four distinct tissue layers are shown from inside out: mucosa (intact except at the incision), submucosa (tunneled space), muscular layers (inner circular and outer longitudinal muscle fibers), and adventitia/serosa. This view demonstrates the precise surgical plane targeted for the subsequent myotomy. LES, lower esophageal sphincter; POEM, per-oral endoscopic myotomy.

botulinum toxin injection of the LES during upper endoscopy inhibits acetylcholine release, leading to decreased LES pressure. Studies have shown an impressive 80% symptom improvement rate; however, this relief is typically short-lived, with 60% of patients experiencing recurrent symptoms within one year.[16,17]

Endoscopic treatment techniques for achalasia include pneumatic dilation (PD) and per-oral endoscopic myotomy (POEM). PD of the LES is performed during upper endoscopy. Several protocols exist,[18] but typically a 3–4 cm balloon is positioned across the LES and inflated to 10–15 pounds per square inch of pressure for up to one minute.[12,16] Perforation rates are generally low, between 1.6% and 4.5%, but can be higher with larger diameter (4 cm) balloons. Initial symptom remission is achieved in 91% of patients; however, only 50% remain in remission at 10 years, and one-third experience recurrent symptoms by four years. Furthermore, multiple dilations are usually required.[19] POEM is a more definitive endoscopic technique for the treatment of achalasia (Fig. 1). The procedure was first described in a porcine model in 2007 and involves creating a myotomy in the inner circular muscle fibers of the LES by working in the submucosal "third space".[20] The first human results in 17 patients were published in 2010, suggesting the procedure was safe and effective.[21] These results were confirmed in a separate series of 18 patients showing significant relief of dysphagia in all patients, with a 46% rate of new-onset gastroesophageal reflux at one year.[22]

The standard of care in achalasia management since the 1990s has been surgical therapy with laparoscopic Heller myotomy (LHM) (Fig. 2).[9,23] Heller's myotomy was first described in 1914 and involved an extramucosal myotomy of the anterior and posterior LES performed via laparotomy.[9,24] A single anterior myotomy was popularized a decade later.[25] While surgical myotomy was successful in relieving dysphagia, many patients developed clinically significant postoperative reflux. Several modifications adding complete or partial gastric fundoplications at the time of myotomy were developed to combat postoperative reflux with favorable results.[26,27] By the 1990s, minimally invasive surgical techniques, specifically laparoscopy, offered improved outcomes and faster recovery in the treatment of foregut diseases. The first LHM for achalasia was reported in 1991.[28] In a series of 206 patients, LHM with partial fundoplication relieved dysphagia in all patients, with only 15% experiencing incomplete relief. There were no mortalities, and morbidity was low, comparable to that of PD.[23]

The aim of this review is to compare POEM and LHM. While LHM was once considered the standard of care, POEM has since been established as an equally safe and effective procedure for the treatment of achalasia. Many recent studies seek to elucidate the strengths and weaknesses of LHM and POEM by comparing outcomes and identifying patient characteristics that may predict favorable treatment response. A better understanding of these treatments will inform patient selection and ultimately improve care for patients with achalasia.

How should patient characteristics influence the choice between POEM and LHM?

Both POEM and LHM have been proven to be safe and effective treatments for achalasia.[29,30] Therefore, treatment decisions should be patient-specific, with the relative advantages and disadvantages of POEM and LHM considered in the context of the individual. One clear pitfall of LHM compared to POEM is that it requires incisions, which intuitively results in worse cosmetic outcomes, increased pain, and a higher risk of surgical site infections. Several other factors may influence the decision to pursue LHM or POEM in select patient populations (Fig. 3).

Does prior surgical or endoscopic intervention impact subsequent treatment selection?

Prior abdominal surgery can complicate achalasia reintervention.[31] POEM, as an endoscopic approach, avoids the peritoneal cavity and may be advantageous in patients with prior upper abdominal surgery by utilizing native tissue planes.

POEM is also an effective rescue option after LHM, with high success rates reported in meta-analyses.[32] While revisional surgery for recurrent dysphagia after LHM is possible, it carries a significant complication rate.[33] Some centers suggest PD as the initial intervention for treatment failure after either LHM or POEM, with consideration of the alternative procedure if dilation fails. Repeat POEM has also been shown to be effective.[34–36]

Are there treatment considerations for achalasia in the elderly/frail population?

Achalasia is a progressive, incurable disease and is often associat-

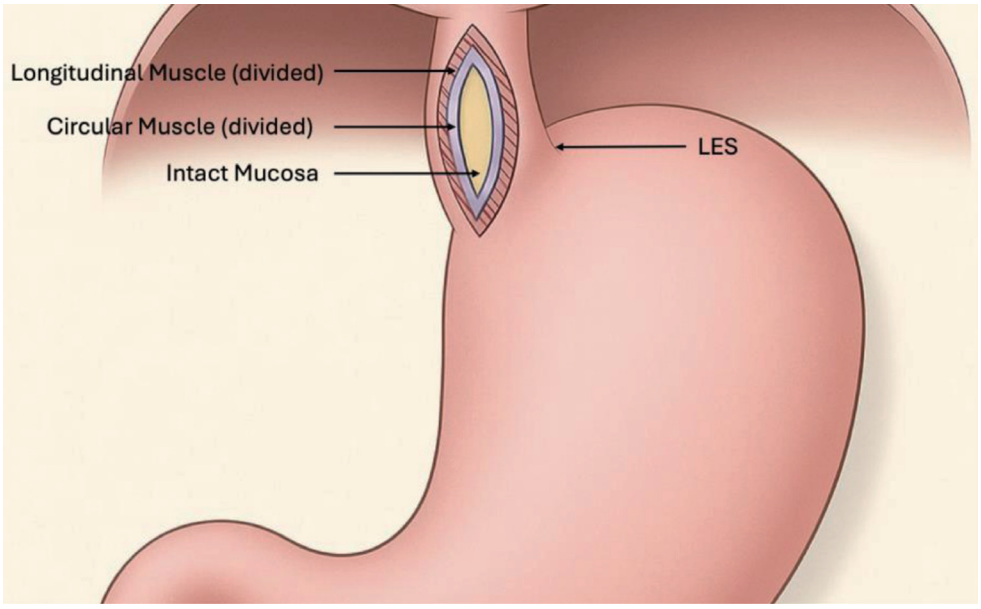

Fig. 2. LHM procedure: Intra-abdominal laparoscopic view of a completed Heller myotomy. The outer longitudinal muscle fibers (striped red) and inner circular muscle fibers (purple) have been divided and gently retracted, exposing the underlying intact mucosa (pale yellow) along the myotomy site. The preserved adventitia/serosa of the esophagus and gastric cardia remains intact. LES, lower esophageal sphincter; LHM, laparoscopic Heller myotomy.

ed with a significant delay in diagnosis due to nonspecific presenting symptoms.[10] Therefore, achalasia disproportionately affects the elderly. Both POEM and LHM require general anesthesia.[37] Elderly and frail patients who are not candidates for anesthesia should instead undergo botulinum toxin injection or PD. Additionally, LHM requires the establishment of pneumoperitoneum, which introduces additional cardiovascular risk for frail patients.[38] For patients who can tolerate anesthesia but not abdominal insufflation, POEM should be considered.

Special populations

Is POEM or LHM preferred in the management of Type III achalasia?

The hallmark of achalasia is failure of relaxation of the LES. However, variations in pressurization and peristalsis of the esophageal body have led to diagnostic subtypes of achalasia.[39] Type I achalasia, the "classic" subtype, is characterized by complete aperistalsis and lack of panesophageal pressurization. Type II achalasia is

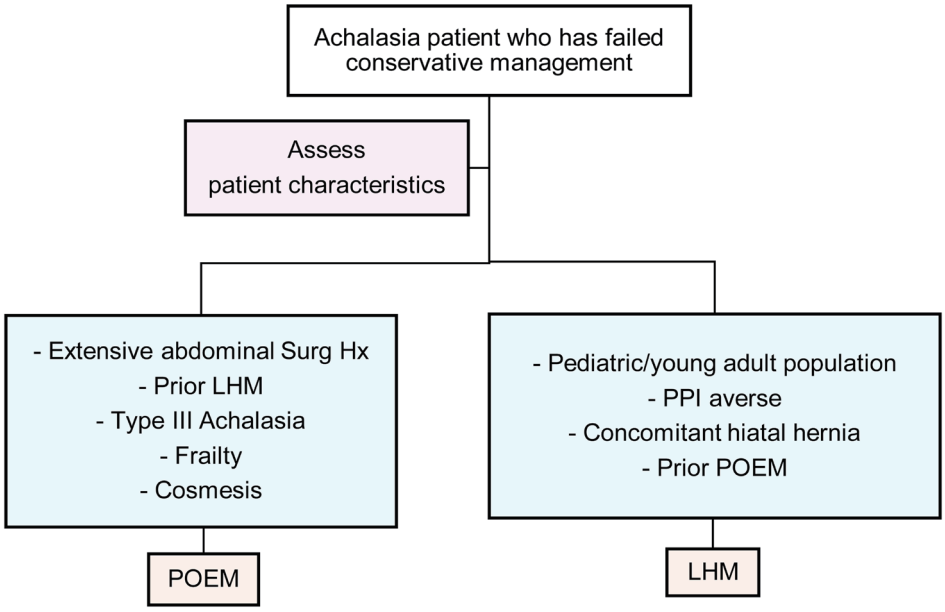

Fig. 3. Treatment algorithm for selecting POEM versus LHM in achalasia patients after failed conservative management. Patient-specific factors guide the choice of myotomy. LHM, laparoscopic Heller myotomy; POEM, peroral endoscopic myotomy; PPI, proton-pump inhibitor.

the most common subtype and features panesophageal pressurization in >20% of swallows. Type III achalasia is rare, occurring in only 5% of patients, and is much more difficult to treat. It is the spastic subtype, characterized by panesophageal pressurization and intense premature esophageal contractions.[39–41] Histologically, Type III achalasia differs in that there is preservation of myenteric ganglionic cells.[39,41] These patients typically report chest pain associated with esophageal spasm. Given the rarity of Type III achalasia, there is a lack of evidence comparing LHM and POEM in this subset of patients, but expert opinion favors the use of POEM due to the ability to perform a longer myotomy, which may alleviate some of the spastic symptoms.[42,43]

How does the presence of hiatal hernia or gastroesophageal reflux disease (GERD) influence the choice of myotomy?

Components of the anti-reflux barrier include a non-effaced LES and an intact hiatus. By definition, an esophageal myotomy disrupts the LES, thereby subjecting patients to post-procedural reflux. It is therefore unsurprising that POEM is associated with higher rates of post-procedural reflux than LHM, given that a partial fundoplication is also created in the latter.[44] Concomitant hiatal hernia in patients with achalasia is rare, occurring in about 4% of patients.[45] Patients with both achalasia and hiatal hernia should undergo Heller myotomy with partial fundoplication and hiatal hernia repair rather than POEM.[46]

Pediatric considerations

Achalasia is a rare disease, and its incidence in the pediatric population is lower than in adults. Data on achalasia treatment in pediatric patients mostly come from a few centers of excellence. POEM also appears to be effective and safe in the pediatric population.[44,47] However, LHM has been established as highly effective and safe and is the historic standard of care for achalasia in children.[48,49] POEM is associated with increased rates of proton pump inhibitor (PPI) use and pathologic reflux, which should be avoided in young patients.[43] However, undesirable anticipated effects of LHM include incisional hernia risk, wound infection rates, and post-fundoplication side effects such as bloating and flatulence. Expert opinion supports the use of both interventions in the pediatric population.[50]

Outcomes

The main outcomes studied in endoscopic and surgical interventions for achalasia are dysphagia relief, post-procedural reflux, perioperative complications, and durability. Dysphagia relief is mostly subjective and therefore assessed through patient-reported outcomes like the Eckardt score, and less commonly via dynamic imaging studies such as fluoroscopic timed barium swallow. Reflux can be intra-esophageal or gastroesophageal, so it is best evaluated with objective pH studies and/or esophagitis seen on EGD. Perioperative complications primarily include gastroesophageal perforation and leaks. Durability is best measured by reintervention, defined as the need for additional endoscopic or surgical treatment. POEM and LHM are both highly effective and safe, but there are some minor differences in outcomes that may impact decision-making (Table 1).[29,51–53,54,55–57]

Are POEM and LHM equivalent in producing dysphagia relief?

Some argue that the addition of a partial fundoplication during LHM may increase postoperative dysphagia rates, though this does not appear significant. In a meta-analysis of 2,342 patients undergoing POEM with two-year follow-up, the pooled clinical success rate was 87%.[58] Werner *et al*.[29] performed a multicenter, prospective, randomized controlled trial comparing LHM and POEM. A total of 221 patients were included, and the primary endpoint was clinical success defined by an Eckardt score of 3 or less. The study showed no significant difference in clinical success (83% for POEM vs. 81.7% for LHM) at two years.[29] In a systematic review comparing LHM and POEM, pooled rates of recurrent dysphagia were 14.5% and 12.2%, respectively.[51] A separate review found no difference in dysphagia after POEM or LHM measured by Eckardt score postoperatively, at one year, or at three years.[52] The data suggest that POEM and LHM are equivalent in providing dysphagia relief.

What are the rates of post-procedural reflux following POEM and LHM?

In a meta-analysis of 2,342 patients undergoing POEM with two-year follow-up, the rate of symptomatic reflux was 22%.[58] A randomized controlled trial comparing LHM and POEM showed higher rates of reflux esophagitis identified at surveillance endoscopy in the POEM group at three months (57% vs. 20%) and two years (44% vs. 29%), as well as higher rates of patient-reported reflux. However, higher PPI use was the only statistically significant finding.[29] Pooled results of several observational studies show no difference in patient-reported rates of reflux after POEM or LHM but significantly higher rates of objective reflux after POEM identified by pH studies, esophagram, or EGD.[52] Accordingly, in agreement with the most recent SAGES guidelines, one should expect PPI use after POEM as part of the therapeutic plan, rather than as a failure. However, the decision to initiate routine PPI therapy post-POEM remains a subject of debate. Some advocate for universal PPI prophylaxis to mitigate GERD risk, while others favor a patient-tailored approach based on individual risk factors and symptom severity. Factors to consider include the presence of pre-existing GERD, hiatal hernia, and the degree of LES relaxation achieved during POEM. Close monitoring for GERD symptoms and objective testing (e.g., pH monitoring, endoscopy) are essential to guide PPI use in these patients. When comparing POEM plus PPI use to LHM with partial fundoplication, postoperative GERD outcomes are quite comparable.[53] However, in PPI-averse patients who do not wish to remain on lifelong PPIs due to fear of side effects, LHM should be favored. It should be emphasized during patient consultation that surgical partial fundoplication lowers the rate of post-procedural GERD but introduces the possibility of gas-bloat syndrome.

Single-session POEM with natural orifice fundoplication was recently described as a technique to reduce post-POEM GERD.[59] A case series of six patients showed it was safe, feasible, and had acceptable early outcomes. From a surgical standpoint, there is some concern about the long-term impact of metal clips remaining in the abdominal cavity after the transmural approach. Another emerging treatment pathway is to perform transoral incisionless fundoplication either concurrently with or subsequent to POEM to create a partial fundoplication without surgical intervention.[60,61]

How do complication rates compare between LHM and POEM?

In a meta-analysis of 2,342 patients undergoing POEM with two-year follow-up, the pooled rate of adverse events was 1.5%.[58] Werner *et al*.[29] found no significant difference in the rate of adverse events between patients undergoing POEM (2.7%) and LHM (7.3%); however, the study was not powered to detect a difference given the relatively low morbidity associated with the procedures. In a systematic review, patients undergoing POEM had marginally lower rates of perforation and leak; however, rates in both groups were very low, and surgeon experience with LHM was not reported in the majority of studies.[51] Serious complications are rare

Table 1. Outcomes comparison of laparoscopic heller myotomy and per-oral endoscopic myotomy

Outcome	Key study (year)	POEM	LHM	Reference
Dysphagia relief	Werner *et al*. (2019) RCT	n ≈ 110; 2 yr f/u; 83 % clinical success	n ≈ 111; 2 yr f/u; 81.7 % clinical success	29
	Meta-analysis of 2,342 POEM patients (2021)	n = 2,342; 2 yr f/u; pooled success 87 %	–	51
	Systematic review (pooled recurrent dysphagia rates)	Recurrent 12.2 %	Recurrent 14.5 %	52
Post-procedural reflux	Werner *et al*. (2019) RCT	57 % esophagitis @3 mo; 44 % @2 yr; ↑ PPI use	20 % esophagitis @3 mo; 29 % @2 yr	29
	Meta-analysis of 2,342 POEM patients (symptomatic reflux)	n = 2,342; 2 yr f/u; 22 % symptomatic reflux	–	51
	Observational studies (objective pH/EGD)	Higher objective reflux by pH/EGD	Lower objective reflux	53
Complications	Meta-analysis of 2,342 POEM patients	n = 2,342; 2 yr f/u; AE rate 1.5 %	–	51
	Werner *et al*. (2019) RCT	AE 2.7 %	AE 7.3 %	29
	Systematic review (perforation/leak rates)	Marginally lower perforation/leak	Slightly higher perforation/leak	52
Reintervention	Systematic review	POEM: 1–7 %	LHM: 9–15 %	52
	Werner *et al*. (2019) RCT	Trend toward fewer re-interventions	Trend toward more re-interventions	29
	Observational cohort (Smith *et al*. 2020)	Re-intervention 27.3 %; interval 2.7 yr	Re-intervention 34.9 %; interval 1.3 yr	54
Learning curve	Meta-analysis of POEM learning (2022)	Proficiency at 25 cases	–	55
	Single-center LHM review (2018)	–	Proficiency in 16 cases	56,57

↑, increase. AE, adverse events; EGD, esophagogastroduodenoscopy; LHM, laparoscopic heller myotomy; POEM, per-oral endoscopic myotomy; PPI, proton-pump inhibitor; RCT, randomized controlled trial.

after LHM or POEM, and both procedures have been established as safe. There may be a slightly higher risk of complications such as perforation or leak after LHM, which likely depends on surgeon experience. Surgeons are now utilizing robotic surgery with tremor reduction and three-dimensional visualization, leading to lower perforation rates, which will be discussed later.[62]

How do reintervention rates compare between LHM and POEM?

Systematic reviews of patients undergoing LHM and POEM identify a higher rate of reintervention in LHM (9–15%) versus POEM (1–7%).[51] In a robust randomized controlled trial, Werner *et al*.[29] found a trend towards lower reintervention rates after POEM compared to LHM, but this did not reach statistical significance. An observational study found a significantly higher rate of reintervention after LHM (34.9%) than POEM (27.3%), as well as a shorter interval to clinical failure after LHM (1.3 vs. 2.7 years).[63] Further study would improve the quality and significance of this data.

Technical considerations

How can endoluminal functional lumen imaging probe (EndoFLIP) be used as an adjunct during myotomy?

Modern technologies, such as EndoFLIP, can assist in guiding the length of myotomy during LHM and decrease the rates of incomplete myotomy and reintervention.[54,64] EndoFLIP is a diagnostic tool that measures distensibility and pressure within the esophagus. During LHM, it can be used intraoperatively to assess esophagogastric junction distensibility after myotomy. This allows the surgeon to tailor the myotomy length to achieve adequate LES relaxation while minimizing the risk of postoperative reflux. Specific metrics, such as the distensibility index, are being studied to guide the extent of myotomy.

What are the learning curves associated with LHM and POEM?

Both LHM and POEM have learning curves influenced by the rarity of achalasia and the proceduralist's experience. Studies suggest proficiency in POEM is gained after approximately 25 procedures for those with advanced endoscopy experience.[65] Proficiency in LHM is gained after 16–20 cases.[55]

Heller myotomy–laparoscopic vs. robotic

Robot-assisted Heller myotomy (RHM) is an increasingly popular surgical technique. RHM was first performed in the early 2000s and has advantages over LHM, including better visualization of the distal esophagus and its layers and wristed instruments that facilitate a safer and longer esophageal myotomy.[56,57] Studies suggest equivalent dysphagia relief and reflux rates between RHM and LHM, with RHM offering reduced blood loss, shorter hospital stays, and lower perforation rates.[66–68] Recent small series have mirrored these findings in children.[69] While most studies investi-

gating laparoscopic versus robotic surgery demonstrate non-inferiority, RHM has emerged as one of the few procedures where there is clear benefit to the robotic platform. There are very few studies comparing outcomes for RHM and POEM.[43,70]

Heller myotomy – type of fundoplication

Richards *et al*.[71] addressed whether to add a fundoplication to LHM in a randomized trial, finding pathologic reflux in 9.1% of patients receiving LHM + Dor versus 47.6% with LHM alone. Historically, a complete (Nissen) fundoplication followed LHM, but caused high rates of long-term dysphagia.[71,72] Partial fundoplication is now standard; a systematic review of nine studies dating back to 2011 reported its universal use.[51] Subsequent randomized trials comparing anterior (Dor) and posterior (Toupet) techniques found no significant differences in postoperative dysphagia or reflux.[73] Dor fundoplication offers mucosal protection and avoids extensive posterior dissection.[74] Toupet may enhance esophageal emptying and therefore quality of life by holding the myotomy open.[75] Ultimately, the choice reflects surgeon preference and clinical context: Dor is favored without a hiatal hernia to minimize retro-esophageal dissection, whereas Toupet is preferred when a posterior dissection is required for hernia repair.

Future directions

Unanswered questions

While both POEM and LHM have demonstrated efficacy in the treatment of achalasia, several key knowledge gaps remain. Long-term data (more than five years) on POEM's durability and the long-term incidence of GERD, particularly in pediatric populations, are currently limited. Further long-term studies are needed to fully assess these outcomes. Additionally, although robotic LHM offers potential advantages over traditional laparoscopic techniques, comparative studies directly comparing RHM against POEM are sparse. Future research should focus on directly comparing these modalities to better define their respective roles in achalasia management. Emerging technologies such as POEM with natural orifice fundoplication and the use of EndoFLIP to guide myotomy length also warrant further investigation.

Cost-effectiveness and resource availability

When choosing between myotomy approaches, it is crucial to weigh both direct procedural costs (capital equipment, disposables, operating-room time) and indirect expenses (training, maintenance, patient recovery).

- RHM offers tremor reduction, three-dimensional visualization, and wristed instruments that may enhance precision. However, studies report a 20–30% higher per-case cost versus conventional laparoscopy once capital purchase, service contracts, and specialized instruments are amortized, without clear long-term outcome advantages in achalasia management.[66–68] These added expenses often exceed the budgets of many middle- and low-income centers.
- LHM with partial fundoplication remains the most cost-effective surgical option. It leverages widely available instrumentation, established training curricula, and lower maintenance overhead, making it accessible in diverse resource settings.
- POEM can shorten hospital stay and accelerate return to diet—factors that may partially offset its higher procedural expendables (advanced endoscopy towers, premium electrosurgical knives, specialized caps) and the need for expert endoscopists and dedicated nursing staff.[59,60] Adoption in centers without established submucosal endoscopy programs often requires phased training through regional centers of excellence or hybrid surgeon–endoscopist mentorship models.

To optimize global access and sustainability:

1. Centralize high-cost platforms (robotic platforms or advanced endoscopy towers) across multiple specialties.
2. Negotiate bulk purchasing and implement safe reprocessing protocols for disposables.
3. Expand tele-mentoring and proctoring to rapidly disseminate skills and ensure procedural quality.

By aligning technique choice with local infrastructure and expertise, institutions can deliver high-quality, cost-effective care for achalasia patients worldwide.

Conclusions

In many ways, POEM and LHM are equivalent. Both procedures provide successful dysphagia relief, and complication rates are largely similar. There are some relative advantages and disadvantages to each; therefore, patient selection and technical considerations should guide individual treatment plans. POEM is best suited for patients with type III achalasia, prior upper abdominal surgical history, failed LHM, or those who cannot tolerate pneumoperitoneum. Newer treatment pathways that include endoscopic fundoplication will likely lower the rate of post-procedural GERD in the future. LHM is best suited for patients who are PPI-averse, have a history of GERD, concomitant hiatal hernia, or prior failed POEM. Modern technologies, such as robotic surgery and EndoFLIP, enhance surgical outcomes. As always, surgeon or procedurist volume dictates outcomes, and achalasia should be treated at centers with adequate experience.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (BCP), acquisition of data (BCP, JWK), analysis and interpretation of data (BCP, JWK), drafting of the manuscript (JWK), critical revision of the manuscript for important intellectual content (BCP). Both authors have made significant contributions to this study and have approved the final manuscript.

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