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EDITORIAL

Realm of hepatitis E: Challenges and opportunities

Jia-Rui Li, Ze Xiang, Shu-Hui Li, Chen-Xi Li, Hong Yan, Jian Wu

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Abstract

Hepatitis E virus (HEV), responsible for widespread viral hepatitis, infects approximately 2.3 billion individuals globally, with a significant mortality burden in Asia. The virus, primarily transmitted through contaminated water and undercooked meat, is often underdiagnosed, particularly in immunocompromised patients. Current HEV treatments, while effective, are limited by adverse effects, necessitating research into safer alternatives. Moreover, HEV's extrahepatic manifestations, impacting the nervous and renal systems, remain poorly understood. This study underscores the imperative for enhanced HEV research, improved diagnostic methods, and more effective treatments, coupled with increased public health awareness and preventive strategies.

Key Words: Hepatitis E; Treatment; Extrahepatic manifestations; Challenges; Opportunities

Core Tip: Diagnosing hepatitis E virus (HEV), especially in immunocompromised individuals, is challenging due to the limitations of standard serological markers, necessitating the use of more sensitive nucleic acid amplification techniques. Existing treatments, mainly ribavirin and interferon-α, play a certain role in controlling the progression of the disease but have notable side effects. There is a lack of safe treatment options for pregnant women and immunocompromised patients. Future research should continue to concentrate on understanding the global prevalence, enhancing surveillance and prevention measures, and exploring innovative treatment approaches for HEV. This focused effort is critical to address the World Health Organization's urgent goal of eradicating viral hepatitis by 2030.

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INTRODUCTION

The identification of hepatitis E virus (HEV), a positive-strand RNA virus, dates back 40 years ago[1], and in 1990, Reyes *et al*[2] successfully sequenced its genome. HEV is a major etiological factor for both chronic and acute viral hepatitis[3]. Among the eight genotypes identified so far, HEV1, HEV2, HEV3, and HEV4 are the predominant genotypes responsible for human infections. Seroprevalence studies indicate that approximately 2.3 billion individuals worldwide are infected with HEV[4], resulting in an annual death toll of 160000 due to hepatitis E solely in Asia[5]. However, the relative threat posed by hepatitis E compared to hepatitis B and C remains significantly underestimated. Limited awareness about this disease has contributed to substantial misdiagnosis or missed diagnosis cases being reported sporadically over time[6]. Given the World Health Organization's vision for eliminating hepatitis by 2030, urgent attention must be given to promoting research on hepatitis E. Currently, there exist numerous challenges pertaining to its diagnosis and treatment including screening protocols management strategies prediction models for severe disease prevention measures addressing extrahepatic manifestations vaccine evaluation efforts as well as establishing suitable animal models.

The incubation period of HEV infection typically ranges from 2 to 6 wk, and the detection of anti-HEV immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies in serum is commonly used as a diagnostic marker for HEV infection[7]. However, immunosuppressed patients with long-term viral infection often have undetectable levels of anti-HEV antibodies. In such cases, the reliable diagnostic method relies on detecting viral RNA in blood and/or stool samples using nucleic acid amplification techniques[8]. Wolski *et al*[9] recently conducted a meta-analysis on global blood donors to test for anti-HEV IgG/IgM or HEV presence. The analysis revealed significant regional variations in both the risk of exposure to HEV and blood-borne transmission rates. In addition, the detection of DNA biomarkers has been reported for clinical diagnosis of hepatitis virus, such as fluorescence[10], mass spectrometry[11], electrochemistry[12], flow tomography[13], *etc*., but there are drawbacks including low sensitivity, poor accuracy, and unsatisfactory stability. Using dark-field microscopic imaging, a novel approach for the simultaneous detection of hepatitis B virus and hepatitis C virus based on automated particle enumeration was established[14], offering a fresh perspective on HEV detection. Ribavirin and interferon-α are commonly used in the treatment of chronic HEV infection, but they are often accompanied by significant side effects. Targeting various stages of the viral life cycle, including attachment and internalization in the early stages, translation and RNA replication in the middle stages, and viral particle formation and release in the late stages, is a necessary prerequisite for the development of novel anti-HEV medications[15]. Netzler *et al*[16] employed a subgenomic replicon strategy to screen 16 compounds from the NA or NNI class of antivirals, leading to the identification of two novel HEV antiviral candidates, namely NITD008 and GPC-N114. These candidates exhibited potent antiviral activity against HEV *in vitro* and demonstrated synergistic effects when used in combination. The entry process of viral particles is also an attractive target for pharmaceutical intervention, and the epidermal growth factor receptor (EGFR) has recently been identified as a novel host factor for HEV, playing a crucial role during HEV infection. Application of EGFR modulators can effectively limit HEV infection[17]. However, there is still a lack of feasible drugs for the treatment of HEV in pregnant women and immunocompromised individuals. Zhang *et al*[18] conducted an unbiased compound library screening on human hepatocytes harboring HEV replications and successfully identified 17 inhibitors targeting HEV-HSP90, providing a promising perspective for the development of new clinical antiviral drugs.

HEV not only poses harm to the liver, but also has the potential to impact various other systems including the nervous system, kidney, and blood system[19-21]. While some cases suggest an incidental association between HEV and these extrahepatic manifestations, the underlying pathophysiological mechanism remains unestablished. Data reveals significant activation of inflammatory cytokines (such as tumor necrosis factor alpha and interleukin-1β) in HEV-infected brain tissue, which promotes mitochondria-mediated apoptosis and provides novel insights into the mechanism of central nervous system injury caused by extrahepatic involvement[22]. El-Mokhtar *et al*[23] demonstrated that renal manifestations associated with HEV infection may primarily be linked to an exacerbated inflammatory response induced by Interferon-gamma produced by peripheral blood mononuclear cells (PBMC), through analysis of inflammatory response markers and renal injury markers in PT cells co-cultured with or without PBMC. Furthermore, direct or indirect associations between HEV replication in human monocytes, macrophages, and human bone marrow derived macrophages may contribute to hematological manifestations associated with HEV infection[24]. Clinically, patients with

systemic manifestations of HEV infection should be given priority to antiviral therapy to help eliminate or improve extrahepatic manifestations of HEV infection.

CONCLUSION

As previously mentioned, there are still numerous challenges and significant obstacles to overcome in the process of translating knowledge into disease prevention and improving clinical outcomes for HEV patients. In order to bridge the existing gap, it is imperative to foster collaboration between researchers specializing in basic science, translational research, and clinical practice while also leveraging the collective efforts of health authorities. This will further advance HEV research and enhance clinical practices. Simultaneously, preventive measures should be directed towards addressing the threat posed by HEV infections. Enhanced emphasis on fundamental hygiene practices and education can effectively contribute to achieving better prevention outcomes.

FOOTNOTES

Author contributions: Wu J designed study and revised the manuscript; Li JR, Xiang Z and Li SH wrote the paper; Li CX and Yan H searched the literature; All authors reviewed and approved the final version; Li JR and Xiang Z contributed equally to this work; Both Yan H conceptualized, designed, and supervised the whole process of the project. Wu J got acquisition of financial support for the project leading to this publication and coordinated the planning and execution of research activities. Wu J and Yan H contributed equally to this work in the manuscript preparation and submission as the co-corresponding authors.

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OPINION REVIEW

Liver surface depressions in the presence of diaphragmatic muscular bands on trans-illumination

Shamir O Cawich, Michael T Gardner, Ramanand Shetty, Jean Pierre Louboutin, Zenica Dabichan, Shaneeta Johnson

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Abstract

Traditional descriptions of liver anatomy refer to a smooth, convex surface contacting the diaphragm. Surface depressions are recognized anatomic variants. There are many theories to explain the cause of the depressions. We discuss the theory that these are caused by hypertrophic muscular bands in the diaphragm.

Key Words: Liver; Anatomy; Depressions; Fissure; Groove; Sulcus; Variant

Core Tip: Surface depressions of the liver are a recognized anatomic variant Transillumination is a method that is useful to evaluate the association with hypertrophic muscular bands in the diaphragm. Using this technique, we determined that hypertrophic bands are associated with surface depressions in 67% of cases. Diaphragmatic muscular bands play a prominent role in the formation of surface depressions.

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INTRODUCTION

Traditional descriptions of liver anatomy refer to a smooth, rounded, convex surface contacting the diaphragm, as demonstrated in Figure 1[1]. The presence of surface depressions is a recognized anatomic variant[2-4].

While the cause for these surface depressions has not been definitively elucidated, several theories have been put forward, including: compression by ribs[5,6], pulmonary emphysema[7], congenital parenchymal weakness zones[8-10], hepatic trauma[11], tumour necrosis leading to desmoplasia and subsequent capsular retraction[10,11,12], regression of liver metastases after chemotherapy[11,13], adjacent inflammatory foci (gallbladder empyema, liver abscesses or cirrhosis) leading to parenchymal scarring and capsular retraction[14], localized iatrogenic injury after trans-arterial chemo-embolization (TACE)[15], fibrous bands and diaphragmatic scars[16]. Although none have been definitively proven as the cause for surface depressions, diaphragmatic muscular bands have gained little attention as an aetiologic factor. These are well-defined fascicles that connect the central diaphragmatic tendon to the rib cage. We discuss our observations during cadaveric dissections regarding the association of diaphragmatic muscular bands and surface depressions on the liver.

WHAT IS THE PREVALENCE OF SURFACE DEPRESSIONS?

The prevalence of surface depressions is difficult to discern, because there is no standardized nomenclature for this variant. A review of the literature revealed that many names have been ascribed to this variant, such as: hepatic surface grooves[5,7,11,16], diaphragmatic grooves[3], accessory sulci[6,8,16], hepatic fissures[3,8], portal fissures[8], accessory fissures[4], and capsular retraction[10,12]. Although there is heterogeneity in nomenclature, a detailed review of the descriptions appearing in the anatomic literature suggest that surface depressions are encountered in 5%[17] to 51%[18] of unselected persons across the globe.

WHAT IS THE RATIONALE FOR TRANSILLUMINATION?

In a prior publication on cadavers with liver surface depressions, we reported that the diaphragm appeared normal in all cases, without evidence of scars, fibrotic slips, ligamentous thickening or thickened muscular bands[16]. In fact, we wrote in our conclusion that our findings "did not support diaphragmatic pathology as a plausible explanation" for surface depressions[16]. Subsequently, however, we had the opportunity to inspect the diaphragm in living persons undergoing surgery, and observed thickened muscular bands *in vivo* while performing open surgery[19] as well as laparoscopic operations[20]. These observations directly challenged our prior statements and we were forced to re-visit this issue.

We postulated that the reason we had not observed diaphragmatic muscular bands in cadaveric studies was because they were not ligamentous thickening, scars or fibrotic slips. Instead, these areas appeared to be composed of hypertrophic muscular bands. Therefore, when muscle tone was absent at the post-mortem examinations, these bands would not be easily visible. However, if the bands were hypertrophic muscle, they should still be discernable by transillumination (Figure 2).

After approval was granted from the local institutional review board (CREC_SA.1034/06/2021), we observed cadaveric dissections for academic medical curricula at two university campuses in the Caribbean. From a total of 120 cadaveric dissections observed, 18 (15%) cadavers had surface depressions on the liver. During academic teachings, the liver and diaphragm were excised en-bloc and studied on the dissecting bench. We took this opportunity to apply a light source against the thoracic surface of the diaphragm and observations were made from the abdominal side (Figure 3). The presence of hypertrophic muscular bands, when present, and their correlation with surface depressions were recorded.

WHAT IS THE RELATIONSHIP OF MUSCULAR BANDS AND SURFACE DEPRESSIONS?

There were hypertrophic muscular bands visible on trans-illumination in 12 (67%) of the cadavers with surface depressions (Figure 4). Apart from hypertrophic muscular bands, there were no other causes of compression in these 12 cases.

These bands were not readily visible upon gross inspection. Admittedly, we did not appreciate differences in the diaphragm in our previous publications[16]. Upon close inspection after transillumination, however, we noted that the diaphragmatic thickness was greater adjacent to surface depressions (Figure 5).

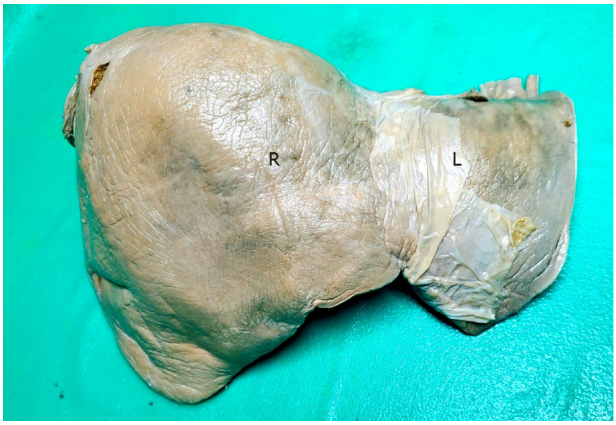

Figure 1 Anatomic specimen of a human liver with conventional surface anatomy. Both right (R) and left (L) hemi-livers have a smooth, convex surface in contact with the diaphragm.

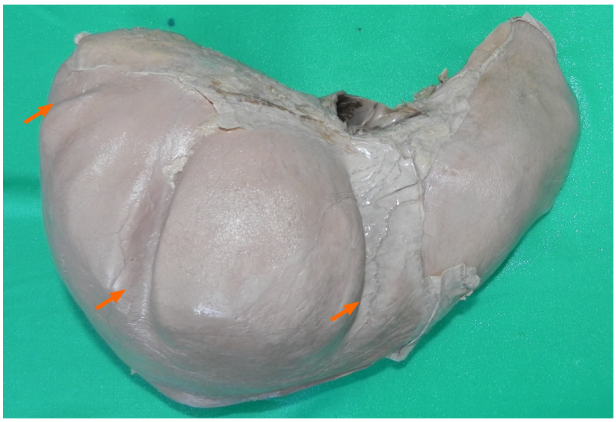

Figure 2 Anatomic specimen of a human liver with variant surface anatomy. Multiple surface depressions (arrows) are demonstrated on the diaphragmatic surface of liver.

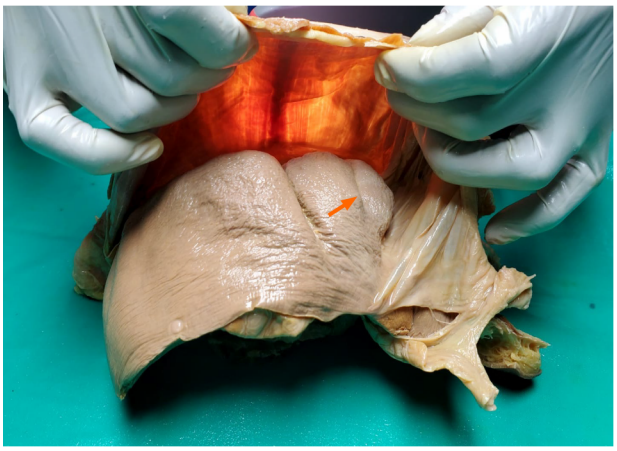

Figure 3 Our technique for observation by trans-illumination. A light source is applied to the thoracic surface of the diaphragm, allowing hypertrophic muscular bands to be identified (arrow).

In addition, we noticed the presence of muscular bands occurring adjacent to surface depressions in all of seven living patients undergoing abdominal imaging for varied indications (Figure 6). These observations support our theory that hypertrophic muscular bands are intimately related to the corresponding surface depressions.

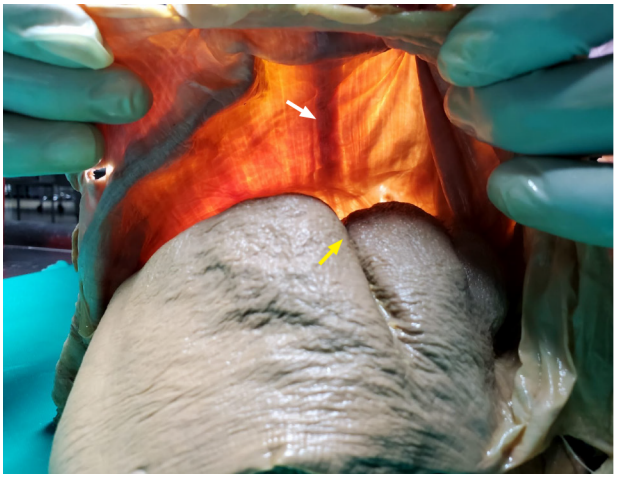

Figure 4 The trans-illumination technique allows hypertrophic muscular bands to be demonstrated (white arrows) in the diaphragm corresponding to surface depressions on the liver (yellow arrows).

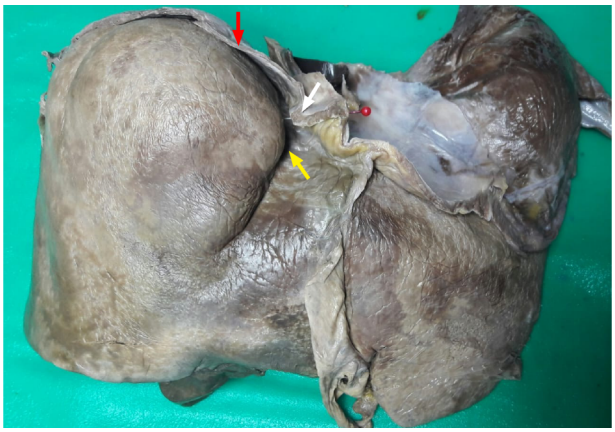

Figure 5 The thickness of the diaphragm is greater (white arrow) adjacent to the surface grooves (yellow arrow), and becomes thin at the smooth liver surface (red arrow).

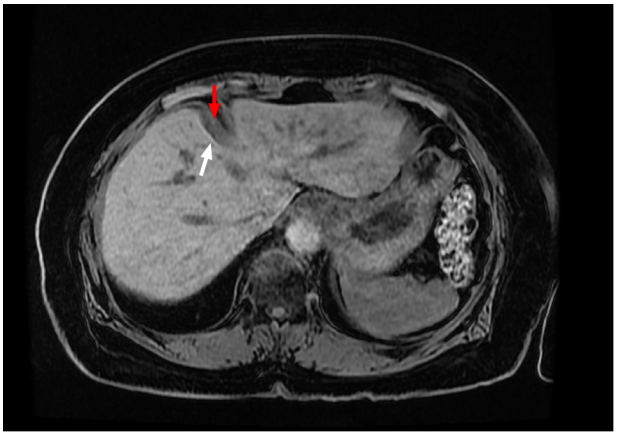

Figure 6 Magnetic resonance imaging of a patient's abdomen. There is a deep surface depression in the right hemi-liver (white arrow) and thickened diaphragmatic band (red arrow) can be seen intimately related to the depression.

IS THERE A MOLECULAR BASIS OF SURFACE DEPRESSIONS?

At the turn of the century, animal studies demonstrated that beta-catenin was an important factor responsible for stimulating hepatocyte growth, and its regulation had the potential to alter the size and shape of the liver[21,22]. Suksaweang *et al*[22] also theorized that there were specific areas in the liver, known as growth zones, that are susceptible to changes in beta-catenin activity.

Beta-catenin has since been shown to be up-regulated in humans with venous congestion secondary to cardiac failure [23]. It is also well known that there are areas between the inter-segmental and inter-sectional planes of the liver where the parenchyma is relatively less-well vascularized[11,24]. These "watershed areas" would be less responsive to the effect of beta-catenin up-regulation, and hence not proliferate as readily as surrounding parenchyma[11]. We suggest that hypertrophic muscular bands in proximity to these "watershed areas" could be responsible for the surface depressions. This could also explain the frequent location of surface depressions closely related to the inter-sectional planes[8,25].

DOES THIS HAVE CLINICAL SIGNIFICANCE?

The presence of surface depressions does have clinical significance, especially in light of the popularity of medical imaging in modern medicine. As more patients are subjected to medical imaging and these variants will be detected increasingly. When detected, these variants can be misinterpreted for metastatic liver secondaries in patients with known malignancies[1,2,4]. In those who have sustained blunt abdominal trauma, the depressions can be mistaken for liver lacerations[2,16]. There are also prior reports of these variants being mistaken for Chilaiditi's syndrome on imaging[13]. In all of these cases, mis-interpretations can negatively affect clinical care decisions[16].

On the other hand, we have found that surface depressions can sometimes be advantageous. For example, in patients who require liver resections, we have modified our practice to routinely attempt to transect the parenchyma where surface depressions are present. This significantly reduces the thickness of the parenchymal transection line, reduces bleeding and so has the potential to improve patient outcomes[16].

CONCLUSION

Surface depressions of the liver are a recognized anatomic variant, with clinical implications when present. We suggest that it is time to adopt standardized nomenclature. We also theorize that diaphragmatic muscular bands play a prominent role in the formation of surface depressions.

FOOTNOTES

Author contributions: Cawich SO, Gardner MT, Shetty R, Louboutin JP and Johnson S designed the research; Cawich SO, Gardner MT and Shetty R performed the research; Cawich SO, Gardner MT and Shetty R wrote the paper; Shetty R, Louboutin JP and Johnson S contributed analytic tools; Shetty R, Louboutin JP and Dabichan Z analyzed the data.

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REVIEW

Management of male obesity-related secondary hypogonadism: A clinical update

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Abstract

The global obesity pandemic has resulted in a rise in the prevalence of male obesity-related secondary hypogonadism (MOSH) with emerging evidence on the role of testosterone therapy. We aim to provide an updated and practical approach towards its management. We did a comprehensive literature search across MEDLINE (*via* PubMed), Scopus, and Google Scholar databases using the keywords "MOSH" OR "Obesity-related hypogonadism" OR "Testosterone replacement therapy" OR "Selective estrogen receptor modulator" OR "SERM" OR "Guidelines on male hypogonadism" as well as a manual search of references within the articles. A narrative review based on available evidence, recommendations and their practical implications was done. Although weight loss is the ideal therapeutic strategy for patients with MOSH, achievement of significant weight reduction is usually difficult with lifestyle changes alone in real-world practice. Therefore, androgen administration is often necessary in the management of hypogonadism in patients with MOSH which also improves many other co-

morbidities related to obesity. However, there is conflicting evidence for the appropriate use of testosterone replacement therapy (TRT), and it can also be associated with complications. This evidence-based review updates the available evidence including the very recently published results of the TRAVERSE trial and provides comprehensive clinical practice pearls for the management of patients with MOSH. Before starting testosterone replacement in functional hypogonadism of obesity, it would be desirable to initiate lifestyle modification to ensure weight reduction. TRT should be coupled with the management of other comorbidities related to obesity in MOSH patients. Balancing the risks and benefits of TRT should be considered in every patient before and during long-term management.

Key Words: Male obesity-related secondary hypogonadism; Androgen therapy; Testosterone replacement therapy; Obesity; Cardiovascular benefits

Core Tip: The obesity pandemic has increased the prevalence of obesity-related health morbidities including male obesity-related secondary hypogonadism (MOSH). Although weight loss is the ideal therapeutic option for MOSH, testosterone replacement therapy (TRT) is often necessary because of the difficulty in achieving significant weight loss through lifestyle interventions or pharmacotherapy which might also improve obesity-related comorbidities. TRT should be coupled with the management of other comorbidities related to obesity in MOSH patients to optimize management which is updated in this evidence-based review.

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INTRODUCTION

Obesity is a chronic progressive or relapsing disease that left untreated results in increased morbidity and mortality[1]. According to the World Obesity Atlas, 38% of the global population (2.6 billion individuals) were either overweight or obese [body mass index (BMI) ≥ 25 kg/m²] in the year 2020, and this is predicted to reach 51% (4 billion) by 2035[2]. Similarly, 14% of the global population had obesity (BMI ≥ 30 kg/m²) in 2020, and this is predicted to touch 24% by 2035. The prevalence of obesity is higher in women than men[3]. In 2020, 18% of all women and 14% of all men globally had obesity and this is expected to reach 27% and 23%, respectively in the year 2035[3]. Obesity is associated with various metabolic and nonmetabolic complications affecting every organ system in the body. Some obese individuals (especially women) may present with Metabolically Healthy Obesity, characterized by excess subcutaneous fat, relatively lower visceral/hepatic fat, normal insulin sensitivity and inflammatory markers, maintained adipose tissue function and preserved cardiorespiratory fitness[4]. In contrast, some other obese subjects (especially men) present with Metabolically Unhealthy Obesity characterized by visceral adiposity, adipose tissue dysfunction, chronic low-grade inflammation, and higher cardiovascular risk. Obese women often tend to have higher rates of depression and they present early with weight-related issues[5].

Another interesting issue in obese men is obesity-related hypogonadotropic hypogonadism, also known by the term male obesity-related secondary hypogonadism (MOSH)[6]. Recent evidence shows that a similar condition known as female obesity-related secondary hypogonadism, which is distinct from polycystic ovary syndrome, may also be present in women[7]. It is important to update the pathobiology and management algorithms for MOSH to inform evidence-based clinical practice decisions, especially when we consider androgen replacement therapy. This review is an attempt with the back-up of the most up-to-date review of current global scientific literature.

PATHOPHYSIOLOGY OF MOSH

There is a complex interplay of various feedback mechanisms with neural and hormonal signaling molecules contributing to MOSH[6]. Obesity with expanded visceral adipose tissue leads to secondary hypogonadism with an 8.7-fold higher risk in patients with a BMI > 30 kg/m². Testosterone (T), even in suboptimal levels, facilitates the differentiation of pluripotent stem cells into adipocytes to increase the aromatization of T into estradiol and to cause a negative feedback mechanism at the hypothalamus and pituitary levels that in turn suppresses the gonadal stimulation and T release. The arcuate nucleus and periventricular nucleus of the hypothalamus release neuropeptide Kisspeptin, which in turn stimulates the release of gonadotrophin-releasing hormone (GnRH)[6].

This link was named the Cohen hypothesis based on the finding that T and obesity have a bidirectional relationship in which obesity acts as a strong independent risk factor for T deficiency and suboptimal T levels can exacerbate obesity[6]. The hypogonadal-obesity-adipocytokine hypothesis is an extension of Cohen's theory, wherein T enhances the activity of lipoprotein lipase enzyme, leading to a rise in triglyceride (TG) uptake into adipocytes. With larger numbers of adipocytes, there is an increase in insulin resistance, production of pro-inflammatory cytokines including tumor necrosis factor-α, interleukin (IL)-1 and IL-6 and increase in leptin and estradiol levels. Leptin, produced by adipocytes, stimulates hypothalamic neurons to release GnRH, and subsequently, luteinizing hormone (LH) from the pituitary gland, and potentiates the release of T. Leptin can directly and indirectly, *via* receptors in testicular tissue, inhibit gonadotrophic actions on Leydig cells to worsen T deficiency. These neurons become resistant to the actions of leptin in obesity. A sustained state of hypogonadotropic hypogonadism is contributed by a reduction in GnRH signals, inhibiting the neuronal release of Kisspeptin and excess estradiol. In circulation, approximately 98% of T is bound to albumin and sex hormone-binding globulin (SHBG). Suboptimal levels of SHBG and T significantly contribute to insulin resistance and a pre-diabetic state. Hyperinsulinemia due to reduced insulin sensitivity in peripheral tissues contributes to central hypogonadism through the modulation of GnRH and gonadotrophin output and secondly to peripheral hypogonadism through direct actions on Leydig cells[6]. A schematic representation of the pathogenesis of MOSH is shown in Figure 1.

Another hypothesis, the Gut Endotoxin Leading to a Decline IN Gonadal function (GELDING) theory, states the role of metabolic endotoxemia[8]. This is the acronym for GELDING. Gut microbiota with an average of 1.5 kg (100 trillion) of bacteria residing in the human bowel produce various proinflammatory cytokines. High-calorie and fat-based diets contribute to the release of bacterial endotoxins from the gut. Exposure to these lipopolysaccharides causes impaired testicular function, thereby contributing to T deficiency.

Adipose tissue plays a major role in glucose homoeostasis and insulin sensitivity. The inverse correlation between visceral fat and testosterone levels is strong[9]. Testosterone increases lipolysis by increasing the number of β-adrenergic receptors. The action of testosterone on subcutaneous and visceral adipose function is different. Subcutaneous fat accumulation in the truncal area is highly predictive of low plasma concentrations of free testosterone rather than visceral adiposity[9]. There are no mechanistic studies that address the differential response to testosterone in different adipose tissues. The hypogonadal-obesity-adipocytokine hypothesis takes into consideration high aromatase activity in adipocytes converting testosterone to oestradiol[6]. TG storage in adipocytes is increased by reduced testosterone by stimulating pluripotent stem cells to mature into adipocytes[9].

DIAGNOSIS OF MOSH

Definition

Though definitions vary, a diagnosis of MOSH is usually made in obese men with BMI of ≥ 30 kg/m^2 with clinical features of hypogonadism (including impaired sexual, physical or mental performance, impaired sexual characteristics, gynaecomastia, breast pain, sleep problems, dysglycemia, flushing, low bone mineral density (BMD) or unexplained anaemia), biochemical evidence of hypogonadism with low total, free or bioavailable testosterone along with low or inappropriately normal LH, with other causes of hypogonadism including hyperprolactinemia having been excluded systematically[10].

Biochemical testing

For the diagnosis of MOSH, a cut-off level of total testosterone (TT) ≤ 12 nmol/L (346 ng/dL) in men with clinical manifestations of hypogonadism is used[11]. Since hyperinsulinemia, type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS) are associated with reduced SHBG concentrations, in the context of MOSH, it is recommended that free testosterone (free T) levels be calculated in order to avoid unnecessary testosterone replacement therapy (TRT). Free T can be determined by multiple methods like physical separation from the protein-bound forms by equilibrium dialysis or ultracentrifugation. Although equilibrium dialysis is the most accurate method among these, it is expensive, time-consuming and practically unfeasible[12]. SHBG and albumin level-based calculations have also been used to estimate free T. For example, the Vermeulen method, though accurate, can slightly overestimate free T. In cases of MOSH, SHBG should be measured during diagnostic workup to calculate free testosterone. According to the European Male Ageing Study (EMAS) study and a longitudinal evaluation of the same study, reduced free T (< 220 pmol/L) is better than total T alone in the detection of MOSH, especially for thresholds between 8.0 and 11.0 nmol per litre[13]. Diurnal variation gets significantly blunted in men > 40 years. However, it is recommended that testosterone be measured in the morning for all age groups. Measurement of testosterone levels should preferably be done while fasting, using a validated technique and not during an acute illness. Low testosterone should be confirmed on two occasions, preferably four weeks apart[6].

In the presence of reduced T concentrations, an LH concentration ≥ 9.4 IU/L is used to define primary hypogonadism, whereas low or low-normal LH concentrations define secondary hypogonadism[14]. MOSH is mostly characterized by a secondary or mixed, rather than primary hypogonadism. While the recent clinical practice guidelines by the Italian Society of Andrology and Sexual Medicine (SIAMS) and the Italian Society of Endocrinology (SIE) consider a cut-off of 12 nmol/L (346 ng/dL) for TRT, the British Society for sexual medicine guidelines recommend TRT in cases with total T < 8 nmol/L (< 231 ng/dL), or free T < 225 pmol/L (< 0.225 nmol/L). Those with total T between 8-12 nmol/L (231-346 ng/dL) may be given a TRT trial for six months based on symptoms[14].

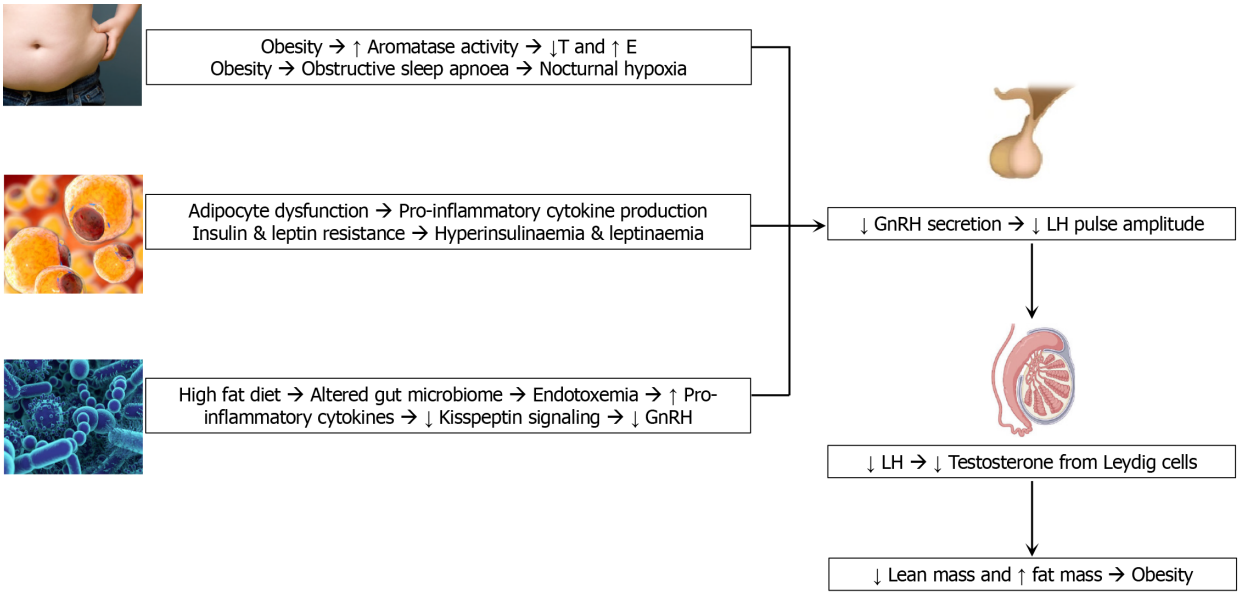

Figure 1 The pathobiology of male obesity-related secondary hypogonadism. E: Estradiol; GnRH: Gonadotropin-releasing hormone; LH: Luteinizing hormone; T: Testosterone.

MANAGEMENT OF MOSH: TO TREAT OBESITY OR HYPOGONADISM OR BOTH?

While obesity and hypogonadism are linked bidirectionally, there is an ongoing debate on whether to focus first on the treatment of obesity which can lead to improvement in gonadal function or to start testosterone replacement to correct hypogonadism first, with expected beneficial effects on body weight and metabolic parameters. In an attempt to address this debate, the following sections focus on the effects of obesity treatment on male hypogonadism and the role of Testosterone therapy on obesity.

Effect of weight loss on hypogonadism

Healthy lifestyle changes can help achieve significant weight loss. Hypothalamic-pituitary-testicular (HPT) axis suppression and testosterone deficiency in MOSH are potentially reversible, without the need for testosterone treatment. A meta-analysis of several studies found that significant weight loss can induce an increase in T levels[15], along with an increase in SHBG, calculated free T, LH , and follicle-stimulating hormone (FSH), and a reduction in estradiol (E2). This has been seen in several observational studies. Low-calorie diet caused a weight loss of 9.8% and therefore induced an average T increase of less than 3 nmol/L, while bariatric surgery, with a weight loss of 32% increased T levels three times higher (almost 9 nmol/L)[16,17].

Interestingly, this is similar to the degree of increase in T levels seen with transdermal T supplementation with a patch or gel in a recent meta-analysis[18]. Thus, consistent weight loss can be as efficient as TRT concerning an increase in T levels. Weight loss intervention, especially, bariatric surgery induces a reduction in estradiol and possibly, the positive effect of these interventions on the HPT axis is the reduction of the estrogen-dependent negative feedback and thereby inhibition of Kiss-1[19].

Lifestyle changes to treat obesity

Functional hypogonadism in MOSH can be managed with lifestyle measures. If lifestyle measures can achieve significant weight loss, it may obviate the need for T treatment. The United States Preventive Services Task Force has recommended effective, intensive, behaviour-based weight loss interventions to help adults with obesity achieve a weight loss of ≥ 5% through changes in diet and physical activity. After one year, an average weight loss of 2-3 kg is observed with such interventions, and can even achieve a weight loss of up to 9 kg[20]. A loss of 5%-10% of initial body weight is a target as well as a measure of successful weight loss as per the European Practical and Patient-Centred Guidelines (2019) for adult obesity management in primary care[21]. In a recent trial, there was a reduction in body weight (compared to baseline) by approximately 4 kg (4%), 7 kg (7%), and 5 kg (5%) within 10 wk, six months, and one year, respectively in the intervention group and good adherence to dietary recommendations even at one year. There were significant reductions in body weight, BMI, waist circumference (WC), remnant cholesterol, and resting heart rate at 10 wk, and these changes were maintained for one year[22]. The dietary interventions, with or without exercise are likely to improve the gonadal function with improvement of T levels[23]. In a study of 68 men who attained a mean loss of 10.3-10.8 kg ± 1.2 kg over the 52-wk study period, there was a significant increase in TT and FT[24].

In the EMAS study, it was shown that around a 20% reduction in BMI is required to produce a significant increase in FT level[25]. Ketogenic diets (KD) can improve the metabolic and weight patterns in obese patients. However, the effect on testosterone levels is less well understood. In a recent meta-analysis comprising eight trials and 230 patients, five trials enrolled subjects on normocaloric KD and three trials enrolled subjects on very low-caloric KD (VLCKD). TT increased in

111 patients, more with VLCKD compared to normocaloric KD. Meta-regression analyses showed significant correlations between the post-KD testosterone rise with patients' age and weight loss[26].

PHARMACOLOGICAL MANAGEMENT OF OBESITY

Obesity is a chronic disease associated with a chronic low-grade inflammatory state and immune dysfunction. Significant improvement in metabolic processes as well as decrease in overall mortality has been reported in several studies with multiple modes of treatment. Glucose-lowering medications have been employed in prediabetic and diabetic individuals. Improvements in erectile dysfunction after anti-diabetic drug therapy may be ascribed to indirect mechanisms such as the reduction of hyperglycaemia, excess body weight, high blood pressure, and the amelioration of other detrimental factors. However, a direct effect of glucose-lowering agents on both endothelial and smooth muscle cells is reasonable.

Metformin

This drug has evidence for anti-obesity, renal, cardioprotective and anticancer roles. There is an anti-androgenic effect as well as a negative impact on testicular and reproductive health[27]. Prolonged duration of metformin-based therapy reduces T levels and counteracts the T elevation accompanied by improved blood glucose[28]. Low T levels have also been observed in patients on metformin, regardless of age, duration of the disease and hemoglobin (HbA1c)[29]. A recent study indicated that for fathers who took one or more prescriptions for metformin during the development of fertilizing sperm, the likelihood of their male offspring having genital birth defects was increased[30]. Mechanisms underlying the dangerous effects of metformin on human testicular health are unclear.

Pioglitazone

This insulin sensitizer appears to improve venous occlusive function through a mechanism independent of glycaemic control[31].

Sodium-glucose cotransporter-2 inhibitors

Regarding gliflozins, animal studies showed that empagliflozin improves erectile function in diabetic rats by increasing NO-mediated relaxation of erectile tissue[32]. Dapagliflozin may protect against diabetes-induced spermatogenic dysfunction *via* GLP-1R/PI3K/Akt-dependent pathway. Treatment with dapagliflozin increases T secretion in obese patients with uncontrolled T2DM and hypogonadism by the extent of weight loss and reduction in testis inflammation. An open-labelled non-randomized pilot study amongst thirty Caucasian patients demonstrated that treatment with dapagliflozin plus tadalafil resulted in improvement in erectile dysfunction, suggesting the ability of dapagliflozin to enhance the efficacy of tadalafil[33].

Glucagon-like peptide-1 receptor analogues

Glucagon-like peptide-1 receptor analogues-based therapy may potentially act on the HPG axis, fostering LH secretion by hypothalamic-pituitary neurons, T production by the testis, ameliorating the semen quality and improving erectile function. Supplementation of liraglutide to metformin therapy ameliorated endothelial functions of the corpus cavernosum of male obese subjects with T2DM, resulting in the recovery of erectile performance[34]. In the Researching Cardiovascular Events with a Weekly Incretin in Diabetes (REWIND) trial, long-term treatment with dulaglutide was also found to reduce the incidence of moderate or severe erectile dysfunction in middle-aged men with T2DM[35]. In a prospective randomized open-label study, the treatment of obese men with liraglutide induced a significant increase in serum TT levels (2.6 nmol/L ± 3.5 nmol/L), improvement of LH and FSH secretion along with an average weight loss of 7.9 kg ± 3.8 kg compared with a 0.9 kg ± 4.5 kg loss only with TRT[36]. Similar encouraging results are expected with other GLP-1 agonists.

BARIATRIC SURGERY

Metabolic and bariatric surgery (MBS) has evolved over the past three decades as a therapeutic strategy for obesity. MBS can reverse obesity-induced hypogonadism in a certain subset of individuals. TRT could be additionally employed if these measures fail to relieve symptoms and normalise testosterone levels. Recent guidelines on MBS and its indications by the American Society for Metabolic and Bariatric Surgery (ASMBS) and the International Federation for the Surgery of Obesity and Metabolic Disorders focused on the currently available surgical treatments for severe obesity and the criteria for selection, efficacy, and risks of surgical treatments for severe obesity and nonsurgical programmes that could be the initial therapy for severe obesity[37].

A BMI of 40 kg/m^2, or 35 kg/m^2 with co-morbidities, is a threshold for surgery that is applied universally. Currently, the dominant procedures are sleeve gastrectomy and Roux-en-Y gastric bypass, and together these procedures account for approximately 90% of all operations performed worldwide. Other procedures are adjustable gastric banding, biliopancreatic diversion with a duodenal switch and one-anastomosis gastric bypass.

A recent clinical study from Spain by Miñambres *et al*[38] revealed that weight loss attained after bariatric surgery among 12 subjects (five Sleeve gastrectomies and seven Gastric Bypass), increases TT, free testosterone and SHBG, resulting in the complete resolution of MOSH in men with severe obesity. The results demonstrate improved sexual

function without an impact on sperm concentration and motility and an overall decline of morphology over time. In another study by Rigon *et al*[39], among 29 men undergoing bariatric surgery with a mean baseline weight of 155.26 kg ± 25.88 kg, there were significant improvements in TT levels from 229.53 ng/dL ± 96.45 ng/dL to 388.38 ng/dL ± 160.91 ng/dL ($P < 0.001$).

A recent systematic review involving 14 studies and 508 patients clearly showed remarkable benefits of improvement of T levels and erectile function in patients following weight loss after bariatric surgery[40]. Trials evaluating the effect of MBS on semen morphology are highly variable and inconsistent, with small prospective studies reporting a decrease in the percentage of sperm with normal morphology. However, a recent meta-analysis described that bariatric surgery had been associated with improved sperm morphology 12 months post-surgery[41].

OTHER THERAPEUTIC OPTIONS

Gonadotrophins such as human chorionic gonadotropin (hCG) or FSH are effective in increasing testosterone levels and semen parameters but are costly and require administration *via* injection. They currently require approval, prescription, and follow-up from specialist centres. Pulsatile GnRH is a less attractive option due to both the cost and the impracticality of continuous infusion. Selective oestrogen receptor modulators (SERMs) such as clomiphene citrate have been shown to increase testosterone levels without a negative impact on fertility, though there is a lack of long-term data regarding their impact on hypogonadal symptoms. The aromatase inhibitors (AIs) may also raise testosterone levels but are associated with reduced oestradiol levels and BMD, and their use requires close, long-term monitoring. Further studies of clomiphene and AIs in conjunction with testosterone therapy are required to confirm whether these agents can be used synergistically. This might mitigate the risk of adverse events of TRT in terms of reduced fertility and symptoms associated with increased oestradiol levels.

Weight reduction and MOSH-summary of the evidence: Overall, results suggest that a significant degree of weight loss does lead to improvement in serum T levels in those with hypogonadism. Lifestyle modification and bariatric surgery for those with severe obesity seem to show the best results in this regard. Some of the pharmacotherapeutic agents have shown a few additional benefits concerning erectile dysfunction or semen motility through unknown mechanisms. The choice of agent should be guided by the presence of other comorbidities like diabetes, or contraindications. The detrimental effects of metformin on sperm production or functioning will have to be elucidated in further studies.

ROLE OF TESTOSTERONE IN THE MANAGEMENT OF MOSH

The management of MOSH should be done with a multipronged approach. The relationship between obesity and T is bidirectional. T exerts multiple effects on body composition, lipid parameters, glycemic parameters, and overall cardiometabolic health. TRT is also fraught with possible adverse effects on cardiovascular events. Ensuring adequate weight loss with an increase in T levels is the cornerstone of therapy in MOSH-the effects of TRT remain controversial. The following section focuses on its role in multiple aspects of MOSH.

Effects on body weight

Some observational, registry-based studies had reported weight loss with TRT. However, analysis of controlled studies revealed alterations in body composition while overall body weight was unaltered. Trials of men with late-onset hypogonadism (LOH) have shown a decline in WC and body fat percentage with TRT but without any change in BMI[42]. One study reported a decrease in body weight by 16 kg, BMI by five points and a reduction in WC by nine cm over an observational period of five years[43].

In one of the oldest meta-analyses on this topic by Isidori *et al*[44], the authors did not find any significant effect of TRT on BMI. There were significant changes in body composition though, with up to 1.6 kg decrease in fat mass and a similar increase in lean body mass. Another meta-analysis, including multiple observational studies as well as RCTs on the effects of TRT in the management of hypogonadism, indicated a decrease in BMI, weight and WC by 1 kg/m^2, 6 kg and 7 cm respectively[45]. However, the difference was only seen in uncontrolled studies, among older and more obese subjects, and studies with longer duration of follow-up[46]. The reduction of fat mass was observed in both placebo-controlled (-2.13%) and uncontrolled (-4.56%) studies. When the meta-analysis was done including only observational studies with 4513 patients, TRT was found to be associated with a time-dependent reduction in body weight by 3.5 kg and WC by 6.23 cm over 24 months.

Effects on body composition

In contrast with the effects of TRT on body weight and BMI, which have mostly been seen in observational studies and among older and more obese subjects, the beneficial changes in body composition have been more consistently seen in RCTs as well, with an increase in lean mass and decrease in fat mass. Since there is an inverse association of muscle mass with diabetes mellitus risk, the anabolic effects of T to increase lean mass and reduce fat mass might be a potential mechanism for metabolic risk reduction with T. A study using data from the 1999-2000 NHANES revealed that men in the highest tertile of total T had higher lower-body and upper-body lean mass and lesser upper body fat mass [β = -6.1%; 95% confidence interval (95%CI): -10.1 to -2.1, $P = 0.005$] than those in the lowest quartile, indicating that even at physiologic levels, an association exists between higher levels of T and favourable lean and fat mass distribution[47].

A meta-analysis of TRT in elderly men reported an increase in lean mass weight from 1.65 kg to 6.20 kg (an effect estimate of 3.59), and a decrease in fat mass (an effect estimate of -1.78). However, the authors admitted to having a high level of heterogeneity between the included studies[48]. An RCT on the effects of TRT in obese men on a hypocaloric diet indicated that those on placebo lost both fat and lean mass whereas the weight loss in those on TRT was almost exclusively due to body fat loss[49]. A group of 202 men received goserelin acetate, placebo or testosterone gel along with anastrozole, the latter to suppress the conversion of testosterone to estradiol[50]. The percentage of body fat increased in groups receiving placebo or testosterone daily without anastrozole whereas lean mass and thigh-muscle area decreased in men receiving placebo and in those receiving testosterone without anastrozole. There was wide variability in the amount of testosterone required to maintain lean mass, fat mass and strength.

Effects on muscle strength

The anabolic effects of T on muscle growth and function are well known, so much so that over the past several decades, T remains one of the most common substances abused by athletes to improve muscle mass, muscle recovery and endurance. In the early meta-analysis by Isidori *et al*[44], the effect of TRT on muscle mass was unequivocally documented compared to placebo. In contrast, in a later meta-analysis, focusing on androgen trials in men aged above 65 years, TRT, especially with parenteral T, was found to cause a moderate increase in muscle strength, with larger effects for lower extremity and total body strength measures[51]. More recent trials on elderly community-dwelling elderly males with hypogonadism have revealed TRT to increase muscle strength measures like leg-press strength, chest-press strength and stair-climbing power[52]. While the results are still controversial, increased muscle strength with TRT, especially in elderly, frail individuals, is a notable finding that will lead to a better exercise ability in such individuals. Finkelstein *et al* [50] highlighted that while androgen deficiency accounted for a decrease in muscle size and strength, estrogen deficiency chiefly accounted for an increase in body fat, and a decline of both contributed to the decline in sexual function.

Effects on lipid profile

Most epidemiologic studies have reported an association of low T levels with an atherogenic lipid profile but higher high-density lipoprotein (HDL) cholesterol[53]. Higher T levels are associated with less atherogenic smaller very-low-density lipoprotein (VLDL) particles and lower ApoB to Apo A-1 ratio[53,54]. TRT reduces plasma HDL cholesterol depending on the dose and route of administration[55]. In interventional studies, TRT was reported to be associated with a modest reduction in HDL cholesterol in hypogonadal men. The reduction in HDL cholesterol is predominantly seen with non-aromatisable oral androgens than injectable T esters or transdermal T. Regarding its effects on LDL, few studies have reported a significant reduction in LDL cholesterol. In a study on 161 men with LOH, the total and LDL cholesterol declined significantly, with no significant changes in HDL cholesterol and TG[56]. In a meta-analysis of 59 RCTs, when the authors included only RCTs enrolling hypogonadal subjects with T levels < 12 nmol/L, TRT achieved a reduction of total cholesterol as well as TG levels without any change in HDL cholesterol[57]. Overall, the results of clinical studies on the effects of TRT on lipid parameters are conflicting.

Effects on glycemic parameters

Several epidemiologic studies found an association of low total T levels with increased risk of T2DM, though the association was weak or absent with free T, hinting at SHBG being the primary determinant of the observed correlation[54,58,59]. The mechanisms linking low T and worsening of insulin resistance could be loss of skeletal muscle mass, increased fat mass, and effects of T on lipid oxidation and mitochondrial function[60]. A prospective study suggested that low T is associated with incident T2DM in men[61].

Clinical trials studying the metabolic effects of TRT in men with T2DM and MetS have yielded conflicting results. In their euglycemic clamp-based studies, Dhindsa *et al*[62] found that three weeks of TRT had no effects on insulin sensitivity or other glucose parameters, but 24 wk of TRT led to changes in body composition and improvement in insulin sensitivity. Only a few RCTs studied the effects of TRT on glucose values or insulin sensitivity indices as primary endpoints. One of the largest studies on T2DM or MetS subjects (n = 220), the TIMES-2, did not find a significant reduction in HbA1c or BMI after 26 wk of topical T, although a slight improvement in homeostatic model assessment for insulin resistance (HOMA-IR) was observed[63]. In the largest RCT conducted exclusively in 199 men with T2DM, parenteral T undecanoate (TU) resulted in significant improvement of HbA1c concentrations, particularly in those with poor glycemic control at baseline with HbA1c ≥ 7.5%[64]. Some other studies failed to find any improvement in HbA1c or HOMA-IR despite an improvement in body composition[65]. A few uncontrolled registry-based observational studies suggest improvement in glucometabolic parameters with TU in men with T2DM and MetS and prevent their progression from pre-diabetes to T2DM[66-68]. However, these studies were mostly short-term, and had methodologic limitations. Their results were inconsistent, and the observed improvements were only modest.

In a meta-analysis of RCTs, TRT reduced fasting plasma glucose (FPG) by 0.34 mmol/L and HOMA-IR by 0.8[57]. A review of RCTs on men with T2DM and baseline T between 12 to 15 nmol/L (346-432 ng/dL) showed a reduction in FPG by 1.2 mmol/L over a period of 3 to 12 months[69]. In a more recent meta-analysis of 833 men with baseline T between 6.7 to 10.1 nmol/L (193-291 ng/dL), TRT improved measures of insulin resistance but did not reduce HbA1c%[70].

In the recent testosterone for prevention of DM trial, 1004 men were randomised to TRT to study the efficacy of TRT in the prevention or reversal of newly diagnosed T2DM in men enrolled in a lifestyle programme[71]. At the end of two years, TRT resulted in a greater likelihood of normal oral glucose tolerance test [odd ratio (OR): 1.20, 95%CI: 1.04-1.38, P = 0.012] and lowered FPG (OR: -0.17 mmol/L, 95%CI: -0.29 to -0.06, P = 0.004). TRT reduced the risk of T2DM by 40% in men with high WC having impaired glucose tolerance or newly diagnosed T2DM. The benefits of TRT were similar for men with baseline T < 11 nmol/L (317 ng/dL) or more. The relationship between low T concentration and obesity and

the risk of T2DM is bidirectional in middle-aged and older men. However, the effects of TRT alone in this regard need further research. A holistic approach, incorporating healthy lifestyle behaviours and optimised management of medical risks, seems to be a more prudent and evidence-based approach to metabolic risk mitigation in men before TRT.

In a recently published sub-study of the TRAVERSE trial, the effect of T on progression from prediabetes to diabetes in men with hypogonadism was evaluated; there were no significant differences in the risk of progression to diabetes between the testosterone and placebo groups at any point of time ranging between six to 48 months of follow-up (P = 0.49)[72]. The rates of prediabetes/diabetes remission and also the changes in glucose and HbA1c levels were similar in testosterone- and placebo-treated men. The encouraging data with TRT on diabetes mellitus prevention is limited to studies with up to a two-year observation period, and in subjects with borderline hypogonadism who were also following a concomitant strict, lifestyle programme.

Effects on blood pressure

Serum levels of T are believed to drive the differences in BP between men and women that become apparent post-puberty [73]. Androgen receptor (AR) signaling increases the activity of the renin-angiotensin-aldosterone system and orchiectomy or the use of AR antagonists reduces plasma renin activity as well as salt-induced hypertension in male rats[74]. T also increases the mRNA expression of renal angiotensinogen[75]. T administration could lead to transient sodium and fluid retention during the first few weeks of treatment, and some older men may manifest edema[76]. Despite all these effects of T, men with hypogonadism have higher systolic blood pressure than eugonadal men[63] and TRT has not resulted in increased BP in most studies[76,77]. However, some recent studies with oral TU and with subcutaneous T enanthate detected a small rise in clinic and 24-h ambulatory BP following 120 d to 180 d of treatment, and the effect was mostly seen on systolic than diastolic BP[78,79].

Effects on cardiovascular events and mortality

T has several potentially beneficial effects on the cardiovascular system. In addition to its effects on lipid parameters, BP and glucose intolerance, T has potent vasodilator action by inhibiting L-type calcium channels causing increased coronary blood flow[79,80]. It also can improve endothelial function and reduce vascular reactivity. TRT, by downregulating *SERCA-2a* expression, reduces the calcium accumulation within the sarcoplasmic reticulum, thereby attenuating cardiac inotropy[81]. T has also been shown to retard atherosclerosis in some preclinical studies but not all[67,75].

Cross-sectional and longitudinal epidemiologic studies revealed an inconsistent relationship between T levels and cardiovascular events[68,82,83]. Epidemiologic studies can only suggest association and not prove causality. Reverse causality is also a possibility, indicating that T is a marker of health, and men at high risk of mortality also have low T levels. Interestingly, Ruige *et al*[83], found that high T levels were associated with low risk for CV events in men > 70 years of age but not in younger men. In the Rotterdam study, men with the lowest quartile of T levels had greater progression of carotid intima thickness than men in the highest quartile of T. However, there was no difference in coronary artery calcium scores[84,85].

A meta-analysis by Corona *et al*[69] reported an association of low T levels with high cardiovascular and overall mortality. Another meta-analysis of 11 RCTs also found that lower T levels were associated with a higher risk of all-cause mortality, especially cardiovascular mortality[86]. Whereas TRT can have positive and negative effects on cardiovascular health, CV concerns also need to be factored in, especially when TRT is planned for elderly men with high CV risk. The therapeutic benefits of androgen therapy in MOSH are shown in Figure 2.

Another multi-centre RCT, the TRAVERSE study[87], on 5246 men between 45 to 80 years of age who had hypogonadism and also pre-existing cardiovascular disease or a high risk for the same, was recently published. The participants were randomly assigned to receive daily transdermal 1.62% testosterone gel (dose adjusted to maintain testosterone levels between 350 ng/dL and 750 ng/dL) or placebo gel. The primary cardiovascular endpoint was the first occurrence of any component among a composite of cardiovascular deaths, nonfatal myocardial infarction, or nonfatal stroke. At a mean follow-up of 33.0 months ± 12.1 months, the occurrence of the primary cardiovascular end-point event was non-inferior in the testosterone group compared to the placebo group (7.0% in the testosterone group *vs* 7.3% in the placebo group [hazard ratio (HR): 0.96, 95%CI: 0.78-1.17; P < 0.001 for noninferiority]. The incidence of each of the events of the composite primary cardiovascular endpoint was similar in the two groups. There was a higher incidence of atrial fibrillation, acute kidney injury and pulmonary embolism in the testosterone group.

Effects on sexual function in middle-aged and elderly males

MOSH is particularly prevalent in middle-aged and elderly males. TRT has demonstrated improvement in sexual function in young men with primary or secondary hypogonadism as well as in middle-aged men with mildly or moderately low T levels[88-90]. While the effects on sexual activity in older men were controversial in older studies, the T trials demonstrated modest improvement in sexual interest and activity with TRT in elderly males with documented hypogonadism, with greater effects seen on libido and sexual activity than on erectile function. A recent meta-analysis showed that TRT is most effective when serum T is < 10.4 nmol/L (< 300.0 ng/dL) and not very effective when T > 12.0 nmol/L (> 350.0 ng/dL)[46,91]. Another meta-analysis found that TRT alone can modestly improve mild, but not severe, erectile dysfunction with an improvement by 2-3 points on the international index of erectile function-Erectile function domain (IIEF-EFD, 2-3 points; effect size 0.30). The degree of improvement in sexual activity is higher in those with lower baseline T and steeper improvement in T levels[92].

Recently, the Sexual Function Study, nested within the parent TRAVERSE trial[93] aimed to find the efficacy of TRT in improving sexual activity, hypogonadal symptoms, libido and erectile function among middle-aged and older hypogonadal men reporting low libido with the primary outcome being change in sexual activity score. TRT with 1.62% gel

Figure 2 Therapeutic benefits of androgen replacement therapy in male obesity-related secondary hypogonadism. CV: Cardiovascular; SBP: Systolic blood pressure.

was associated with significant improvement in sexual activity compared to placebo with a mean between-group difference of 0.49 and 0.47 acts per day at 6 and 12 months, respectively, omnibus $P = 0.011$. The beneficial effects of TRT were maintained at 24 months. On analyzing individual secondary outcomes, TRT improved hypogonadal symptoms and sexual desire, but not erectile function.

Beneficial effects of TRT on mental health

TRT is thought to affect mood, energy, and health in multiple ways. Some trials have demonstrated significant improvements in the quality of life (QoL) with TRT when validated questionnaires were used like the Q-LES-Q or SF-36[92,94]. However, there are also trials showing no significant effects of TRT on health-related QoL, and therefore, no definite conclusions can be drawn[95,96]. Basaria *et al*[97] reported negative effects of TRT in hypogonadal males with subthreshold depression, which they propose might have been due to the use of insensitive Howl measures. In one large RCT from Japan, improvement with TRT was seen in the physical subdomain of the SF-36 scale with slight improvement in the emotional subdomain[98]. The effect on the physical subdomain is of particular interest in the context of MOSH, as the increased exercise capacity can translate into improved weight loss.

Concerns with the use of TRT in MOSH

The chief concerns about TRT relate to the pro-stimulatory and pro-differentiating effects of T on prostate and breast cancers. An increased risk for incident prostate cancer, prostate-related adverse events, increase in lower urinary tract symptoms (LUTS) or prostate volume and excess risk in breast cancer are suggested, though not convincingly seen in short-term studies following TRT in men with low T. The Endocrine Society recommendations listed in Table 1 are against the use of TRT in those at very high risk of adverse effects including metastatic prostate cancer or breast cancer, or those at moderate to high risk. However, the CVD risk and fertility concerns related to TRT are discussed in detail, since they are the most relevant in the context of MOSH. Formulation-specific adverse effects and monitoring are tabulated in Table 2.

Adverse effects of TRT on fertility

While TRT is used to induce secondary sexual characteristics and to improve libido and sexual functioning, TRT does not support spermatogenesis due to negative feedback on GnRH and gonadotroph secretion. One study showed that regular TRT in 271 healthy fertile men induced azoospermia after 4 months, and restoration of spermatogenesis after stopping TRT took nearly six months[99]. There have been concerns about persistent suppression of spermatogenesis after discontinuing TRT. TRT is not recommended for hypogonadal men desiring fertility in 6-12 months[99,100]. A prior meta-

Table 1 Characteristics of available testosterone formulations

Formulation	Available strengths	Dose regimen	Advantage	Formulation-specific adverse effects	Monitoring frequency
Parenteral preparations					
Testosterone enanthate	50, 75, 100 mg in 0.5 mL and 200 mg in 1.0 mL sesame oil	Start with 75 mg weekly subcutaneous/intramuscular injections and up-titrate dose to reach target T	Relatively inexpensive, flexible dosing	Pain of injections; fluctuations in symptoms due to peaks and troughs in serum T	50, 75, 100 mg in 0.5 mL and 200 mg in 1 mL sesame oil
Testosterone cypionate	100 and 200 mg/mL in cottonseed oil	Deep intramuscular injection to gluteal muscles once in 2 wk or subcutaneous injections in abdominal adipose tissue weekly		Pain of injections. Fluctuations in symptoms due to peaks and troughs in serum T	50, 75, 100 mg in 0.5 mL and 200 mg in 1.0 mL sesame oil
Testosterone undecanoate	750 mg/3 mL in castor oil	Start with 750 mg deep intramuscular injection deep in the gluteal muscle repeat after 4 wk and then every 10 wk	Infrequent administration	Painful, large-volume intramuscular injection; some report coughing immediately after injection. Possible risk of pulmonary oil micro-embolism	750 mg/3 mL in castor oil
Implants					
Testosterone pellets	75 mg/pellet	Inserted subcutaneously into fat in the hip area; 2 to 6 months will last 3 to 4 months; 6-10 implants last for 4-6 months	Infrequent administration	A surgical incision is required for insertions; local hematoma and infection; spontaneous extrusion of pellets	Measure T concentrations at the end of the dosing interval; adjust the number of pellets and/or dosing interval to maintain serum T concentrations in the mid-normal range
Topical/transdermal					
Testosterone patch	2 or 4 mg patches daily	4 mg starting dose, to be applied to back, abdomen, and upper arms. Do not apply the patch to the same area within 7 d	Easy application	Serum T concentrations are sometimes in the low-normal range. May need applications of two patches daily. Skin irritation at the application site	Assess serum T 3-12 h after application; adjust the dose to achieve T levels in the mid-normal range
Testosterone gel	1.00% gel-50 to 100 mg T/d	25-50 mg T packets to apply to the shoulder or upper arms; 20.25 mg T per 1 pump actuation, or a 20.25 mg packet	Flexibility of dosing; easy application; good skin tolerability; less erythrocytosis	Potential of contact transfer to female partners or children; skin irritation in some	Assess serum T 2-8 h following gel application, after the patient has been on treatment for at least 1 wk; adjust the dose to achieve serum T in the mid-normal range
	1.62% gel-40.5 to 81 mg T/d	40.5 mg T. 2 pump actuation or a 40.5 g packet; apply to shoulders or upper arms			
	2.00% gel 10 mg/0.5 g per pump actuation	40.0 mg (4 pump actuation)/d starting dose; apply to inner thighs			
	2.00% lotion 30 mg/pump actuation	Start with 60 mg, apply to axilla	Good skin tolerability	Potential of contact transfer to female partners or children. Dripping/wet sensation in the axilla	
Buccal/nasal					
Buccal tablets	30 mg twice/d	Apply to gums	Convenience and discreet	Gum-related adverse events; dislodgment	
Nasal gel	11 mg gel intranasal two or three times daily	Start with one actuation (5.5 mg) into each nostril-a total of 11 mg; apply to nose three times daily	Rapid absorption and avoidance of first-pass metabolism	Multiple daily intranasal dosing; local nasal irritation; not appropriate for men with nasal disorders	

Oral					
Testosterone undecanoate capsules	40 mg capsules 2-3 times daily. 158 to 396 mg twice daily	80 to 120 mg/d. Start with 237 mg twice a day with food	Convenience of oral administration	Variable response; must be administered along with a fatty meal; fat content of meals may increase bioavailability	Monitor serum T 3-5 h after ingestion of the tablet

T: Testosterone.

analysis reported, however, that previous TRT did not negatively impact treatment outcomes with gonadotropin/GnRH fertility induction[101].

Cardiovascular risks with TRT

In a retrospective review of men with low T undergoing angiography, TRT was found to be associated with an increased risk of composite CV outcome of MI, stroke and death (HR: 1.29), as also reported in an insurance database-based study [102,103]. However, post-hoc analysis of the AIM-HIGH trial showed low T levels to be associated with an increased risk for primary composite CV outcome in men with MetS and low HDL cholesterol[15]. All observational studies have limitations including heterogeneous study population and differences in treatment regimens, durations, baseline risk factors and study design. One of the first RCTs, designed to investigate functional immobility following TRT in older men with sarcopenia [testosterone replacement for older men with sarcopenia, (TOM trial)], had to be stopped prematurely due to an unexpected increase in CV events with TRT[76].

Subsequent RCTs excluded men with high baseline CV risk. Therefore, most trials reported very few MACEs. However, meta-analyses of several RCTs failed to show a statistically significant association of TRT with CV events[104, 105]. In most of the RCTs, CV events were not a prespecified outcome. Two RCTs-the Cardiovascular Trial of the T Trials and the Testosterone Effects on Atherosclerosis in Ageing Males Trial-reported that the carotid intima-media thickness and the coronary calcium scores did not change with TRT, though there was an increase in the volume of noncalcified plaque with TRT in the former[76,85]. Overall, the FDA notified that these trials have significant limitations that weaken their value. It nevertheless directed pharmaceutical companies to include a label, warning about the cardiovascular risks of TRT. Further trials are ongoing that might lead to more clarifications.

Risks of venous thromboembolism

By stimulating erythropoiesis and increasing hematocrit, TRT runs the theoretical risk for VTE. One case-control study and a registry-based study have demonstrated up to two-fold increased risk of VTE within six months after TRT[106, 107]. However, the number of events was too small, and most cases occurred in men with preexisting hypercoagulable states.

RECOMMENDATIONS ON THE USE OF TRT IN MOSH

The Endocrine Society (ES), European Association of Urologists and Andrologists (EAU, European Academy of Andrology) and the American Association of Clinical Endocrinologists (AACE) recommend TRT to be used in symptomatic hypogonadal men to induce/maintain secondary sex characteristics and to improve symptoms of sexual satisfaction like libido, erectile function, and emotional satisfaction. These guidelines do not recommend TRT for the sole

Table 2 Cautions with testosterone replacement therapy and monitoring for adverse effects

High-risk population for TRT	Special considerations in monitoring
Very high risk of serious outcomes: prostate cancer; breast cancer	For patients who opt for prostate monitoring: Men aged 55-69 yr & those aged 40-69 yr who are at increased risk for prostate cancer and choose monitoring; perform DRE and measure PSA at baseline, at 3-12 months after starting treatment, and then as per local prostate cancer screening guidelines
Moderate to high risk of adverse outcomes	Urologic consultation should be sought if: (1) Increase in serum PSA > 1.4 ng/mL within 12 months of starting TRT; (2) PSA > 4 ng/mL at any time; (3) DRE detected new onset prostate abnormality; and (4) significant worsening of LUTS
Unevaluated prostate nodule or induration	To check Haematocrit at baseline, then at 3-6 months following TRT, and then annually. If Hct > 54%, stop therapy until it decreases to a safer level; evaluate for other causes of erythrocytosis (sleep apnoea, COPD), re-initiate at lower doses when Hct falls below normal
Baseline PSA > 4 ng/mL or > 3 ng/mL in men at high risk for prostate cancer	
Severe lower urinary tract symptoms	
Haematocrit > 48% (> 50% for men living at high altitudes)	
Uncontrolled or poorly controlled heart failure	
Myocardial infarction or stroke in the preceding 6 months	
Untreated severe obstructive sleep apnoea	
Wants fertility in the near future	
Formulation-specific adverse effects (Table 1)	

TRT: Testosterone replacement therapy; PSA: Prostate specific antigen; DRE: Digital rectal examination; Hct: Haematocrit; COPD: Chronic obstructive pulmonary disease; LUTS: Lower urinary tract symptoms.

purpose of weight reduction in obese men, for improved glycemic control or metabolic outcomes in men with MetS or T2DM, to improve exercise capacity, physical functioning, or cognitive functioning in elderly males or as an anti-ageing agent. The recently published SIAMS and SIE guidelines recommend starting TRT in all symptomatic hypogonadal men and older men with hypogonadism for improvement in sexual functioning, to improve BMD and prevent bone loss, to improve major depressive symptoms, to reduce WC and improve body composition and also to reduce fasting and post-load glucose status[11].

The EAU recommends lifestyle changes for weight reduction in overweight and obese men with functional hypogonadism as weight loss can lead to increased serum T. It recommends against TRT for weight reduction in obese men or for glucometabolic outcomes[108]. The AACE and American College of Endocrinology recommend consideration of TRT in men with symptomatic hypogonadism and obesity not for fertility, but only as an addition to lifestyle intervention. These guidelines recommend against TRT in men to improve glycemic control, although they do not make any recommendation for or against TRT for weight reduction. The recent SIAMS and SIE recommend TRT to reduce WC and improve body composition by reducing fat mass and increasing lean mass in subjects with hypogonadism with or without MetS or T2DM[11]. They also recommend TRT to improve fasting and post-prandial glycemia in subjects with hypogonadism with MetS or prediabetes to reduce the risk of progression to T2DM. However, they recommend against considering TRT to control dyslipidemia or to improve HbA1c% in patients with or without T2DM.

Thus, it might be a prudent decision to start TRT in obese men with MOSH, especially if they have dysglycemia. However, if used in the elderly or those at high risk for cardiovascular events, this must be weighed against potential risks and done under close supervision, as outlined in section 8.

AVAILABLE T PREPARATIONS

The available formulations of T, dosing schedule and formulation-specific adverse effects are listed in Table 1. The SIAMS guidelines recommend gel formulation of T for the treatment of older adults with hypogonadism, especially if potentially reversible conditions like obesity are present.

Do different formulations of T have different effects and side effects?

The heterogenous results of TRT from multiple trials lead to speculations about whether the type and route of adminis-

tration of TRT can play a role in this, and this has also been substantiated by sensitivity and meta-regression analyses. The potential for aromatization might explain the heterogeneity in findings, especially the effects on bone density and HDL cholesterol. Observational studies employing the long-acting parenteral TU, indicate a consistent effect of TRT on BMI and central obesity[109]. Both transdermal and parenteral preparations of T have been demonstrated to improve fasting glycemia and body composition. However, no improvement in body composition was observed in trials using oral T preparations. In a study, both transdermal and parenteral T significantly improved FPG. Among the parenteral preparations, TU produced greater reductions in fat mass and lean mass. Transdermal and oral TRT lead to increased serum dihydrotestosterone (DHT) due to increased 5-alpha reductase activity present in the skin and liver. In a study, serum DHT is associated with incident CVD and stroke risks and all-cause mortality[16,110].

Contraindications of TRT

Both the ES and the EAU recommend against TRT in men with prostate or breast cancer or if there is a suspicion of prostatic malignancy in the form of a palpable prostate nodule or palpable induration or a prostate-specific antigen (PSA) level > 3 ng/mL, combined with other risk factors, severe LUTS as evidenced by International Prostate Symptom Score > 19. Other contraindications are listed in Table 2.

MONITORING OF PATIENTS RECEIVING TRT

Monitoring for efficacy

Those on TRT should be assessed for clinical response and adverse effects at the third and twelfth months after initiation and thereafter every year. Biochemical monitoring for efficacy should be done with measurement of serum T at different time intervals while on TRT (Table 2), targeting T levels in the mid-normal range, usually 280-873 ng/dL.

Monitoring for adverse effects

Before initiation of TRT in men > 40 years of age, the potential benefits, and risks along with the need for prostate cancer screening must be discussed, and the patient must be engaged in shared decision-making. This includes all men between 55-69 years of age with life expectancy > 10 years and those between 40-69 years at high risk for prostate cancer. Further details about monitoring of prostate-related adverse effects and haematocrit are outlined in Table 2.

ROLE OF OTHER THERAPEUTIC INTERVENTIONS FOR MOSH

SERMs and AI

AI and SERMs can be an alternative for TRT but have not yet been established as common clinical practice[111]. Due to the worsening of bone density without any improvement of body composition, AIs are not an ideal alternative to TRT in obese hypogonadal men[112].

hCG with TRT

The use of hCG in hypogonadal men is promising, given recent evidence that it stimulates recovery of spermatogenesis from TRT-induced azoospermia[113]. However, the main disadvantage is frequent injections with pain and discomfort. Further studies are needed to provide evidence of improved hypogonadism and erectile function in the setting of non-testosterone-based treatment in patients with MOSH.

CONCLUSION

Treatment of MOSH should involve approaches in an integrated fashion combining lifestyle modifications with TRT. Diet, along with a combination of aerobic and muscle-strengthening exercises, forms the cornerstone of obesity management. Given the effects of TRT on body composition, particularly an increase in muscle mass and strength, TRT facilitates the ability to exercise more. A combination of TRT along with appropriate lifestyle modifications is expected to result in better outcomes. A limited number of studies have, however, addressed this issue. Small RCTs have suggested that a combination of TRT and LSM can lead to improvements in glycemic control, insulin resistance indices, atherogenic lipid profile, blood pressure, body composition, fatty liver indices and even reversal of MetS. Randomised controlled trials comparing the effects of obesity management, TRT and other agents like SERMs on different aspects of MOSH are necessary to make recommendations regarding their pragmatic use. Patients who are on TRT should be regularly monitored as per the current international guidelines to ensure they receive the benefits of therapy and to detect potential complications on time. Prompt selection of the appropriate individuals and optimal management strategies for the underlying conditions including obesity should be planned in curtailing the problems posed by MOSH.

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FOOTNOTES

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MINIREVIEWS

Ovarian function in patients with systemic lupus erythematosus: Pathogenesis, drug application and prospective therapies

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Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease in which multiple organs are damaged that prevails in fertile women. Currently, glucocorticoids and immunosuppressants are widely used to treat SLE patients. However, ovarian dysfunction occurs following the use of these drugs in women with SLE. Here, we summarize recent progress in terms of understanding ovarian injury, the effects of drug application and strategies to improve ovarian function in women with SLE. This review could be helpful to precisely cure SLE in women desiring to have offspring.

Key Words: Systemic lupus erythematosus; Ovarian reserve; Ovarian insufficiency; Mesenchymal stem cells; Fertility; Autoimmune disease

Core Tip: Systemic lupus erythematosus (SLE) is an autoimmune disease that often occurs in women of childbearing age. Disorders of the immune system and clinical treatment drugs can affect the ovarian and reproductive functions of female patients. Herein, we summarize the research progress on SLE combined with ovarian dysfunction, hoping to provide a reference for the clinical treatment of patients with SLE for ovarian function and fertility needs.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoreactive activity of the immune system, the production of a variety of autoantibodies, abundant deposition of immune complexes, and damage to multiple tissues and organs. The ovaries, as female gonads, are the organs from which estrogen is secreted. Follicles mature and ovulate, playing an important role in endocrine and reproductive functions. SLE commonly occurs in women between 20 and 40 years of age. If immune complexes are deposited in ovaries, they can cause ovarian damage, manifesting as low ovarian reserve[1]. Currently, drugs such as tacrolimus, mycophenolate mofetil, cyclophosphamide, and prednisolone are the main treatment options for patients with SLE. However, some of these drugs, such as cyclophosphamide, have ovarian toxicity. They may cause less menstruation, amenorrhea, infertility, low ovarian reserve, premature ovarian failure and other clinical manifestations in women with SLE. Therefore, this review briefly summarizes the abnormal changes in ovarian function indicators, the ovarian damage caused by clinical medications, and the measures to improve ovarian function in female patients with SLE in recent years, hoping to provide insights into the ovarian reserve function of patients with SLE and the clinical treatment for patients with SLE with reproductive needs.

ABNORMAL OVARIAN FUNCTION IN PATIENTS WITH SLE

Ovarian reserve refers to the quantity and quality of oocytes in the ovary and is closely related to female fertility[2]. Clinically, the most commonly used indicators to evaluate ovarian reserve include the menstrual cycle, antral follicle count (AFC), ovarian volume (OV), follicle stimulating hormone (FSH), corpus luteum luteinizing hormone (LH) and anti-Müllerian hormone (AMH).

Menstrual cycle

A woman's menstrual cycle can reflect her ovarian function and reproductive capacity. The length of the menstrual cycle, regular or disordered cycle, the amount of menstrual flow, and amenorrhea can all reflect ovarian function and hormone secretion levels. Female patients with SLE have been suggested to be more likely to suffer from menstrual disorders and even persistent amenorrhea. In a recent study of 40 SLE patients, 45% of the patients (18 patients) were reported to have irregular menstruation, including 11 patients with oligomenorrhea, 1 patient with irregular menstruation, 4 patients with oligomenorrhea and irregular menstruation, and 1 patient with amenorrhea, and the rate of menstrual abnormalities was significantly greater in these patients than in healthy control individuals[3]. This is consistent with the studies carried out by Shabanova[4] and Girbash[5]. In addition, the systemic lupus erythematosus disease activity index (SLEDAI) score of SLE patients with irregular menstrual cycles was notably greater than that of SLE patients with regular menstrual cycles.

Ovarian morphology

The ovarian morphology can indicate its reserve function. The number of primordial follicles in the ovary represents a woman's ovarian reserve, and the AFC is generally used to quantify ovarian function[6]. A decrease in the number of AFCs often indicates that the responsiveness and reserve function of the ovary are reduced[6]. In addition, a smaller OV will cause lower sex hormone levels and ovarian responsiveness. Therefore, the OV is also an important indicator of ovarian reserve[7]. Girbash *et al*[5] compared 50 SLE patients with 50 healthy control individuals of similar age and found that the AFCs and OVs of SLE patients were markedly reduced, and both were related to age.

Sex hormones

Follicles are mainly composed of granulosa cells and eggs. Granulosa cells play a vital role in egg development, differentiation and maturation. Estradiol (E2) is secreted by follicular granulosa cells and can be used as an indicator of ovarian reserve. However, this indicator is susceptible to ovarian diseases and other indicators, and thus, it cannot be simply evaluated alone. Since the ovaries are regulated by the hypothalamic-pituitary-gonadal (HPG) axis, when the ovarian reserve is reduced, negative feedback occurs to promote FSH and LH secretion. Therefore, in clinical practice, FSH, FSH/LH, and E2 are often jointly tested as biochemical indicators for evaluating ovarian function[7]. If a patient's ovarian reserve decreases, her E2 levels decrease, and her FSH and FSH/LH values increase accordingly. This phenomenon

corresponds to the results after ovarian damage in SLE patients. In a prospective study, researchers measured FSH, E2, and LH on the third day of the menstrual cycle. They found that E2 levels were reduced in SLE patients, and FSH and LH levels were significantly greater than those in healthy control individuals[8].

Anti-Müllerian hormone

AMH is also secreted by follicular granulosa cells and remains relatively stable throughout the menstrual cycle. It is more sensitive and specific than other biochemical indicators and is therefore widely used as a direct indicator of ovarian reserve. Lawrenz *et al*[9] measured the AMH values of 33 premenopausal SLE patients and compared them with the AMH values of 33 age-matched healthy people. The authors found that the AMH levels in the SLE group were notably lower than those in the healthy group. Furthermore, they found that there was no correlation between the AMH value and the SLEDAI score. Conversely, in another case-control study of 40 SLE patients of childbearing age, the AMH level was significantly lower in patients with SLE than in healthy control individuals and was negatively correlated with SLEDAI[3]. Thus, the correlation between AMH levels and disease activity in SLE patients still needs further large-scale testing to draw an accurate conclusion.

EFFECTS OF SLE MEDICATION ON OVARIAN FUNCTION

Glucocorticoids

Glucocorticoids (GCs) are the preferred medication for treating SLE patients and can quickly suppress immune responses and reduce the damage of inflammatory reactions to the body, thereby rapidly alleviating the condition of SLE. However, GCs (especially high-dose GCs) can decrease levels of ovarian-related sex hormones and AMH secretion, decrease AFC and OV, and cause amenorrhea, and thus, GCs can impair ovarian function in patients with SLE. All these changes may be related to the inhibition of the HPG axis function by GC[10].

Whirledge *et al*[11] assumed that GCs affect ovarian function through the following three mechanisms. GCs influence ovarian function indirectly through altering the levels of circulating gonadotropins by acting on the hypothalamus and pituitary. Excessive GC levels inhibit gonadotropin-releasing hormone secretion, leading to hypogonadotropic hypogonadism. The second mechanism of glucocorticoid regulation is also indirect and affects the levels of metabolic hormones and growth factors, such as insulin-like growth factor-1. Finally, GCs regulate ovarian function by binding to GC receptors on ovarian cells, affecting LH activity and steroid biosynthesis. It can be concluded that GCs can extensively act on all links of the HPO axis to cause damage to ovarian function.

Cyclophosphamide

Cyclophosphamide (CTX), a commonly used immunosuppressant, unfortunately has strong gonadal toxicity and can lead to amenorrhea and infertility[12]. Boumpas *et al*[13] reported that 28% of SLE patients had persistent amenorrhea after CTX treatment, and 8% of the patients had temporary amenorrhea. SLE patients receiving high-dose CTX treatment were more likely to have persistent amenorrhea than were those receiving low-dose CTX treatment (39% *vs* 12%, respectively). In addition, amenorrhea occurs in 12% of patients younger than 25 years and in 62% of patients older than 31 years. The above studies have shown that the ovarian damage caused by CTX is closely related to the age of SLE patients at the start of CTX treatment and the cumulative dose over treatment time, which has been corroborated by the results of others[14,15]. Therefore, CTX should be used with caution in SLE patients of childbearing age who are willing to have children.

CTX can activate the PI3K-AKT-mTOR signaling pathway and increase the phosphorylation of AKT and other related proteins. Thus, it can directly affect the oocytes and granulosa cells of primordial follicles and accelerate the activation of primordial follicles[16]. Moreover, CTX-induced ovarian toxicity may be mediated by an inflammatory response, which can be caused by increasing the levels of proinflammatory cytokines [interleukin (IL)-6, IL-8, TNFα, *etc*.] and decreasing the levels of anti-inflammatory factors such as IL-10[17]. In addition, CTX changes the expression of pro- and antiapoptotic genes, leading to increased apoptosis in the ovary[18]. In conclusion, CTX induces oocyte damage and apoptosis in granulosa cells and follicles, resulting in premature depletion of follicular reserves.

In addition, other drugs used to treat SLE, such as hydroxychloroquine, tripterygium glycosides, mycophenolate mofetil and tacrolimus, are rarely reported to have effects on the ovary and need to be further studied.

MEASURES TO IMPROVE OVARIAN FUNCTION IN PATIENTS WITH SLE

The recurrent attacks of SLE can significantly exacerbate the damage to affected tissues and organs. In addition, the ovarian damage resulting from the long-term application of glucocorticoids and immunosuppressants cannot be ignored. For patients with SLE of childbearing age, it is essential to protect their ovarian function and maintain fertility, thereby necessitating the urgent development of a treatment strategy to improve ovarian damage.

The clinical application of CTX enhances the recruitment of follicles, eventually leading to ovarian dysfunction. Therefore, inhibiting ovulation reduces the ovarian toxicity of CTX during treatment. Gonadotropin releasing hormone (GnRH) is secreted from the hypothalamus to the pituitary portal circulation, where it stimulates the pituitary to secrete FSH and LH and maintain the normal menstrual cycle. GnRH analog (GnRH-a) is a synthetic agonist of GnRH that can competitively bind to the gonadotropin-releasing hormone receptor on the pituitary gland and inhibit the secretion of

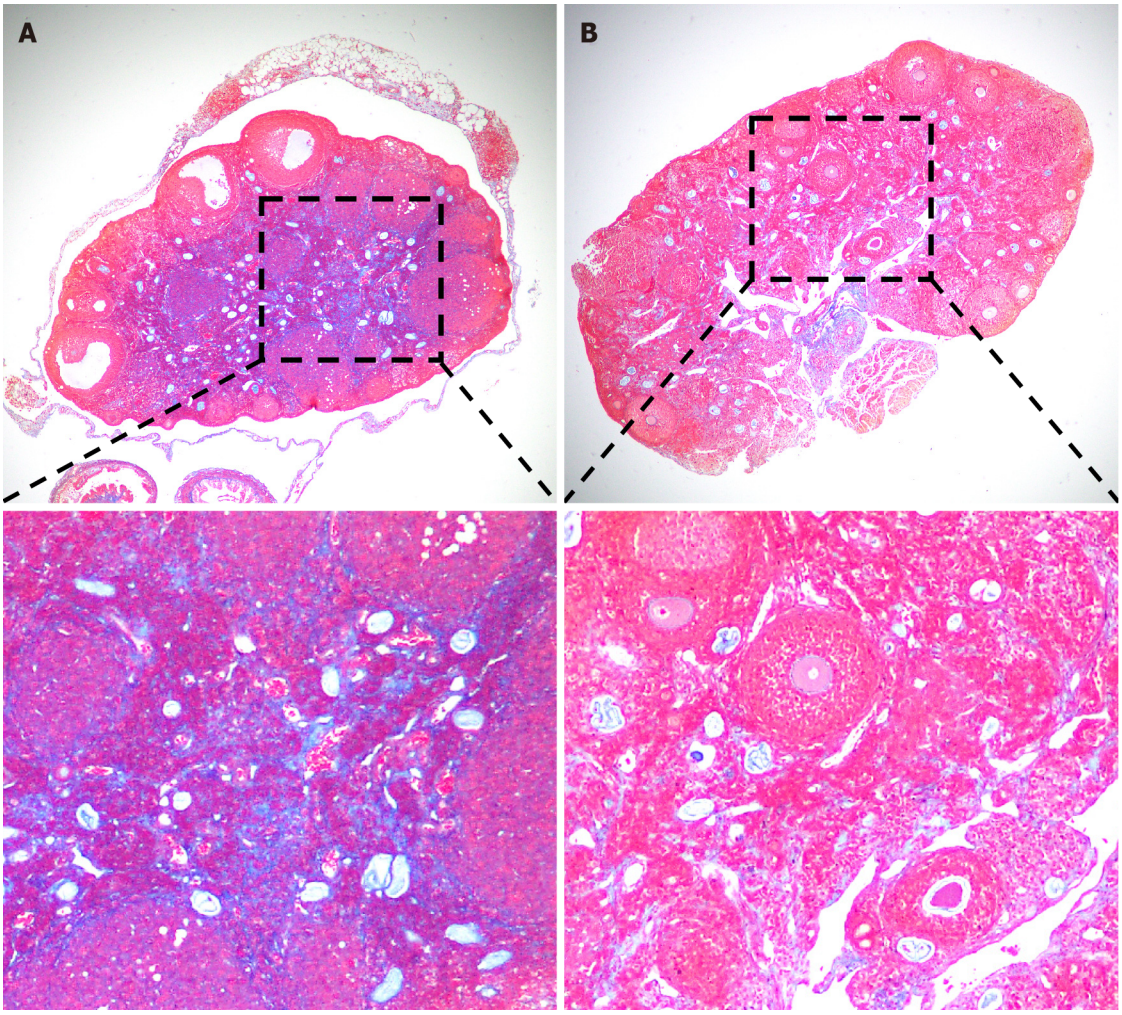

Figure 1 Umbilical cord mesenchymal stem cell (UC-MSC) transplantation reduced ovarian fibrosis in MRL/Lpr (MRL/Mpj-Faslpr/J, #000485, The Jackon Laboratories, United States) mice. A: Representative masson staining of ovaries from MRL/Lpr mice in the PBS group; B: Representative masson staining of ovaries from MRL/Lpr mice in the umbilical cord mesenchymal stem cell transplantation group. Magnification: 40 × (top) and 100 × (bottom).

FSH and LH, thus inhibiting ovulation[19]. In addition, clinical studies have reported that GnRH-a combined with CTX in the treatment of SLE reduces ovarian exposure to alkylation reagents, resulting in a reduction in ovarian damage[20,21].

Severe ovarian damage profoundly impacts the entire reproductive system and can even result in infertility in severe cases. Currently, the cryopreservation of embryos and oocytes is considered a promising approach for preserving fertility. However, the social, ethical, and technical challenges associated with this strategy cannot be neglected. Mesenchymal stem cells (MSCs) are multipotent adult stem cells with low immunogenicity, self-renewal to maintain their stem cell properties, and multidirectional differentiation ability[22]. MSCs can migrate into the damaged ovary to increase the production of bioactive factors[23,24], such as vascular endothelial growth factor, hepatocyte growth factor, insulin-like growth factor, and other growth factors that promote immunomodulation, anti-inflammation, angiogenesis, anti-apoptosis, and anti-fibrosis. In recent years, MSC therapy has gradually been applied to treat ovarian insufficiency[25-27], and its efficacy has been confirmed by clinical trials. Using a polycystic ovary syndrome (PCOS) mouse model, Chugh *et al*[27] confirmed that intraovarian injection of bone marrow mesenchymal cells significantly reduced steroid gene expression, thereby inhibiting the inflammatory response and restoring ovarian function. Transplantation of human umbilical cord MSCs (UC-MSCs) into patients with ovarian insufficiency can increase E2 levels and the number of follicles, improve follicular development, and result in successful clinical pregnancy[27]. Furthermore, our recent experiments demonstrated that UC-MSC transplantation improves ovarian function by inhibiting ovarian fibrosis in SLE model mice (Figure 1). Hence, MSCs are of great significance in the treatment of immune-related ovarian insufficiency, such as pregnancy complicated with SLE. Clinical data from trials carrying out MSC therapy to treat women with SLE are expected to validate this new approach in the future.

CONCLUSION

In summary, SLE damages ovarian and reproductive function in female patients of childbearing age. Disorders of the

immune system can lead to autoimmune oophoritis, which injures the ovaries and reduces the ovarian reserve. Additionally, when treated with glucocorticoids, cyclophosphamide, triptolide polyglycosides, *etc*., gonadal toxicity will occur, resulting in HPO axis disorders and even adverse pregnancy outcomes. To better address ovarian dysfunction in SLE patients, the key unanswered question is how proinflammatory niches cause ovarian damage and loss of function. Therefore, it is important to seek safe and effective treatments for SLE and ovarian insufficiency. MSCs have been widely used to treat SLE and have great potential for treating ovarian insufficiency-related diseases. They are expected to become an ideal and reliable treatment for SLE combined with ovarian insufficiency in the near future.

FOOTNOTES

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MINIREVIEWS

Innovation in pathogenesis and management of aortic aneurysm

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Abstract

Aortic aneurysm (AA) refers to the persistent dilatation of the aorta, exceeding three centimeters. Investigating the pathophysiology of this condition is important for its prevention and management, given its responsibility for more than 25000 deaths in the United States. AAs are classified based on their location or morphology. various pathophysiologic pathways including inflammation, the immune system and atherosclerosis have been implicated in its development. Inflammatory markers such as transforming growth factor β, interleukin-1β, tumor necrosis factor-α, matrix metalloproteinase-2 and many more may contribute to this phenomenon. Several genetic disorders such as Marfan syndrome, Ehler-Danlos syndrome and Loeys-Dietz syndrome have also been associated with this disease. Recent years has seen the investigation of novel management of AA, exploring the implication of different immune suppressors, the role of radiation in shrinkage and prevention, as well as minimally invasive and newly hypothesized surgical methods. In this narrative review, we aim to present the new contributing factors involved in pathophysiology of AA. We also highlighted the novel management methods that have demonstrated promising benefits in clinical outcomes of the AA.

Key Words: Aortic aneurysm; Abdominal aneurysm; Thoracic aneurysm; Immunotherapy; Surgical management; Pathophysiology; Inflammation and molecular pathways

Core Tip: This manuscript explores diverse pathophysiologic pathways (inflammation, atherosclerosis and immune system), varied treatment methods (pharmacological, radiation and surgical), and associated factors like inflammatory markers [transforming growth factor-β, interleukin (IL)-1β, tumor necrosis factor-, matrix metalloproteinase-2, IL-6, IL-8]. Genetic disorders linked to aortic aneurysms (AA) include Marfan syndrome, Ehler-Danlos syndrome, Loeys-Dietz syndrome, Cantu syndrome, and JAK-2 mutation. Approaches such as Low laser irradiation, photobiomodulation, UV-B irradiation may impact AA prevention and shrinkage. Medications like Canakinumab, Paricalcitol, peroxisome proliferator-activated receptor-γ agonist and mesenchymal stem cell transplantation are currently under investigation. Additionally, Different minimally invasive, endovascular surgical methods are highlighted.

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INTRODUCTION

A persistent dilatation of the aorta with a diameter of three centimeters or more is termed as an aortic aneurysm (AA)[1]. In a given year, AA is responsible for more than 25000 deaths in the United States[2]. According to reports, the prevalence of AAA ranges from 2% to 12% and affects 8% of males over the age of 65[3]. Aneurysms often remain asymptomatic until a rupture, which is frequently fatal with a mortality rate between 85% and 90%. For those seeking medical treatment post-rupture, survival rates vary between 50% and 70%. Hence, the principal aim in the management of aneurysms is the timely identification and implementation of interventions to mitigate the risk of rupture[4]. In contrast to the observed occurrences in thoracic aneurysms, abdominal variations of these aneurysms are substantially more common. Aortic diseases continue to have a significant global impact despite notable improvements in both diagnostic techniques and treatments[5]. The United States Preventive Services Task Force's most recent guidelines state that male smokers between the ages of 65 and 75 ought to undergo a single ultrasonography examination[6]. Aortic aneurysms can be categorized according to their location distinguishing between thoracic aortic aneurysm (TAA), abdominal aortic aneurysms (AAA) or a combination of both. Morphologically, aneurysms may present as saccular, fusiform, or pseudoaneurysm. Etiological classifications encompass those associated with atherosclerosis, inflammation, genetic disorders, trauma, infection, and autoimmune conditions[7]. This review aims to explore various aspects of the literature on aortic aneurysms. It includes a detailed examination of the underlying pathology, the molecular pathways, trials related to immunotherapeutic interventions, the role of radiation therapy, and surgical methods within the field of aortic aneurysm. This review is driven by two main goals: firstly, to comprehensively understand and analyze the common causes and pathophysiology of aortic aneurysms, and secondly, to offer insights into the latest treatment modalities. This endeavor aims to inform and guide future research practices in the field.

DIFFERENCE BETWEEN ABDOMINAL *VS* THORACIC AORTA

AA encompasses two main types, TAA and AAA, with distinct risk factors and pathophysiological mechanisms. Notably, the origin of vascular smooth muscle cells (VSMCs) in TAA is from the neural crest[8]. whereas in AAA, they originate from the endothelium and mesoderm, emphasizing the fundamental difference in their pathogenesis[9]. The diameters of the aorta, in both the thoracic and abdominal regions, were significantly impacted by age, gender, body surface area, and modifiable risk factors such as diastolic blood pressure and cigarette smoking. These findings highlight the multifactorial nature of aortic aneurysm development, demonstrating the combined influence of both intrinsic and modifiable factors [10]. TAA typically exhibit a more robust genetic basis, often linked to autosomal gene mutations in conditions like Marfan and Loeys-Dietz syndrome. In contrast, AAA is primarily associated with atherosclerosis, indicating a divergence in the underlying pathophysiological mechanisms between the two types of aneurysms[11]. AAA tends to expand more rapidly (0.3-0.45 cm annually) compared to TAA, which expands slower (up to 0.3 cm annually in non-bicuspid aortic valve patients). The pathogenesis of AAA is associated with elevated levels of matrix metallopeptidases, contributing to degradation of the extracellular matrix. Meanwhile, an overactive transforming growth factor-beta is a major factor in the development of TAA[12]. While TAA and AAA exhibit distinct genetic backgrounds and etiologies, they share several common pathological characteristics. Both types display a pathologically dilated aortic phenotype, often characterized by the loss of smooth muscle cells, an inflammatory response within the arterial wall, and alterations in the extracellular matrix composition[2]. This study identified CX3CR1 and HBB as shared biomarkers for TAA and AAA while highlighting the significant infiltration of innate and adaptive immune cells in aortic aneurysm development and progression. The close correlation of CX3CR1 and HBB with immune cell infiltration suggests their potential as targets for immunotherapeutic interventions[13]. AAAs exhibited downregulation of the blood coagulation pathway and upregulation of the integrin signaling pathway. In contrast, TAAs showed the opposite trends, suggesting these differences contribute to the distinct pathogenesis of AAAs and TAAs[14]. Findings revealed that 22.5% of TAA patients also had AAAs, with notable associations between AAAs and age ≥ 65, smoking history, hypertension, and specific TAA locations,

suggesting the importance of AAA screening in TAA patients, especially those over 55 years old with systemic hypertension, a smoking history, or TAAs in the descending thoracic aorta[15]. This study induced TAA and AAA in mice by combining hypertension and elastic lamina degeneration. Hypertension was a crucial factor in aneurysm formation. At the same time, the direct effects of angiotensin II on the vascular wall were not the primary cause, indicating distinct pathophysiological mechanisms for AAA and TAA[16].

PATHOPHYSIOLOGY; ROLE OF ATHEROSCLEROSIS AND INFLAMMATION

The relationship between atherosclerosis and AA)has been debated, given their shared risk factors and the belief that atherosclerotic lesions may contribute to some aneurysms. Recent studies, however, suggest that while they share some similarities, there are distinct local responses to atherosclerosis in humans, with plaque deposition potentially leading to aneurysm formation in one scenario and lumen narrowing in another, indicating that the vascular response to atherosclerotic lesions can vary widely[17]. Despite these differences, AAA and atherosclerosis share many similar biomarkers, such as fibrinogen, C-reactive protein, and low high-density lipoprotein (HDL) levels[18]. Additionally, a locus on chromosome 9p21 is associated with both conditions, suggesting shared genetic risk factors[19]. These findings are further supported by the observation that some mice (*e.g.*, Apolipoprotein E deficient) prone to atherosclerosis are also more susceptible to AAA induction[20]. AAA and atherosclerosis are distinct diseases despite their shared risk factors and pathophysiological aspects. However interventions that protect against AAA also frequently reduce atherosclerosis, suggesting that these two conditions are interconnected[21]. Inflammatory processes are evident in both conditions, with inflammatory cells in advanced AAAs and inflammatory pathways implicated in atherosclerosis progression. Matrix degradation occurs in the arterial wall of both AAA and atherosclerosis, contributing to aneurysm expansion and plaque formation. Thrombosis is a critical factor, leading to complications in AAAs and plaque rupture in atherosclerosis. Hemodynamic forces like shear stress drive arterial remodeling in both conditions. Signaling molecules influence inflammation, matrix remodeling, and vascular cell behavior in both AAA and atherosclerosis. Both conditions are associated with coronary heart disease, suggesting shared risk factors or mechanisms. While the precise relationship between AAA and atherosclerosis is intricate and influenced by common factors, their interplay underscores the need for comprehensive understanding to develop effective therapies[22]. The involvement of dysregulations in key angiogenesis and inflammation-related factors is potentially identified in AAA formation. Expression levels of ANGPT1, CXCL8, PDGFA, TGFB1, VEGFB, and VEGFC and plasma levels of transforming growth factor (TGF)-alpha, TGF-β1, VEGF-A, and VEGF-C were found to be significantly altered in the AAA group compared to the control subjects without AAA. These findings have implications for identifying diagnostic biomarkers and therapeutic targets. Elevated production of pro-inflammatory cytokines hastens atherosclerosis. Similarly, in AAA, inflammatory cytokines are expressed in affected tissues of symptomatic patients and mouse models. However, direct proof of cytokine involvement in AAA through knockout animal studies is lacking and needs further investigation. Comparisons of atherosclerosis and AAA advancement in available animal models and human studies propose that certain cytokines, like interferon (IFN)-γ, have conflicting roles in these diseases, suggesting differing origins and mechanisms between atherosclerosis and AAA. Conversely, other cytokines yield analogous effects in both diseases, as seen with interleukin (IL)-1β (encouraging disease) and TGF-β (mitigating disease). This underscores the intricate interplay of cytokines in these vascular conditions[23]. Most macrophages found in AAAs come from circulating monocytes, but some are aortic tissue-resident macrophages. Throughout AAA development and progression, macrophages have destructive and reparative roles in extracellular matrix remodeling and promotion and resolution of inflammation, depending on the microenvironmental conditions and the chemokines and cytokines present[24]. The presence of TNF-alpha and IL-1β in tissue extracts of AAA is indicative of that infiltrative inflammatory process[25]. IL-1β plays a central role in the inflammatory process of aneurysm formation *via* cascading of signaling pathways and various mechanisms of action[26]. Endothelin-1 (ET-1) contributes to atherosclerosis and AAA development by reducing protective HDL levels, increasing oxidative stress, and prompting inflammation through immune cell infiltration. ET-1 enhances matrix metalloproteinase-2 (MMP-2) activity, leading to structural protein degradation in perivascular fat, vascular walls, and atherosclerotic lesions. This weakening of tissues fosters aneurysm formation and plaque instability. Overall, ET-1's involvement encompasses diminished HDL, heightened oxidative stress, inflammation, and MMP-2 activation, significantly impacting atherosclerosis and AAA progression[27]. The NLRP3 inflammasome, a cytosolic multiprotein complex, serves as a pivotal regulator in the activation of caspase-1, orchestrating the controlled release of pro-inflammatory cytokines IL-1β and IL-18. Additionally, it plays a role in instigating apoptosis, thereby contributing to the inflammatory processes observed in both atherosclerotic disease and AA[28]. Metabolomics identifies succinate as a biomarker and therapeutic target in aortic aneurysm and dissection[29].

The general inflammatory conditions contributing to AAA formation also involve activating the following pro-inflammatory transcription factors, NF-κB, AP-1 and markedly increased expression of IL-6 and IL-8 and the involvement of neutrophils[30]. Inflammatory cytokines promote the degeneration of the vascular wall by inducing increased MMP-9 expression in macrophages, which degrade the components of the extracellular matrix (ECM) of the arterial wall, contributing to the expansion process[31]. This process also involves an immune response, notably CD4+ T cells, which aggregate within the AAA lesion and secrete Th1 cytokines like IL-4, which continue to stimulate MMP secretion by macrophages[32]. The release of neutrophil extracellular traps by activated neutrophils in the presence of IL-1β is also believed to play a central role in AAA formation, as their suppression in mice models stopped aneurysm formation[33]. AAA formation results from inflammatory injury that leads to an imbalance in proteolysis. Experimental models that try to stop this vicious cycle have shown promise, but preclinical studies have also shown worsened disease progression,

This raises doubts on the accuracy of mice models to portray human disease[34]. The ECM is a dynamic structural component of the aortic wall that plays a crucial role in AAA formation, notably through modification by MMPs. Various MMPs have different ECM collagen substrates. A set of processes involving individual risk factors, molecular mechanisms, and triggers leads to degradation of the ECM in the tunica intima and media, all of which contribute to inflammatory infiltration[35]. Loss of vascular smooth muscle cells (vSMCs), predominant in the middle aortic wall layer, causes weakening and contributes to AAA formation and progression. This condition occurs due to inflammatory cell infiltration that induces apoptosis, mechanical wall stress, and detachment of the ECM[36].

IMMUNE SYSTEM IMPLICATIONS

The development and progression of AAAs heavily rely on the immune system's responses, involving both cell-mediated and antibody-based reactions[37]. Numerous studies have demonstrated a robust association between immune factors and AAA. IgG antibodies extracted from the AAA wall exhibited significantly elevated levels compared to normal aortas, with IgG1, IgG2, IgG3 and IgG4 showing marked increases of 193, 160, 389, and 627 times, respectively. Moreover, IgG antibodies targeting HDL were more prevalent in both tissue and plasma of AAA patients, showing a correlation between aortic size and AAA presence. This suggests a potential contribution of these autoantibodies to AAA formation [38]. Research indicated a substantial increase in IL-17 and IL-23 cytokine production in AAA patients compared to controls. These cytokines, associated with inflammatory autoimmune disease, were proposed as significant players in AAA pathophysiology[39]. Studies linking bioactive peptides and AAA revealed higher concentrations of IL-1B, IL-6 and IL-17 in individuals with AAA, with an observed association between bioactive peptides and aortic diameter. IL-1α levels are typically detectable in healthy individuals, primarily concentrated in the cell membrane and nucleus. However, in AAA patients, IL-1α was detected in serum, correlating with affected vessel diameter in vitro. This indicated a potential role of IL-1a in AAA enlargement *via* neutrophil mobilization. Conversely, other research found no statistically significant association between serum IL-1a levels and AAA size or growth rate[40-42]. A comprehensive 2018 investigation delved into genome-wide expression patterns to understand perivascular adipose tissue (PVAT) involvement and immunological aspects in AAA. Comparison between dilated and non-dilated areas of the aortic areas revealed an overrepresentation of innate and peripheral immune tolerance-breaking factors, leading to clonal T cell proliferation in dilated PVAT compared to non-dilated PVAT. These findings suggest an immunological aspect in AAA, potentially involving an autoimmune component, especially triggers like a high-fat diet or smoking[43].

MOLECULAR PATHWAYS

The onset of AAA stems from vascular inflammation, marked by infiltration of inflammatory cells from adventitia into the intima, gradually upregulating MMPs. MMPs, a group of extracellular enzymes, play a pivotal role in various physiological processes, including tissue remodeling and resorption. Elevated MMP9 Levels, responsible for serving elastin, collagen Type 1 and IV, and fibrinogen, have been observed in both plasma and aortic aneurysmal tissue. Persistent elevation of MMP9 Levels post-endovascular repair might indicate an increased risk of developing leaks[44, 45]. Studies in humans and mice highlight high levels of cytokines released by Th2 cells in aneurysm tissues, contributing significantly to AAA formation. The absence of IFN-γ intensifies AAA development in mice, while IL-4 deficiency prevents AAA development. In contrast, in human fibroblasts, IL-4 influences ECM protein production differently. Two major groups of macrophages, M1 (proinflammatory) and M2 (repair of ECM) play significant role in AAA formation. GM-CSF, a crucial cytokine moderating macrophage infiltration, regulates MMP9 secretion, reducing it when the GM-CSF pathway is blocked[35]. Dysregulated TGF-Beta signaling and mast cells contribute to AAA formation and inflammatory processes within the adventitia[46,47]. Distinct immunological characteristics exist between small AAAs (< 55 mm) and large (> 55 mm) AAAs. Notably, the upregulation of the key T-cell regulatory gene, cytotoxic T-lymphocyte-associated protein 4 (CTLA4 or CD152) is exclusive to small AAAs. Biomarker identification remains crucial for understanding pathogenesis and developing targeted therapeutics. G0S2 has been identified as a highly accurate biomarker for early AAA diagnosis[48,49]. Macrophage ADAR1 has been implicated in aneurysm formation through Drosha protein degradation, eliciting macrophage-mediated vascular inflammation in AAA by inhibiting microRNAs targeting NF-kB signaling[50]. Osteoprotegerin (OPG), found in elevated concentrations in AAA, holds promise as a diagnostic marker and potential treatment target. Inactivation of OPG in mice induced AAA development, suggesting its crucial role[51].

GENETIC DISORDERS

TAA correlates with syndromes linked to connective tissue defects. Mutations in molecular pathways, such as activation of TGF-, notch ligands, and angiotensin II, contribute to AA[52]. Genetic syndromes associated with aortic aneurysm include Marfan syndrome, Ehler-Danlos syndrome, Loeys-Dietz syndrome, familial thoracic aortic aneurysm and dissection, bicuspid aortic valve disease, autosomal dominant polycystic kidney disease and Turner syndrome[53]. In Marfan syndrome, mutation in Fibrillin-1 disrupts TGF-B, leading to tissue integrity impairment. Prophylactic beta blockers are administered to slow aortic aneurysm progression[54]. Loeys-Dietz-syndrome exhibits a triad of hyperte-

lorism, bifid uvula, AA with tortuosity. Genetic causes involve pathways enhancing TGF-β activity, including TGFBR1, TGFBR2, TGFB2, TGFB3, SMAD2, SMAD3[55]. Meester-Loer-syndrome represents an x-linked form of TAA with multiorgan involvement including craniofacial malformation, cardiovascular, neurological and cutaneous system issues. It is caused by BGN loss of function, encoding small leucine-rich proteoglycan biglycan which increases TGF-B pathway activity[56]. Autosomal recessive cutis laxa type 1B, involving aortopathy, vascular tortuosity and aneurysm formation, results from a mutation in EFEMP2, that encodes fibrillin-4[57]. Cantu syndrome, an autosomal dominant overgrowth syndrome, manifests congenital hypertrichosis, facial dysmorphism, cardiomegaly, and AA. This syndrome is caused by the *ABCC2* gene, a regulatory subunit for ATP-sensitive potassium channels in the cardiac, vascular smooth muscle and skeletal system[58]. The presence of JAK-2 mutation in bone marrow-transplanted mice has shown an increased incidence of AA by accelerating the degradation of aortic elastic lamina and activation of matrix metalloproteinase[59]. Elevated homocysteine levels have been associated with aortic aneurysm progression, though it remains a weak risk factor given the multifactorial causes of aortic aneurysm[60]. A brief summary of pathophysiologic etiologies involved in AA is illustrated in Figure 1.

TREATMENTS; IMMUNOLOGICAL IMPLICATIONS

Numerous therapies targeting the immune system have been developed in recent years. In a study spanning 12 months, administration of Canakinumab, a monoclonal antibody neutralizing IL-1B, showed similar progression to AAA compared to the placebo group[61]. Another approach involving selective suppression of inflammation in the aneurysm wall used perioperative treatment of paricalcitol, a specific vitamin D receptor agonist, and demonstrated selective disruption of nuclear factor of activated T cells-2-mediated inflammation[62]. Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist is recognized for suppressing atherosclerosis progression. Perioperative administration of the PPAR-γ agonist, pioglitazone, significantly reduced macrophage infiltration and adiponectin expression in retroperitoneal periaortic fat and aortic wall. This suggests a potential avenue for preventing or delaying aortic aneurysm progression [63]. However, in the FAME-2 trial, administration of fenofibrate did not impact AAA growth rate or inflammatory markers such as osteopontin and kallistatin[64]. Tyrosine kinase inhibitor, Imatinib, may inhibit AAA formation and growth by preventing pathological vascular formation[65]. A Mendelian randomization (MR) analysis was aimed to identify therapeutic targets associated with AAA. Four drug targets (BTN3A1, FASN, PLAU, and PSMA4) exhibited significant MR results across two independent datasets. Proteasome 20S subunit alpha 4 (PSMA4) and plasminogen activator, urokinase (PLAU), showed compelling evidence of colocalization with aortic aneurysms, particularly in AAAs. The study suggests that targeting PLAU and inhibiting PSMA4 through drug interventions may potentially lower the risk of developing AAs[66]. Nanotherapy could be an ideal method of delivering medications to target cells. A Rapamycin-loaded nanotherapy, involving macrophage cell membrane implication by creating reactive oxygen species to AAA cells, demonstrated efficacy in preventing aneurysm expansion[67]. Despite numerous promising interventions identified in preclinical studies, this substantial investment has not yielded any clinical applications, and currently, there is no medical therapy available for stabilizing growing AAAs. Nevertheless, ongoing clinical studies like IMAGEN trial to evaluate effect of myo-inositol and ARREST trial to investigate the effect of mesenchymal stem cells, are underway to explore potential treatments[68,69].

TREATMENTS; IMPLICATION OF RADIATION

Certainly, investigating the potential positive effects of radiation on regressing AA is an intriguing avenue. The use of Radiation is commonly employed during the endovascular intervention for aortic repair, impacting both patients and medical staff[70]. However, exploring whether radiation might exhibit positive effects on AA regression warrants careful investigation. In an in vivo study involving simulated aortic dilatation in apolipoprotein-E deficient mice, low level laser irradiation exhibited a noteworthy outcome. It showed a significant reduction in the maximum diameter of suprarenal aorta. Moreover, it effectively prevented further dilation, restraining it to less than 50% of cross sectional diameter of aorta compared to the non-treated control group[71]. A recently hypothesized method involves photobiomodulation of the abdominal aorta. This approach entails delivering treatment through percutaneous insertion of LED-studded patch onto the posterior abdominal wall. It is intended for small aneurysms (3-4.5 cm in anteroposterior diameters) that have proven refractory to pharmacological intervention. Both *in vivo* and *in vitro* studies have indicated that this method can effectively prevent progression to urgent surgical intervention or impending rupture[72]. In an interventional study on angiotensin II infused hypercholesterolemic mice, it was discovered that irradiation using Ultraviolet B (UVB) had the potential to prevent the progression and lower the mortality rate associated with aortic aneurysm. The effect was attributed to a significant reduction in CD4 T cells and macrophage, along with a systemic expansion of CD4+Foxp3+ regulatory T-cells, effectively restricting the growth of AA[73]. Additionally, experiments employing irradiation in combination with bone marrow transplantation demonstrated a reduction in the inflammatory process linked to atherosclerosis and the development of aortic aneurysm in angiotensin II infused mouse models[74].

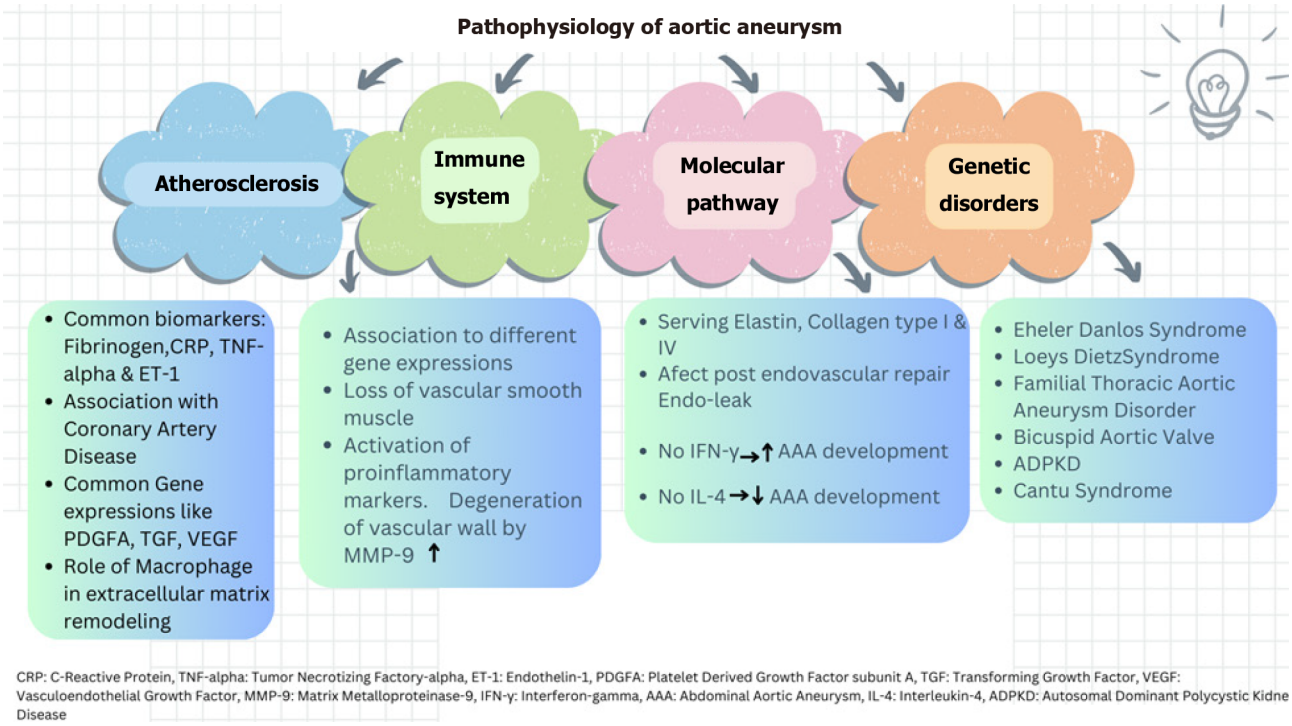

Figure 1 Pathophysiologic etiologies of aortic aneurysm.

TREATMENTS; SURGICAL IMPLICATIONS

The surgical approach to aortic aneurysms has a dynamic landscape of treatment strategies, with exploration of open thoracoabdominal repair, endovascular techniques, and the new innovative integration of 3D printing technology. The surgical process involved in open thoracoabdominal repair, characterized by midline transabdominal or retroperitoneal incisions, aortic, and iliac artery clamping, is considered one of the most invasive procedures. But contrary to belief, an open approach is not inferior and is effective, and durable in terms of the graft integrity with preservation of the renal function, even in patients with increased cardiovascular risk[75-77]. An approach to prevent complications during the surgery using a left heart bypass (LHB) which is a circulatory support system used to perfuse the distal aorta during TAAA operation. The advantages of LHB ensure distal perfusion, decreasing the use of heparin, and mitigating the risk of bleeding and postoperative neurological deficits, yielding favorable outcomes[78]. Some of the main side effects of surgical aortic aneurysmal repair include hemorrhage, acute renal failure, ischemic colitis, distal emboli, graft thrombosis, infection, pseudoaneurysm formation, aorto-cava and aorto-enteric fistula, neurologic deficit, ureteral obstruction, sexual dysfunction, chylous ascited and prigraft seroma[79]. The endovascular aneurysm repair (EVAR) has revolutionized the management of AAA. However, later studies showed lower long term benefit compared to open surgery, yet it remains the most commonly utilized approach. It is the go-to option for patients unfit for an open approach, offering a survival benefit and proving to be cost-effective[80]. The primary reason for EVAR's reduced survival benefit is attributed to secondary aneurysm ruptures caused by endo-leaks and a high rate of secondary intervention. Advancements aim to mitigate these complications by limiting the leaks and focusing on shrinkage of aneurysms. One approach involves pre-emptive embolization, such as embolization of aneurysm sac side branches (ASSB) and using aneurysm sac coil embolization (ASCE), which has shown promise in decreasing the development of endovascular leaks[81]. A comprehensive meta-analysis by Wu *et al*[82] showcased enhanced clinical outcomes in EVAR clinical through implementation of prophylactic pre-emptive embolization targeting the inferior mesenteric artery (IMA-ASSB). This approach notably curbed aneurysm sac expansion and minimized the requirement for re-intervention. Additionally, it was noted that non-selective embolization of ASSB (NS-ASSB) proved more effective in decreasing the incidence of leaks[82]. Doumenc *et al* [83] examined novel surgical approaches for resolving Type IA endoscopic leaks during EVAR, with Custom-made fenestrated endovascular aortic aneurysm repair and open explanation. Many physician modified endografts including fenestrated-branch EVAR for complex AAAs showed acceptable long term outcomes however, demonstrated low survival rates due to underlying comorbidities[84]. Thoracoabdominal branch endoprosthesis (TAMBE) device is also used to offer endovascular treatment for all types of TAAAs and pararenal aneurysms. TAMBE is a four-branch device that can be used for a range of visceral anatomic configurations and showed promising results with more ongoing clinical trials[85]. There is another similar graft called the Valiant Navion stent graft showing promising results with rare complications such as endo-leak and access/deployment failures[86]. Advancement in making 3D models of visceral artery aneurysms with more accurate visualization and analysis of vascular anatomy could assist operators in attempting minimally invasive treatment with good results. The imaging studies using 3D printing models that allow for the assessment of the position, morphology and geometry of the aneurysm sac, particularly of vessel branches, could encourage surgeons to perform endovascular procedures[87]. 3D printing technology has been collaborating utilizing

artificial intelligence to update the map throughout the patient journey, and has been attempted in one center so far with promising results[88].

Studies showed EVAR has better long term outcomes on shrinking AAAs rather than stable AAAs. Factors such as older age and larger infra-renal angle were found to be associated with this finding. However, underlying factors which are contributing to this shrinkage, are still under investigation[89]. Additionally, Studies have shown that shrinkage of > 10 mm are less prone to developing subsequent aneurysm growth and have significantly lower risk of requiring surgery for endo-leaks[90]. On the other hand, smaller population studies didn't show any significant association between aneurysm shrinkage ≥ 5 mm and survival or reintervention rate within one year[91]. Inflammatory markers such as neutrophil to lymphocyte ratio and platelet to lymphocyte ratio emerged as negative predictors of post-EVAR shrinkage of AAAs[92]. Some of the major side effects of endovascular repair are Endo-leaks, stent migration, endograft infection, limb kinking or occlusion, endograft collapse, and systematic complications like ischemia of major branches[93]. There are some promising studies focused on minimally invasive approaches, to be non-inferior compared to gold standard approaches. However, more trials are required to determine the benefit of both the device and technology of less-invasive EVAR[94].

CONCLUSION

The development of aortic aneurysms, regardless of their location in the thorax or abdomen, has been shown to be associated with inflammatory process, atherosclerosis, immune system dysregulation, and genetic disorders. The potential implications of immune-suppressive therapies, such as canakinumab and pioglitazone, as well as radiation therapies such as photbiomodulation, may play a role in both prevention and regression of aortic aneurysms. Several newer surgical methods, including open thoracoabdominal aortic repair and endovascular aortic repair techniques have demonstrated promising results in recent years. However, these techniques may carry certain risks such as endo-leaks and endograft infection or collapse. Nevertheless, the overall benefits compare to previous methods highlights the innovated approach to the prevention and treatment of aortic aneurysm.

FOOTNOTES

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MINIREVIEWS

Sodium-glucose cotransporter-2 inhibitors protect tissues *via* cellular and mitochondrial pathways: Experimental and clinical evidence

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Abstract

Mitochondrial dysfunction is a key driver of cardiovascular disease (CVD) in metabolic syndrome and diabetes. This dysfunction promotes the production of reactive oxygen species (ROS), which cause oxidative stress and inflammation. Angiotensin II, the main mediator of the renin-angiotensin-aldosterone system, also contributes to CVD by promoting ROS production. Reduced activity of sirtuins (SIRTs), a family of proteins that regulate cellular metabolism, also worsens oxidative stress. Reduction of energy production by mitochondria is a common feature of all metabolic disorders. High SIRT levels and 5′ adenosine monophosphate-activated protein kinase signaling stimulate hypoxia-inducible factor 1 beta, which promotes ketosis. Ketosis, in turn, increases autophagy and mitophagy, processes that clear cells of debris and protect against damage. Sodium-glucose cotransporter-2 inhibitors (SGLT2i), a class of drugs used to treat type 2 diabetes, have a beneficial effect on these mechanisms. Randomized clinical trials have shown that SGLT2i improves cardiac function and reduces the rate of cardiovascular and renal events. SGLT2i also increase mitochondrial efficiency, reduce oxidative stress and inflammation, and strengthen tissues. These findings suggest that SGLT2i hold great potential for the treatment of CVD. Furthermore, they are proposed as anti-aging drugs; however, rigorous research is needed to

validate these preliminary findings.

Key Words: Sodium-glucose cotransporter-2 inhibitors; Cardiovascular diseases; Sirtuins; Oxidative stress; Inflammation; Mitochondrial dysfunction

Core Tip: Sodium-glucose cotransporter-2 inhibitors, diabetes drugs, unlock tissue protection *via* diverse pathways. They boost mitochondrial efficiency, curb oxidative stress and inflammation, and enhance autophagy. Clinical trials show cardiovascular benefits, suggesting immense potential beyond diabetes and even towards anti-aging therapy.

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INTRODUCTION

Despite ongoing research and preventive efforts, cardiovascular disease (CVD) remains the leading cause of global mortality, a sobering statistic highlighted by the American Heart Association in its 2021 update[1]. In individuals with metabolic syndrome and diabetes, this grim reality is heavily influenced by mitochondrial dysfunction, which acts as a crucial driver of disease progression and persistence, ultimately contributing to adverse cardiac remodeling and events. The renin-angiotensin system plays another pivotal role, with elevated angiotensin II (Ang II) activity fueling oxidative stress and localized inflammatory responses. Furthermore, a decline in sirtuins (SIRTs) appears to be a key player in this complex interplay. These proteins exert intricate control over cellular responses to environmental cues, impacting oxidative stress levels and interacting with Ang II in various pathways linked to fibrosis, apoptosis, inflammation, and cardiac and vascular remodeling[2-6]. Recent research has revealed a fascinating interplay between sodium-glucose cotransporter-2 inhibitors (SLGT2i) and the sirtuin-renin-angiotensin-aldosterone system (RAAS) crosstalk, which has profound implications for mitochondrial function.

EXPERIMENTAL EVIDENCE: CELLULAR AND MITOCHONDRIAL PROTECTION

Beyond their established role as deacetylases[7], SIRTs wield another hidden power: modulating oxidative stress through intricate regulation of cellular adaptive responses[8]. This means that SIRTs not only remove acetyl groups from proteins but also adjust how cells respond to oxidative stress, akin to a team of tiny biochemists fine-tuning the cellular environment. Specifically, Ang II, a key player in the RAAS, acts as a kind of conductor, stimulating SIRTs *via* their type 1 receptor and the production of reactive oxygen species (ROS)[9]. Think of it as Ang II turning up the volume on SIRT activity, leading to increased ROS production. However, it is not one-sided. Ang II also throws a wrench in the works, down-regulating SIRT1 and 2 in the heart, like a dimmer switch lowering their brightness[10]. In contrast, SIRT3 adopts a different approach, inducing forkhead box O3 (FOXO3) to move into the cell nucleus, which ultimately leads to reduced catalase levels and increased ROS[10]. Imagine SIRT3 as a mischievous elf sneaking FOXO3 into the nucleus, causing some oxidative stress mischief.

Diving deeper into the mitochondrial realm, we find SIRT3, 4, and 5 holding court. Among them, SIRT3 reigns supreme, partnering with cyclophilin D to unlock the powerhouses' hidden doors (the transition pores), influencing how freely things flow and ultimately impacting cell function. However, lurking in the shadows, Ang II orchestrates a nefarious play, wielding specific tools to adorn FOXO3a with an acetyl group[11-13]. This glamorous attire, sadly, mutes the antioxidant heroes like superoxide dismutase and catalase, allowing ROS to run rampant and culminate in a magnified heart (cardiac hypertrophy). Yet, SIRT3 stands as a beacon of hope, meticulously fine-tuning complex I, the conductor of the mitochondrial orchestra, ensuring smooth energy production[14]. This highlights the intricate waltz of SIRTs, each playing a unique melody within the symphony of cellular health. However, the plot thickens with SIRT4, a rogue in the story of cardiac remodeling. Unlike its benevolent brethren, it throws its lot in with Ang II, unleashing a torrent of destructive effects[15]. This alliance silences manganese-dependent superoxide dismutase, the valiant shield against oxidative stress, leading to a cascade of damage and, ultimately, a hypertrophied heart.

Concerning vascular reactivity, potassium and calcium channels have long been recognized as playing central roles. However, it has recently been reported that dapagliflozin promotes vasodilation by activating the protein kinase G pathway without altering the activity or expression of calcium or potassium channels[16]. Conversely, in adipose tissue, SGLT2i exert multiple actions that promote a healthy phenotype with reduced secretion of inflammatory adipokines such as leptin and increased secretion of adiponectin[17]. Furthermore, both adiponectin and the induction of macrophage

polarization toward the macrophage 2 phenotype lead to adipocyte browning and increased brown adipose tissue activity, which promotes greater utilization of lipid substrates in a context in which inflammatory and lipotoxic effects are attenuated[17,18]. In addition, SGLT2i activate the adenosine monophosphate-activated protein kinase (AMPK)/SIRT1/peroxisome proliferator-activated receptor γ co-activator 1 α (PGC1α) pathway in adipose tissue, associated with changes in mitochondrial morphology and function[19]. Beyond their established glucose-lowering prowess, SGLT2i can shrink epicardial adipose tissue (EAT)[20]. This targeted attack on the fatty culprit dampens the flames of myocardial inflammation, offering dual protection against heart disease.

SGLT2i act like metabolic magicians in the liver, increasing the production of fibroblast growth factor 21[18-21], which boosts lipid oxidation and prevents the activation of the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome. This leads to pyroptosis, a form of programmed cell death linked to insulin resistance and obesity. By preventing pyroptosis, SGLT2i improve insulin sensitivity and promote adipocyte browning. At the pancreatic level, SGLT2i protect from damage by inhibiting the activation of the NLRP3 inflammasome. Its direct effect on pancreatic α cells is controversial, as it leads to increased glucagon secretion and a consequent increase in hepatic gluconeogenesis[22,23].

There is considerable evidence about the positive cellular and mitochondrial effects of SGLT2i on the renal system. Specifically, less fibrosis, organ damage, and inflammatory damage have been demonstrated through the modulation of the SIRT1/AMPK/PGC1α pathway[24,25]. Inhibition of the mammalian target of rapamycin complex 1 -possibly secondary to elevated ketone bodies- was interestingly linked to autophagy, lower stress, and prevention of podocyte and endothelial injury[24]. Furthermore, SGLT2i can mitigate renal fibrosis by modulating transforming growth factor beta, autophagy, and peroxisome proliferator-activated receptor alpha through fatty acid oxidation[26,27]. Another beneficial effect at the tubular level is the reduction of serum uric acid levels, possibly attributed to the alteration of uric acid transport activity induced by glycosuria[28].

In addition to their classic pharmacological effects, SGLT2i have manifested pleiotropic actions that involve signaling pathways with SIRTs, especially SIRT3 and SIRT1, reducing oxidative stress, inflammation, and fibrosis[29,30]. SGLT2i constitutes a multifaceted defense against heart failure, striking on two fronts[31]. They wield a molecular scalpel, inhibiting the Na^+/H^+ exchanger to calm the storm of sodium and calcium overload within heart cells. Simultaneously, they ignite the metabolic spark, promoting a fasting-like state that fuels the engine of ketone production, providing an optimal energy source for the struggling heart. This mechanism led to the postulation that SGLT2i could activate SIRTs linked to the autophagy pathway and innate immunity. SGLT2i choreograph a wonderful cellular ballet (beclin 1, toll-like receptor 9, SIRT3, and mitochondria), weaving together threads of autophagy, oxidative stress, and mitochondrial health [32]. With SIRT3 as the key piece, they stimulate mitochondrial respiration, quiet the whispers of oxidative stress, silence the shouts of apoptosis and inflammasome signaling, and build a defensive wall against cardiac injury. In this regard, it is worth noting that SIRT3 deficiency -both in mice and in patients- caused the loss of the cardiac protective effects of SGLT2i.

Specifically, at least three central processes are presently known by which SGLT2i can activate SITRs: One of them is that SGLT2i stimulate the fasting process cellularly by promoting gluconeogenesis through activating the sensitive element binding protein cyclic adenosine monophosphate. Thus, the SIRT1 promoter regulates its transcription[33]. SGLT2i engages in a clever cellular game, manipulating nicotinamide adenine dinucleotide (NAD^+) levels to turn on SIRTs, the molecular maestros[34].

Furthermore, the AMPK and SIRT1 signaling pathways are reciprocally activated. Their complementary functions demonstrate that AMPK promotes mitochondrial biogenesis and mitochondrial DNA replication and can activate SIRT1 by increasing intracellular NAD^+[35]. SIRT1 and AMPK, the cellular power couple, engage in mutual crosslink activation. Fueled by caloric restriction and SGLT2i, they synergistically boost each other's power, modulating a symphony of metabolic efficiency and stress resilience[19].

CLINICAL EVIDENCE: CELLULAR AND MITOCHONDRIAL PROTECTION

In the realm of clinical information, the signaling pathways involving SGLT2i and SIRT1, 3, and 6 take center stage. In this regard, several clinical studies are of particular interest. The EMPEROR-Preserved trial marks a seismic shift in the landscape of heart failure management, demonstrating that SGLT2i's protective umbrella extends beyond diabetes[36]. This landmark study opens doors for a wider population struggling with preserved ejection fraction heart failure, offering them a powerful weapon against CVD. In this context, Packer elucidates heart failure mechanisms, focusing on hyperinsulinemia-driven EAT expansion and its pro-inflammatory consequences, and also highlights the beneficial effects of SGLT2i on EAT, reducing inflammation and improving cardiac health[20].

Emerging evidence suggests that SGLT2i may hold promise as an anti-aging agent. These drugs appear to target key pathways involved in aging, including inflammation, cellular energy regulation, and the harmful effects of senescent cells. Similar to the established anti-aging drug metformin, SGLT2i may offer benefits through mechanisms such as reducing free radical production, activating autophagy, and modulating the inflammatory response. Remarkably, SGLT2i may also positively impact the gut microbiome, further contributing to their potential anti-aging effects. This multifaceted action against inflammaging, a chronic low-grade inflammation linked to accelerated aging and age-related diseases, makes SGLT2i particularly interesting candidates for therapeutic repurposing[37]. However, robust clinical studies are crucial to validate the anti-aging potential of SGLT2i beyond their established role in diabetes management. While preliminary results are promising, further research is needed to confirm their efficacy and safety in this context.

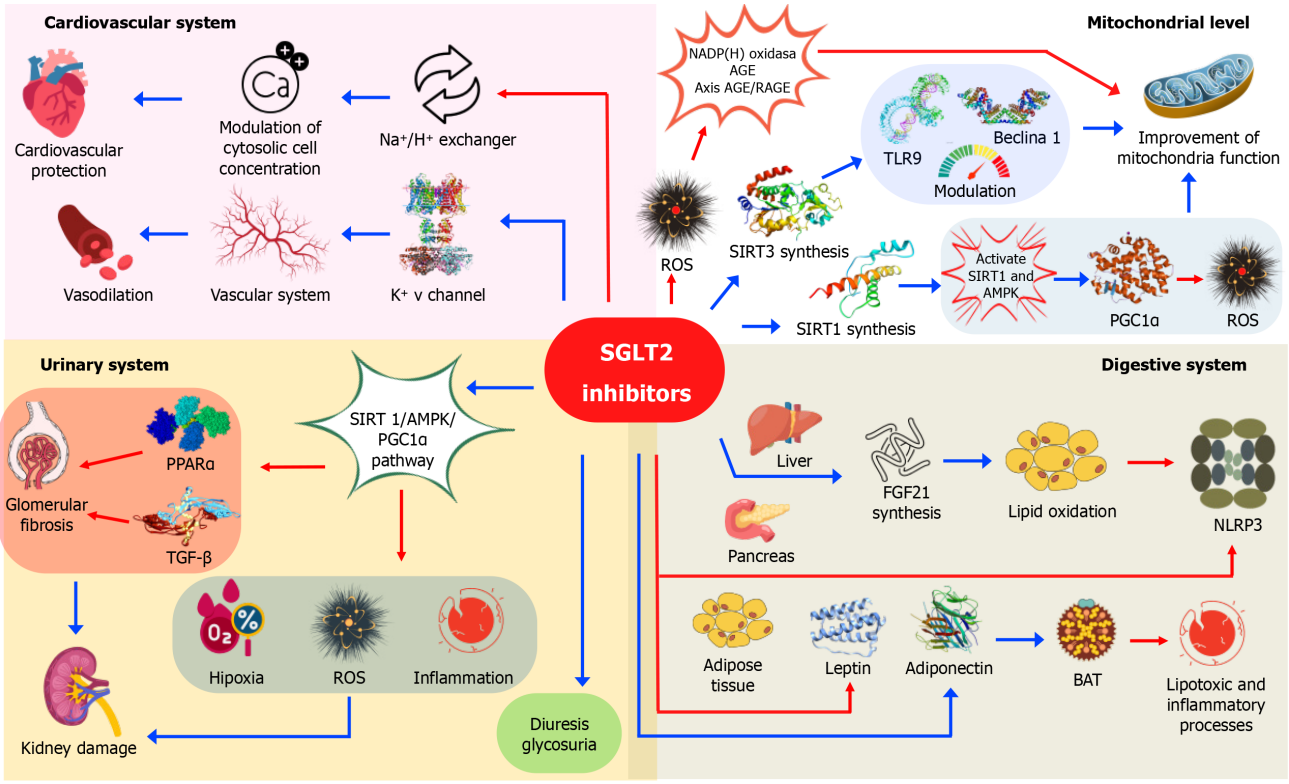

Figure 1 Graphical overview of the beneficial effects of sodium-glucose cotransporter-2 inhibitors across various systems and at the mitochondrial level. These actions include modulation of cardiovascular function, improvement of renal function, and enhanced insulin sensitivity. Furthermore, sodium-glucose cotransporter-2 inhibitors improve mitochondrial function, thereby reducing oxidative stress in different cells and their inflammatory responses. Blue arrows indicate stimulation, while red arrows indicate inhibition. AGE: Advanced glycation end product; AMPK: Adenosine monophosphate-activated protein kinase; BAT: Brown adipose tissue; ERK: Extracellular signal-regulated kinase; FGF21: Fibroblast growth factor 21; NADP(H): Nicotinamide adenine dinucleotide phosphate; NLRP3: Nucleotide-binding oligomerization domain-like receptor protein 3; PGC1α: Proliferator-activated receptor gamma coactivator 1 alpha; PPARα: Peroxisome proliferator-activated receptor alpha; RAGE: Advanced glycation end product receptor; ROS: Reactive oxygen species; SIRT: Sirtuin; SGLT2i: Sodium-glucose cotransporter-2 inhibitors; TLR9: Toll-like receptor 9.

Finally, a lack of nutrients, as well as low energy production at the cellular level, can mitigate a large number of cardiometabolic disorders. In particular, the SIRT and AMPK pathways induce the hypoxia-inducible factor 1 beta and promote ketosis[38]. Consequently, autophagy/mitophagy are stimulated with beneficial effects on cardiac cells, reducing oxidation-inflammation[39]. Specifically, SGLT2i encourage no less than three critical SIRTs present in mitochondria. Furthermore, recent evidence demonstrates that SLGT2i mimic mitochondrial function, reducing inflammation and oxidative stress. Figure 1 provides a graphical overview of the beneficial effects of SGLT2i across various systems and at the mitochondrial level.

CONCLUSION

Added to the classic action of SGLT2i, which is linked to both inhibiting renal glucose reabsorption and triggering metabolic reprogramming through increased glycosuria and reduced glucotoxicity, a growing body of research demonstrates their pleiotropic effects across various cell types and organs, mediated by distinct signaling pathways that contribute to their beneficial outcomes. Table 1 summarizes the main studies showing tissue protection by SGLT2i. The pleiotropic effects of SGLT2i on diverse cellular and mitochondrial signaling pathways in multiple organs and tissues are well-documented by metabolomics studies. However, the possibility of accompanying epigenetic modifications requires further investigation[40,41].

The introduction of SGLT2i holds the potential to transform the clinical prognosis of cardio-reno-metabolic diseases based on the aforementioned mechanisms. Therefore, the findings presented in this review, beyond encouraging results from large clinical trials, also raise significant expectations for future advancements. Conceptually, SGLT2i are understood to act in various target organs as a protective agent, regulating the delicate balance between oxygen consumption and energy production. Their effects at the mitochondrial level, particularly on oxidative stress and cell protection, are crucial determinants of their efficacy.

Table 1 Major scientific studies demonstrating tissue protection by sodium-glucose cotransporter-2 inhibitors

SGLT2i	Study design	Animal/human	Protected tissue	Effect	Ref.
Dapagliflozin	Animal study	Rabbits	Blood vessels	Activation of Kv channels and PKG	[16]
Empagliflozin	Animal study	Mice	Adipose tissue, and liver	Induction of anti-inflammatory macrophage 2 phenotype of macrophages	[18]
Canagliflozin	Animal study	Mice	Adipose tissue	Induction of AMPK-SIRT1-Pgc-1α signalling pathway	[19]
Canagliflozin	Animal study	Mice	Liver	Enhancing FGF21-ERK1/2 pathway activity	[21]
Empagliflozin	Animal study	Mice	Pancreas	Activation of the NLRP3/caspase-1/GSDMD pathway	[23]
Canagliflozin	Animal study	Mice	Kidney	Normalized Pin1 expression and AMPK activation	[25]
Canagliflozin	Animal study	Mice	Kidney	Autophagy modulation	[26]
Empagliflozin and canagliflozin	Cell culture	Renal cells	Kidney	Block basal and TGF-β1-induced expression	[27]
Luseogliflozin	Human study and animal study	Human and xenopus laevis oocytes	Kidney	Uric acid transport activity	[28]
Empagliflozin	Animal study	Mice	Heart	Improving mitochondrial homeostasis	[30]
Empagliflozin	Animal study	Mice	Heart	Modulation of the Beclin 1-TLR9-SIRT3 complexes in the mitochondria	[32]
Empagliflozin	Human study	Human	Heart	Reduced the combined risk of cardiovascular death or hospitalization for heart failure	[36]
Dapagliflozin	Animal study	Mice	Kidney, liver, and heart	Induction of the AMPK-mTORC1 signaling	[41]

AMPK: Adenosine monophosphate-activated protein kinase; ERK: Extracellular signal-regulated kinase; FGF21: Fibroblast growth factor 21; GSDMD: Gasdermin D; mTORC1: Mechanistic target of rapamycin complex 1; NLRP3: Nucleotide-binding oligomerization domain-like receptor protein 3; SIRT: Sirtuin; SGLT2i: Sodium-glucose cotransporter-2 inhibitors; TGF-β: Transforming growth factor beta; TLR9: Toll-like receptor 9.

FOOTNOTES

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MINIREVIEWS

Potential and application of abortive transcripts as a novel molecular marker of cancers

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Abstract

Abortive transcript (AT) is a 2-19 nt long non-coding RNA that is produced in the abortive initiation stage. Abortive initiation was found to be closely related to RNA polymerase through *in vitro* experiments. Therefore, the distribution of AT length and the scale of abortive initiation are correlated to the promoter, discriminator, and transcription initiation sequence, and can be affected by transcription elongation factors. AT plays an important role in the occurrence and development of various diseases. Here we summarize the discovery of AT, the factors responsible for AT formation, the detection methods and biological functions of AT, to provide new clues for finding potential targets in the early diagnosis and treatment of cancers.

Key Words: Abortive transcript; Abortive initiation; RNA polymerase; Transcription; Tumor marker

Core Tip: Abortive transcript (AT), as a special non-coding RNA, is the transcription product of abortive initiation. Abortive initiation occurs before normal transcription initiation and may be influenced by many factors. If its expression can be monitored normally, it will be of great significance for diagnosis and treatment of cancer. Though there are many difficulties and challenges in the study of AT in diseases, in-depth exploration of the role and mechanism of AT in cancers will provide a new potential target for early diagnosis and treatment of cancers and clinical prognosis.

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INTRODUCTION

Abortive transcript (AT), is a special non-coding RNA, which is the transcription product of abortive initiation. Abortive initiation is an essential step during transcription initiation, where short nascent RNAs are synthesized and released by RNA polymerase (RNAP)[1]. AT is closely related to the structure of RNAP and the initiation process of RNA transcription[2]. AT was identified in 1976, and has been extensively studied since then by domestic and international scholars. AT not only plays a role in transcription, but also can be used as a new marker for early diagnosis and treatment of tumors. Here we summarize the research progress of AT in recent years.

DISCOVERY OF ABORTIVE TRANSCRIPT

Abortive initiation generally occurs in the body. At the initiation stage of transcription, RNAP cannot escape from the promoter region, but repeatedly synthesizes RNA with a fragment size of < 10 nt. RNAP plays a real transcription role until the > 10 nt RNA is synthesized. ATs are 2-8 nt long, and were first identified *in vitro* in 1976. When the extension reaction of RNAP lacks both NTPs (CTP and UTP), the transcription system is blocked and nascent RNA is released[3]. However, when all four NTPs (ATP, GTP, CTP and UTP) are present, RNAP transcription produces RNAs of varying lengths, which was initially called "abortive RNA". In 1980, Carpousis *et al*[4] officially confirmed the existence of ATs when they detected 2-6 nt long abortive RNA through *in vitro* experiments and named it "abortive transcript". In addition, it was found that 2 nt AT accounted for 50% of all abortive products. In 2009, Goldman first detected AT in *Escherichia coli* (*E. coli*). His team used plasmid transformation to express the N25anti gene (a mutant of the T5 phage N25 gene that produces 11-19 nt AT) and detected 11-19 nt AT in *E. coli* for the first time using a lock-in probe.

Researchers have found that the occurrence of abortive initiation is related to RNAP through *in vitro* experiments[2,5]. The length distribution of AT and the scale of abortive initiation are related to the promoter, discriminator and transcription initiation sequence, and are influenced by transcription elongation factors[6,7]. For example, the chain loops between the functional domains of RNAP σ3 and σ4 are close to the active site of RNAP, and located in the expulsion channel of RNA products in the whole enzyme, which can prevent the extension of RNA products, thus leading to abortive initiation[8]. Studies have found that the length of AT produced by abortive initiation is typically 2-10 nt, while a few ATs can reach 19 nt[1,2]. These studies provided important evidence in an emerging field of research.

FACTORS INFLUENCING ABORTIVE TRANSCRIPT FORMATION

RNA polymerase

RNAP is a complex enzyme composed of multiple subunits, which catalyzes transcription and can perform various functions. The holoenzyme form of RNAP is $\alpha_2\beta\beta'\sigma$, which consists of five subunits. The α subunit is related to the formation of $\alpha_2\beta\beta'$, the tetramer core of RNAP, and can determine which genes are transcribed. The β subunit contains the binding site of nucleoside triphosphate, which can catalyze polymerization. The β′ subunit contains a binding site for the DNA template. The σ subunit can recognize the start position of transcription and facilitate stable binding of RNAP to the promoter site[9]. Enzymes without σ subunit are called core enzymes, which can only catalyze chain elongation but have no effect on initiation[10]. Different σ subunits can respond to different signals and environmental conditions to identify specific promoter sequences of different genomes[11]. In addition, the CRE pocket formed by the β subunit of RNAP can make contact with the +2G position of the non-template DNA strand. However, this contact has no direct impact on the escape of the RNAP promoter, so the synthesis efficiency of abortive products will not change significantly[12].

In addition, the distance between the leading edge of the RNAP and its downstream DNA can affect the synthesis of ATs, which leads to a small decrease in abortive initiation that is reproducible and can affect the length of abortive products[13].

Promoter and promoter escape

Promoter is a region of the DNA sequence that can activate RNAP. It is located upstream of the 5′ end of a structural gene, which enables accurate binding of the RNAP to the template DNA and has specificity of transcription initiation[9]. Promoters T7A1, T7A2, T7A3 and T7D exist on isolated restriction fragments of the phage template[14], and all three phage promoters T7A1, T5N25 and T5N25 (antiDSR) can produce numerous ATs in the late stage of transcription initiation. DNA signals in the promoter recognition region and the initial transcription sequence region are key factors affecting the production of these ATs[15]. In addition, the initial T7N25 transcription sequence can undergo certain mutations that prolong abortive initiation and release 16-19 nt abortive transcripts. Among them, the hexamers at -35 and -10 positions can be altered, and the 17 base pairs in the middle gradually move away from the mutation, which inhibits the production of 16-19 nt long ATs[16]. In addition, when the promoter, RNAP, and 7-9 nt RNA product form a complex, abortive initiation can be inhibited to a certain extent and the synthesis rate of long RNA products can be increased[17]. Thus, the initiation of transcription is a critical stage of gene expression, which involves the interaction of RNAP with the promoter. The structure of the promoter affects its affinity for RNAP, and thus the level of gene expression.

In late transcription, promoter escape is the main biochemical reaction. In the abortive initiation curled model, RNAP is fixed on the promoter at the initiation stage of transcription and immobile. Instead, DNA downstream of the polymerase is collected into the polymerase, and the extension stage of transcription begins after the RNAP escapes from the promoter[18]. The strong interaction between RNAP and promoter recognition region can reduce the rate of promoter escape, thereby promoting abortive initiation[19]. Studies have shown that lacUV5 is the first escape rate-limiting promoter. By measuring the ratio of synthesized invalid RNA to productive RNA, it was found that lacUV5 promoter participates in more invalid synthesis and is more difficult to escape than T7A1 promoter[17].

Transcription factor Gre

RNAP can be involved in abortive initiation *in vitro* and synthesizes 2-15 nt transcripts. Hybridization with lock-in nucleic acid probes has shown that AT production can be directly detected *in vivo*. Gre transcription elongation factors (GreA and GreB) can combine with pore structures of secondary channels to enhance RNA cleavage by polymerases[20], which can reduce the occurrence of abortive initiation and affect the length of AT[21-23]. Further studies demonstrated that abortive initiation *in vivo* could reflect promoter strength and RNAP function and was regulated by GreA. *E.coli* GreA can also induce cleavage of transcripts as short as 4 nt at the initiation stage, thereby inhibiting the generation of ATs[24]. In addition, reducing the number of ATs at 6-10 nt and 11-15 nt and supplementing the transcription cycle with GreB protein, while the number of ATs at 2-5 nt and 16-20 nt was unchanged, increased RNA product synthesis by 2-5 times. Among them, 16-20 nt long ATs were not affected by GreB, suggesting that they were not products of RNAP backdating [23]. In addition, Gre may also affect promoter escape. Some studies have investigated the factors influencing promoter escape of *E. coli* RNAP, involving phage promoters T7A1, T5N25 and T5N25 (antiDSR). *In vitro*, the addition of *E. coli* transcription cleavage factor GreA or GreB can significantly increase the clearance rate of T5N25 (antiDSR), but has little effect on normal T7A1 and T5N25 regulated transcription. *In vivo* experiments with *E. coli* GreA and GreB knockout strains also showed that Gre factor derived from T5N25 (antiDSR) had a stimulating effect on transcription[7].

DETECTION METHODS FOR ABORTIVE TRANSCRIPTS

Detection of the special structure, function and biological role of AT depends on the advancements in biotechnology and new methods. At present, there are four main methods to detect short ATs.

Autoradiography technique for AT detection

It is an *in vitro* transcription technique based on a specific transcription template and p32-labeled NTP. This method can only detect the length and content of AT by autoradiography *in vitro*. However, it cannot identify the sequence of AT, or conduct qualitative and quantitative analyses of naturally produced ATs in the body[1].

The probe method

Since AT is a type of short-stranded RNA, it can be prolonged or the stability of its binding to the probe can be enhanced when using a locked nucleic acid probe. For example, SYBR Green is economical[25], rapid and highly sensitive, and TaqMan-MGB is highly specific, accurate and reliable[26]. Goldman *et al*[2] performed direct detection of 11 nt AT produced by T5 phage N25 gene with promoter mutation with a nucleic acid probe, which proved for the first time that plasmids could produce ATs in the body. However, due to technical difficulties and the special structure of AT, the above methods cannot achieve specific qualitative and quantitative analyses. Meanwhile, the existence of naturally generated ATs < 10 nt in the body has not been confirmed experimentally.

BSH-ABC model

According to the base packing hybridization principle, a base packing hybridization assisted ligation reaction is designed, that is, a ssDNA probe A and two auxiliary RNA strands B and C are designed to provide strong base packing force as well as prolong ATs. This method is called BSH-ABC mode[27]. After the extension of ATs in BSH-ABC mode, real-time reverse transcriptase-polymerase chain reaction and TaqMan-MGB probe can be used for the specific detection of ATs of different lengths with high sensitivity. However, since ATs produced in the body are not modified by fluorophores, BSH-ABC mode also has disadvantages in detecting ATs.

BSH-ABCD model

Based on the principle and technology of base packing hybridization and strand displacement reaction, studies have further explored the simultaneous and stable combination of two 8 nt oligonucleotide chains using their own double helix structure, which is called BSH-ABCD mode[28]. This method permits quantitative detection of ATs to some extent, but needs to be further explored.

BIOLOGICAL FUNCTIONS OF ABORTIVE TRANSCRIPTS

AT inhibits transcription

ATs could not only inhibit the transcription of the parent gene, but also interfere with the transcription of other related genes, and the inhibition rate was up to 7.5 times. The interference ability of ATs with different lengths and different types of genes is different. The length distribution of ATs and the scale of abortive initiation are related to the promoter, discriminator and transcription initiation sequence, and influenced by transcription elongation factors. ATs may inhibit transcription by interacting with RNAP[29]. Abortive initiation can affect the transcription termination of phage T7 gene 10. ATs derived from promoter φ10 can exhibit a trans-acting anti-termination activity against terminator Tφ. When the abortive initiation cycle of T7 RNAP on φ10 begins, oligomeric and polymeric short RNAs of G can be generated, which can specifically sequestrate the 5 nt and 6 nt C+U extension sequences and ultimately interfere with the formation of the terminator hairpin. This anti-termination activity depends on sequence-specific hybridization of ATs to the 5′-end of the half TφRNA. *In vivo*, excessive accumulation of RNAP and loss of ribonuclease can increase the AT content and enhance its anti-termination activity. In *E. coli* T7 infection, the anti-termination activity of ATs against Tφ promotes the expression of downstream promoter-free genes 11 and 12. Other studies have shown that abortive initiation can also act as a checkpoint for promoter proofreading and structural transformation, thereby regulating gene expression[30].

AT is involved in the synthesis of DNA and RNA

Transcription initiation in all cells is generally thought to occur as a function of NTP alone. However, it is well known that prokaryotic and eukaryotic RNAPs can initiate transcription *in vitro* using 2-8 nt long oligonucleotides. Goldman *et al* [31] found that using sequences in the 2-4 nt range are complementary to the coding strand. In addition, the artificially synthesized ATs with the 5′-end between -3 and +1, and the 3′-end between +1 and +3 of this segment can be used as primers to initiate transcription.

AT CAN BE USED AS A NOVEL MOLECULAR MARKER OF DISEASES

Liver injury

Yan *et al*[32] used high-throughput sequencing to detect the expression profile of 8 nt-long ATs in the serum of mice with or without acute liver injury induced by carbon tetrachloride. The results showed a total of 661 ATs in the experimental group and the control group, among which 16 ATs were differentially expressed in the two groups. Quantitative polymerase chain reaction was used to verify the high-throughput sequencing results, and the results of both techniques were consistent. The source of these 16 differentially expressed ATs was traced, and indicating that these 16 ATs may be derived from genes related to liver injury. Thus, AT may become a novel molecular marker of liver injury.

Ovarian cancer

CA125 protein has been identified as a biomarker for various cancers. The gene muc16 expressing CA125 protein can release numerous relevant ATs at the transcription initiation stage. Some studies have successfully detected the expression of muc16 related ATs[28]. Felder *et al*[33] found that CA125 was a repeat peptide epitope of muc16 in ovarian cancer, which can promote cancer cell proliferation and inhibit anticancer immune response. Muc16 is expressed in non-mucinous epithelial ovarian cancer[34]. It has become the most widely used and biomarker in the screening of ovarian cancer.

Pancreatic cancer

Haridas *et al*[35] detected the expression pattern of muc16 in pancreatic cancer tissues. The results showed that muc16 was not expressed in normal pancreas, but was significantly up-regulated in pancreatic cancer and pancreatitis tissues. These results suggest that muc16 may play a role in the progression and metastasis of pancreatic cancer.

Breast cancer

Lakshmanan *et al*[36] analyzed the expression of muc16 in breast cancer tissues and found that normal tissues did not express muc16, but 54% of breast cancer tissues showed positive muc16 expression. Their results indicate that muc16 is more commonly expressed in breast cancer tissues, and plays an important role in breast cancer occurrence and development.

Lung cancer

Muc16 is highly expressed in patients with multiple brain metastases from non-small cell lung cancer (NSCLC) and is

Table 1 Protein tumor biomarkers

Biomarker	Alteration	Related tumor	Detection method	Advantage	Disadvantage	Ref.
AFP	Up-regulation	Primary liver cancer, viral hepatitis, liver cirrhosis, gonad embryonic tumor, *etc.*	Blood	The most sensitive and specific index for early diagnosis of primary liver cancer, and suitable for large-scale census	For patients with early liver cancer, the detection rate of AFP is low and the misdiagnosis rate is high	[43]
CEA	Up-regulation	Colon cancer, rectal cancer, pancreatic cancer, gastric cancer, lung cancer, *etc.*	Blood	A broad-spectrum tumor marker	The specificity is not strong, the sensitivity is not high, and the early diagnosis of tumor is not obvious	[44-46]
PSA	Up-regulation	Prostate cancer	Blood	Avoid some unnecessary biopsies	Benign prostatic hyperplasia and prostatitis can also show positive PSA	[47]
SCCA	Up-regulation	Cervical cancer, lung squamous cell carcinoma, head and neck cancer, *etc.*	Blood and tissue	High specificity	Low sensitivity	[48-49]
CYFRA21-1	Up-regulation	Lung cancer, esophageal cancer, hepato-cellular carcinoma, *etc.*	Blood	The sensitivity and specificity of the diagnosis for bladder cancer are good	Screening for lung cancer is not highly specific	[50-52]
TPA	Up-regulation	Lung cancer, breast cancer, ovarian cancer, bladder cancer	Blood	High detection rate for malignant tumors, and high sensitivity in the observation of curative effect	Has no correlation with tumor site or tissue type	[53-56]
HE4	Up-regulation	Ovarian cancer	Blood	High sensitivity and specificity for the diagnosis of ovarian cancer, especially in early ovarian cancer, and related to the stage and metastasis of ovarian cancer	A high positive rate in endometrial cancer	[57]

AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; PSA: Prostate specific antigen; SCCA: Squamous cell carcinoma antigen; CYFRA21-1: Cytokeratin 19 fragment; TPA: Tissue polypeptide antigen; HE4: Human epididymal protein 4.

associated with poor prognosis[37]. Other studies have shown that muc16 is elevated in serum samples from patients with stage I NSCLC, and may be a good biomarker for lung cancer[38].

Liver cancer

Xia *et al*[39] found that *GPC3* is a differentially expressed gene in hepatocellular carcinoma that produces numerous ATs during the transcription process, and the stage and quantity of AT production can predict the efficiency and results of normal transcription to a certain extent. A recent study found that REXO2, a key enzyme responsible for cleaving the short fragment RNA of ATs[40,41], is closely related to the occurrence and development of liver cancer, and can promote the proliferation, migration and invasion of liver cancer cells[42].

Given these preliminary findings on the roles of ATs in various diseases, ATs have the potential to become biomarkers for cancer diagnosis and treatment. At present, there are several markers that can be used for cancer diagnosis. Some commonly used cancer markers are summarized in Tables 1, 2 and 3[43-65]. Therefore, exploring new, non-invasive, highly sensitive and specific early detection methods, and systematically and rigorously exploring the biological functions and mechanism of cancers, will provide new biomarkers for the early diagnosis and treatment of cancers.

Table 2 Glycochain antigen tumor biomarkers

Biomarker	Alteration	Related tumor	Detection method	Advantage	Disadvantage	Ref.
CA125	Up-regulation	Ovarian cancer	Blood	High sensitivity of diagnosis	Poor specificity, nearly half of early cases are not elevated	[58]
CA15-3	Up-regulation	Lung cancer, colon cancer, pancreatic cancer, ovarian cancer, breast cancer, *etc.*	Blood	The most important specific marker for breast cancer	Low sensitivity in the early stages of breast cancer	[59]
CA19-9	Up-regulation	Pancreatic cancer, gallbladder cancer	Blood	The sensitivity and specificity can reach more than 90%	The elevation of CA19-9 is susceptible to many benign diseases	[60-61]
CA50	Up-regulation	Pancreatic cancer, colon cancer, liver cirrhosis, lung cancer	Blood	Double identify Lewis antigen negative and positive tumors, a broader broad-spectrum tumor marker than CA19-9	Inflammation of the gastrointestinal tract may cause mild or transient elevation of CA50	[62]

CA125: Glycochain antigen 125; CA15-3: Glycochain antigen 15-3; CA19-9: Glycochain antigen 19-9; CA50: Glycochain antigen 50.

Table 3 Enzymatic tumor biomarkers

Biomarker	Alteration	Related tumor	Detection method	Advantage	Disadvantage	Ref.
NSE	Up-regulation	Small cell lung cancer	Blood	The diagnostic sensitivity can reach 80% and the specificity can reach 80%-90%	NSE alone is not sufficient to accurately differentiate SCLC from NSCLC	[63]
ProGRP	Up-regulation	Small cell lung cancer	Blood	High sensitivity and specificity	Insufficiency of kidney function can also lead to elevated ProGRP values	[64]
PAP	Up-regulation	Prostate cancer	Blood	An auxiliary index for tumor grading	Serum PAP may be elevated in some patients with benign prostatic hyperplasia	[65]

NSE: Neuron specific enolase; ProGRP: Gastrin releases peptide precursors; PAP: Prostatic acid phosphatase.

CONCLUSION

The normal transcription process may be affected by many factors and lead to abortive initiation, resulting in ATs. At is a short non-coding RNA that has been shown to affect the normal transcription process. Studies have demonstrated that ATs have the potential to become novel markers for liver injury and tumor, which can facilitate the early detection, diagnosis and treatment of cancer. For example, some lncRNAs, miRNAs and other non-coding RNAs have been confirmed as diagnostic and prognostic markers of cancers, and can affect their occurrence and development. As a special non-coding RNA, AT also has the potential to be a novel marker. At present, there are many challenges in the study of ATs in diseases. Given that REXO2 is a key enzyme required for AT cleavage and has a cancer-promoting effect, it is important to explore whether ATs play more biological roles and functions in the transcription process, and affect the occurrence, development and prognosis of cancers. Moreover, whether ATs can be employed for the early diagnosis of cancers needs further exploration, and its role and mechanism require rigorous study. Therefore, in-depth exploration of the role and mechanism of ATs in cancers will certainly provide a new strategy to search for potential targets for early diagnosis, treatment and clinical prognosis of cancers.

FOOTNOTES

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MINIREVIEWS

Eribulin in breast cancer: Current insights and therapeutic perspectives

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Abstract

Eribulin is a non-taxane synthetic analogue approved in many countries as third-line treatment for the treatment of patients with metastatic breast cancer. In addition to its mitotic property, eribulin has non-mitotic properties including but not limited to, its ability to induce phenotypic reversal of epithelial to mesenchymal transition, vascular remodelling, reduction in immunosuppressive tumour microenvironment. Since approval, there has been a surge in studies investigating the application of eribulin as an earlier-line treatment and also in combination with other agents such as immunotherapy and targeted therapy across all breast cancer sub-types, including hormone receptor positive, HER2 positive and triple negative breast cancer, many demonstrating promising activity. This review will focus on the application of eribulin in the treatment of metastatic breast cancer across all subtypes including its role as an earlier-line agent, its toxicity profile, and potential future directions.

Key Words: Eribulin; Breast cancer; Metastatic breast cancer; Chemotherapy; Efficacy; Safety

Core Tip: Eribulin is a non-taxane chemotherapeutic agent which is utilised for the treatment of locally advanced or metastatic breast cancer patients who have progressed after 2-3 lines of taxane or anthracycline-based regimen. Eribulin's non-mitotic properties which include its anti-mesenchymal, immunomodulating and vascular remodelling features could make it a perfect candidate in becoming adjuncts to standard treatment regimen for breast cancer across different subtypes. In the era of targeted therapy, immunotherapy and antibody-drug conjugates, we review current evidence to elucidate whether eribulin still has a role to play in earlier and later-line settings both as a single agent and in combination with other agents in patients with metastatic breast cancer across all subtypes of breast cancer.

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INTRODUCTION

Breast cancer is the most common cancer affecting females with around 2 million cases annually worldwide[1]. Despite improvement in survival rates of patients with breast cancer, survival outcomes for patients with advanced and metastatic breast cancer remains dismal, reflecting the need for novel and alternative therapies. While novel agents such as cyclin-dependent kinase (CDK) 4/6 inhibitors and antibody-drug conjugates have revolutionised the treatment of metastatic breast cancer, particularly estrogen-receptor positive (ER+) and triple negative breast cancer (TNBC) respectively, survival outcomes especially for patients with metastatic TNBC remains poor. Many factors account for the poor survival including but not limited to lack of apparent tumor-specific receptor or pathway to target in TNBC, drug intolerance and development of treatment resistance. Mechanistic insights into the pathophysiology of TNBC is crucial to allow development of novel therapeutic strategies. With increasing understanding of the complexity of the tumour microenvironment, there is developing interest in the utilisation of agents that are not only cytotoxic but also ones that could modulate the tumour microenvironment to mount an effective anti-tumoral response[2]. One such therapeutic agent is eribulin.

Eribulin is a synthetic analogue of halichondrin B (Figure 1) that binds to the positive ends of the microtubule through the vinca domain on the polymerization surface of b-tubulin[3-5]. Halichondrin B is a natural large polyether macrolide originally isolated in 1985 from *Halichondria okadai*, a rare black sea sponge off the coast of Japan[6]. Due to its complex structure and the presence of contaminants, it was difficult to use until 1998 when the complete synthesis of halichondrin B was developed by Dr. Yoshito Kishi at Harvard. Kishi discovered that the anticancer activity of halichondrin resided on the right side macrolactone as macrolactone 1 shows inhibitory activity against certain cancer cell lines[7]. Afterwards, Eisai Research Institute licensed the technology and carried out the synthesis and future development of eribulin[5].

Eribulin inhibits polymerization and consequent microtubule extension without affecting microtubule shortening[8-10]. It binds to the plus ends of microtubules, halts microtubule growth in the interphase cells without affecting the shortening phase, and forms non-productive aggregates of tubulin, leading to G2/M cell-cycle block and eventually apoptosis after prolonged mitotic blockage[11]. This mechanism is in contrast to other microtubule-targeting agents such as taxanes, epothilones and vinca alkaloids, which influence both microtubule growth and shortening[8]. This averts the assembly of mitotic spindle during prometaphase, leading to cell cycle arrest and, ultimately, to apoptosis[8]. As cancer cells divide at a more rapid rate than normal cells, they have increased susceptibility to this microtubule-targeted mechanism relative to healthy cells. In addition to its anti-mitotic mechanism of action, eribulin possesses several non-mitotic activities, many affecting the tumour microenvironment (Figure 2). This includes the phenotypic reversal of epithelial to mesenchymal transition (EMT), potentially *via* suppressed transforming growth factor-beta (TGF-β)/Smad signalling, with consequent inhibition of tumour invasion and metastasis. Also potentially interlinked with EMT reversal, in-vitro studies reveal substantially decreased proportions of cancer stem cells (CSC) in both ER+ and ER-negative breast cancer cell lines after eribulin exposure[12,13]. Additionally, reduction of the immunosuppressive tumour microenvironment and vascular remodelling have been described[14-17].

Apart from breast cancer, eribulin is approved for the treatment of advanced or metastatic liposarcoma after progression on anthracycline therapy, where impressive survival benefits have also been observed[18]. Furthermore, significant antitumor activity has been observed against a range of preclinical cancer models, including colorectal, non-small cell lung cancer, melanoma, glioblastoma and ovarian cancer[19-23]. However, this review will concentrate on eribulin activity in breast cancer across different stages and subtypes, including discussion on its utilisation as an earlier-line treatment, toxicity profile and future directions.

ERIBULIN IN METASTATIC BREAST CANCER

The evidence supporting the approval of eribulin for the treatment of metastatic breast cancer is based on results from several trials summarized in Table 1. In an open-label, randomised multicentre phase 3 study – EMBRACE trial[24] – 762 patients who received 2-5 prior lines of chemotherapy, including a taxane and anthracycline were randomised in a 2:1

Table 1 Summary of studies investigating the role of eribulin in metastatic breast cancer

Clinical study	Type of study	Number of patients analysed	Number of previous chemotherapy lines	Treatment groups (number of patients)	OS		PFS		ORR (%), *P* value
					Median (months)	HR (95%CI), *P* value	Median (months)	HR (95%CI), *P* value	
Metastatic breast cancer – all subtypes combined									
Cortes *et al* [24], 2011	Phase III, randomised, open-label, multicentre	762	2-5 (≥ 2 for locally recurrent or MBC)	E (508) *vs* TPC (254)	13.1 *vs* 10.6	0.81 (0.66-0.99), *P* = 0.041	3.7 *vs* 2.2	0.87 (0.71-1.05), *P* = 0.137	12 *vs* 5, *P* = 0.002
Yuan *et al*[27], 2019	Phase III, randomised, open-label, multicentre	530	2-5	E (264) *vs* vinorelbine (266)	13.4 *vs* 12.5	1.03 (0.80-1.31), *P* = 0.838	2.8 *vs* 2.8	0.80 (0.65-0.98), *P* = 0.036	30.7 *vs* 16.9, *P* < 0.001
Kaufman *et al* [26], 2015	Phase III, randomised, open-label, multicentre	1102	1-3 (1-2 for advanced and/or metastatic disease)	E (554) *vs* capecitabine (548)	15.9 *vs* 14.5	0.88 (0.77-1.00), *P* = 0.056	4.1 *vs* 4.2	1.08 (0.93-1.25), *P* = 0.30	11.0 *vs* 11.5, *P* = 0.85
Pernas *et al* [28], 2018	Phase I, single-arm, multicentre	54	1-3	E + balixafortide (CXCR4 antagonist)	16.8	NA	4.6 (95%CI: 3.1, 5.7)	NA	29.6
Metastatic breast cancer – eribulin as an earlier agent									
Ortega *et al* [48], 2019	Phase II, single-arm, multicentre	53	0	E	NA (not reached)	NA	4.1 (95%CI: 3.2, 6.6)	NA	20.8 (95%CI: 9.8, 31.7)
Hayashida *et al* [63], 2018	Phase II, open-label, single-arm, multicentre	32	0-1	E	NA	NA	8.3 (95%CI: 7.1, 9.4)	NA	43.8 (95%CI: 26.5, 61.0)
Takashima *et al* [64], 2016	Phase II, open-label single-arm, multicentre	35	0	E	35.9	NA	5.8 (95%CI: 4.8, 8.1)	NA	54.3 (95%CI: 37.8, 70.8)
McIntyre *et al* [65], 2014	Phase II, open-label, single-arm, multicentre	56	0	E	NA	NA	6.8 (95%CI: 4.4, 7.6)	NA	28.6 (95%CI 17.3, 42.2)
Triple Negative Breast Cancer (TNBC)									
Twelves *et al* [38], 2016	Phase III, randomised, open-label, multicentre	284	1-3 (1-2 for advanced and/or metastatic disease)	E (150) *vs* capecitabine (134)	14.4 *vs* 9.4	0.70 (0.54-0.91), *P* = 0.006	2.9 *vs* 2.3	0.80 (0.61-1.05), *P* = 0.112	NA
Pivot *et al*[39], 2016	Phase III, randomised, open-label, multicentre	352	305 study (*vs* TPC): 2-5 (≥ 2 for locally recurrent or MBC); 301 study (*vs* capecitabine): 1-3 (1-2 for advanced and/or metastatic disease)	E (199) *vs* TPC/capecitabine (153)	12.4 *vs* 8.1	0.72 (0.57-0.90), *P* = 0.005	2.8 *vs* 2.5	0.77 (0.60-0.97), *P* = 0.028	NA
Tolaney *et al* [37], 2021	Phase Ib/ II, open-label, single-arm, multicentre	167	≤ 2; 0 (*n* = 66); 1-2 (*n* = 101)	E + pembrolizumab	16.1	NA	4.1	NA	23.4 (17.2-30.5)
Yonemori *et al* [36], 2019	Phase I/II, open-label, single-arm, multicentre	48 (Phase I: 24; Phase II: 24)	≥ 2	E + olaparib	14.5	NA	4.2	NA	37.5 (18.8-59.4)

Lee *et al*[42], 2019	Phase I, single-arm, single-centre	25	0-3	E + everolimus	8.3 (95%CI: 5.5, undefined)	NA	2.6 (95%CI: 2.1, 4.0)	NA	NA
ER-positive									
Twelves *et al* [38], 2016	Phase III, randomised, open-label, multicentre	537	1-3 (1-2 for advanced and/or metastatic disease)	E (259) *vs* capecitabine (278)	18.2 *vs* 16.8	0.90 (0.74-1.09), *P* = 0.283	4.3 *vs* 5.3	1.11 (0.89-1.38), *P* = 0.367	NA
Pivot *et al*[39], 2016	Phase III, randomised, open-label, multicentre	945	305 study (*vs* TPC): 2-5 (≥ 2 for locally recurrent or MBC); 301 study (*vs* capecitabine): 1-3 (1-2 for advanced and/or metastatic disease)	E (544) *vs* TPC/capecitabine (401)	15.7 *vs* 13.5	0.87 (0.75-1.00), *P* = 0.058	4.1 *vs* 3.4	0.84 (0.72-0.98), *P* = 0.031	NA
HER2-positive									
Twelves *et al* [38], 2016	Phase III, randomised, open-label, multicentre	169	1-3 (1-2 for advanced and/or metastatic disease)	E (86) *vs* capecitabine (83)	14.3 *vs* 17.1	0.97 (0.69-1.35), *P* = 0.837	4.0 *vs* 5.1	1.36 (0.93-1.98), *P* = 0.115	NA
Pivot *et al*[39], 2016	Phase III, randomised, open-label, multicentre	254	305 study (*vs* TPC): 2-5 (≥ 2 for locally recurrent or MBC); 301 study (*vs* capecitabine): 1-3 (1-2 for advanced and/or metastatic disease)	E (150) *vs* TPC/capecitabine (104)	13.5 *vs* 11.7	0.75 (0.57-1.00). *P* = 0.051	3.7 *vs* 4.2	1.00 (0.75-1.35), *P* = 0.970	NA
Sakaguchi *et al* [55], 2018	Phase II, single-arm, multicentre	28	0	E + trastuzumab	NA (not reached)	NA	11.3 (344 d)	NA	53.6 (95%CI: 36.62, 69.93)
Lutrino *et al* [54], 2016	Phase II, single-arm, single-centre	24	2-9	E + trastuzumab	8 (range 1.3-14.8)	NA	5.4 (range 1-10.5)	NA	41.7%
Inoue *et al*[56], 2019	Phase II, open-label, single-arm, multicentre	25	0	E + trastuzumab + pertuzumab	NA	NA	23.1 (95%CI: 14.4, 31.8)	NA	80 (95%CI: 59.3, 93.2)

CI: Confidence interval; E: Eribulin; HR: Hazard ratio/hormone receptor; NA: Not available; ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival; TNBC: Triple-negative breast cancer.

ratio to receive either eribulin mesylate 1.4 mg/m^2 days 1 and 8 every 21 d, or treatment of physician choice (TPC), which was defined as any single-agent chemotherapy, targeted therapy, radiotherapy or symptomatic treatment[24]. Ninety-seven percent (97%) of patients in the TPC arm received chemotherapy – 26% vinorelbine, 18% gemcitabine, 18% capecitabine, 16% taxane, 22% other chemotherapy[24]. While median progression free survival (PFS) was not significantly different between eribulin and TPC arms (3.7 months *vs* 2.2 months respectively; *P* = 0.137), overall survival (OS) was significantly longer in the eribulin arm (13.1 months *vs* 10.6 months respectively; *P* = 0.041)[24], making eribulin one of the few single agents available today, in addition to antibody-drug conjugates, demonstrated to prolong OS in the refractory setting and defying the presumption that improved OS is an improbable outcome from chemotherapy in refractory metastatic breast cancer[24-26].

In another study by Yuan *et al*[27], eribulin displayed statistically significant superior PFS, although similar OS relative to vinorelbine in Chinese women with locally recurrent or metastatic breast cancer previously treated with 2-5 prior chemoregimens (PFS: HR 0.8, *P* = 0.036; Median OS: HR 1.03; *P* = 0.838). Moreover, toxicity profile of eribulin was more favourable compared to vinorelbine as the number of treatment-emergent adverse events leading to discontinuation was significantly lower in eribulin than vinorelbine (14% *vs* 7.2%).

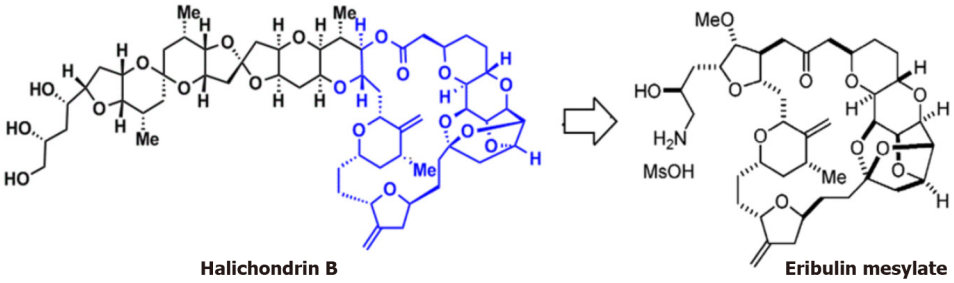

Figure 1 Chemical structures of halichondrin B and eribulin mesylate. Eribulin is a structurally-simplified, synthetic, macrocyclic ketone analogue of halichondrin B Citation: Swami U, Shah U, Goel S. Eribulin in Cancer Treatment. *Mar Drugs* 2015; 13: 5016-5058. Copyright© The Authors 2015. Published by MDPI. The authors have obtained the permission for figure using from the copyright holder (Supplementary material).

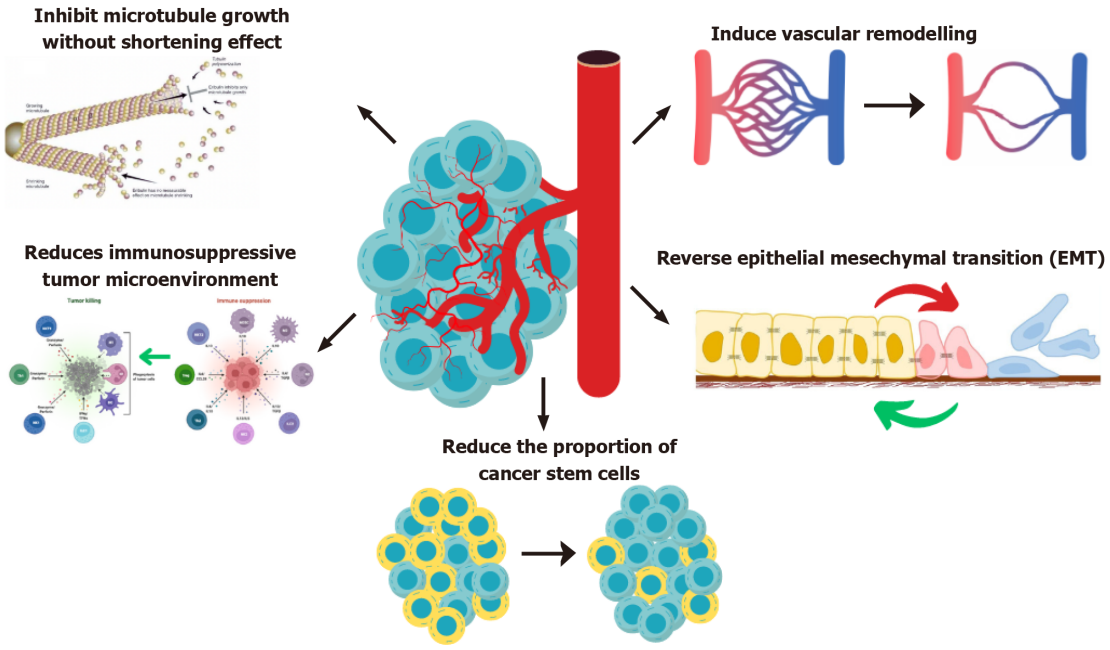

Figure 2 Eribulin non-mitotic mechanisms of action in cancer. Eribulin induces phenotypic reversal of epithelial to mesenchymal transition, decreases proportions of cancer stem cells, reduces the immunosuppressive tumour microenvironment and promotes vascular remodelling.

Eribulin was also studied in combination with balixafortide, a chemokine receptor CXCR4 antagonist to unravel any synergy between these agents in HER2-negative metastatic breast cancer patients[28]. Balixafortide has been shown in several pre-clinical studies to exert anti-tumour, anti-metastastic and immunomodulatory effects, promoting a more favourable tumour microenvironment which is more sensitive to chemotherapy and immunotherapy[29-31]. As such, it has similar mechanism of action to eribulin and therefore could display a synergistic effect when used in combination. This is supported by the more superior PFS and clinical benefit response rate in the combination arm [PFS: 6.2 months; ORR: 38%; clinical benefit rate (CBR): 63%; 1-year OS: 75%][28] compared to the single-agent eribulin arm (PFS: 3.6 months; ORR: 13%; CBR: 28%; 1-year OS: 54%), although noting participant characteristics discrepancy in this inter-trial comparison[24]. Further clinical trials are warranted to confirm these findings.

Eribulin's superior efficacy could be attributed to the non-mitotic effects of eribulin, most of which are absent in other alternative agents. Firstly, eribulin induces vascular remodelling leading to increased tumour perfusion and diminished hypoxia, as evidenced by the increased microvessel density and decline in hypoxia-associated protein expression of CA-9 and vascular endothelial growth factor following eribulin treatment in MX-1 and MDA-MB-231 human breast cancer xenograft models[16]. Diminished hypoxic conditions decreases the likelihood of extensive extracellular matrix remodelling, such as increased collagen deposition, which is associated with metastasis[32]. Additionally, increased tumour perfusion implies that an increased drug concentration would reach tumour cells and thus would be more susceptible to subsequent lines of therapy. Secondly, treatment with eribulin in MX-1 *in vivo* experimental mouse model reversed EMT as characterised by the increased expression of epithelial markers and decreased expression of mesenchymal markers[14]. EMT reversal may lead to reduced CSC regeneration and tendency to metastasise[12]. Simultaneous with EMT reversal was diminished lung metastasis and prolongation of OS in eribulin-treated mice compared to controls[14]. Combining eribulin's non-mitotic properties together with its well-known mitotic mechanism, it is no surprise that eribulin improves survival outcomes.

DIFFERENTIAL EFFICACY OF ERIBULIN ACROSS SUB-TYPES

Eribulin in TNBC

Metastatic TNBC represents an aggressive and difficult to manage form of breast cancer, with a significant risk of distant relapse, after which median OS is only 13 months with treatment[33]. While antibody-drug conjugates such as trastuzumab-deruxtecan and sacituzumab-govitecan have recently been approved for use in metastatic TNBC given their promising effect on overall survival, these agents are expensive and display a fairly adverse toxicity profile, with grade 3-4 adverse events of up 52%[34]. Additionally, immune checkpoint inhibitors, also approved for use in metastatic TNBC in combination with chemotherapy produced fairly low objective response rate of less than 20%[35]. Efforts are currently ongoing to identify biomarkers to tailor therapy and identify the most optimal sequence and combination regimen for the treatment of TNBC.

Eribulin remains an important agent in TNBC either as a single-agent or in combination with other agents such as chemotherapy, immunotherapy and targeted therapy. Given the lack of receptor-specific targets and diverse heterogeneity in TNBC, agents that modulate the tumor microenvironment and target signal transduction, epigenetic modifications such as eribulin are urgently needed. Eribulin with its immunomodulatory effects has the potential to prime the tumor microenvironment to facilitate their counterpart agent utilised in combination to work more effectively. Several agents such as Olaparib and pembrolizumab have been explored in clinical trials in combination with eribulin[36,37].

Subgroup analyses from Study 301, a phase 3 trial investigating the efficacy of eribulin compared to capecitabine in taxane and anthracycline pre-treated patients who had ≤ 3 prior chemotherapies revealed a statistically significant improvement in OS for metastatic TNBC patients treated with eribulin compared to those with capecitabine (14.4 months *vs* 9.4 months respectively; $P = 0.01$)[38]. However, the PFS was not significantly different between the two groups (2.9 months *vs* 2.3 months respectively; $P = 0.112$)[38]. Similar results were observed in a pooled subgroup analysis of study 301 and 305, involving 352 TNBC patients conducted by Pivot *et al*[39]. In this analysis, OS and PFS were significantly longer in the eribulin arm relative to the control arm (OS: 12.4 months *vs* 8.1 months respectively; $P < 0.01$) (PFS: 2.8 months *vs* 2.5 months; $P < 0.05$)[39].

The interest in combining eribulin with immune checkpoint inhibitor in TNBC came about from the IMPASSION130 trial which demonstrated a significant improvement in PFS in metastatic TNBC patients with PD-L1+ tumours treated using a combination of atezolizumab, an anti-programmed death-ligand-1 (anti-PD-L1), and nab-paclitaxel relative to nab-paclitaxel only (7.5 *vs* 5 months respectively; $P < 0.0001$), leading to the accelerated United States Food and Drug Administration approval[40]. Eribulin was combined with pembrolizumab in a phase Ib/II study – ENHANCE1/KEYNOTE-150 – involving 106 patients with metastatic TNBC pre-treated with ≤ 2 lines of chemotherapy[37]. Outcomes were promising, with a CBR of 36.8%, median PFS and OS of 4.1 months and 16.1 months respectively[37]. Safety for the combination was acceptable and comparable to the two drugs when used as monotherapy[37]. While clinical activity was preserved regardless of PD-L1 status, RR in PD-L1+ive tumours was superior at 30.6% compared to 22.4% in PD-L1-negative disease[37]. These results were consistent with work by Goto *et al*[15] demonstrating that eribulin mitigates PD-L1 and FOXP3 expression in eribulin-treated breast cancer samples, likely *via* EMT-reversal, as shown by the inverse correlation between the two proteins and the epithelial marker, E-cadherin. Considering that the anti-programmed cell death protein 1 (PD-1) pembrolizumab modulates the activity of cytotoxic T lymphocytes *via* its immune-checkpoint blockade, the combination with eribulin warrants further exploration in larger trials. It is crucial to remember that population response rate (RR) to immunotherapy is variable and as such, utilising a composite index which incorporates biomarkers such as tumour-infiltrating lymphocytes number, tumour mutational burden and PD-L1 expression, that predict response to immunotherapy can be helpful.

Promising efficacy has also been demonstrated for eribulin when combined with targeted therapy in metastatic TNBC. In a phase 2 Japanese study involving 24 patients previously treated with both anthracycline and taxanes treated with a combination of olaparib and eribulin, the median PFS and OS were 4.2 (95%CI, 3.0-7.4) and 14.5 (95%CI, 4.8-22.0) months, respectively[36]. These survival data were superior to data from a similar Japanese patient population treated with eribulin alone[41]. However, there was some concern on toxicity with 83.3% of patients experiencing neutropenia, 41.7% anemia and 33% febrile neutropenia[36]. A phase 1 trial investigating the combination of everolimus and eribulin in metastatic TNBC patients who had ≤ 4 Lines of prior chemotherapy showed modest efficacy, with 36% of patients achieving partial response, 36% of patients having stable disease and 27% of patients progressing. The median OS was 8.3 months (95%CI: 5.5 to undefined) and PFS was 2.6 months (95%CI: 2.1 to 4.0)[42].

Eribulin in estrogen receptor-positive breast cancer

While selective ER modulators, aromatase inhibitors, and selective ER down-regulators, especially in combination with CDK4/6 inhibitors provide substantial clinical benefit by reducing the risk of disease recurrence and mortality, a subset of patients with ER+ breast cancer particularly luminal B tumor experience more aggressive disease with poorer survival outcomes relative to luminal A tumor[43]. Furthermore, resistance to endocrine therapies represents a major challenge, limiting the success of management of ER + breast cancer. Fortunately, *in vitro* studies have revealed that eribulin has the potential to induce luminal type conversion and expression of genes implicated in hormonal sensitivity, thereby potentially improving response and survival in ER+ breast cancer patients[14]. In regards to the latter, eribulin has also been shown to overcome resistance in hormone-resistant breast cancer cells. Previous clinical trials have investigated the role of eribulin in combination with endocrine therapy demonstrated promising results[44-48].

A phase II single-arm study has shown that eribulin could be beneficial especially among luminal B breast cancer patients by its ability to induce a phenotypic shift to the luminal A subtype[44] which tends to be less aggressive, lower grade and more sensitive to anti-estrogen therapy than luminal B subtype[45,46] with a consequently better prognosis[46,

47]. In the SOLTI1007 study, Prediction Analysis of Microarray 50-gene classifier (PAM50) gene-expression profiling conducted on breast tumours obtained from 101 early-stage ER+, HER2-negative breast cancer patients post-treatment with eribulin for 4 cycles revealed 44.1% of luminal B subtype tumour underwent complete (100%) phenotypic transformation to the luminal A subtype. This phenotypic change was associated with the increased expression of luminal-related genes (ESR1 and NAT1), genes implicated in negative regulation of apoptosis (BCL2 and IL6), and angiogenesis (ANGPTL4 and HIF1A), and reduced expression of cell-cycle related genes (CCNB1, RAD17, MKI67) and genes related to microtubule cytoskeleton organization (AURKA, CENPA, KIF23)[48]. This is consistent with an earlier study which also showed upregulation in expression of genes implicated in hormonal sensitivity – ESR1, BCL2 and ERBB4[44]. Hence, eribulin may induce increased hormonal sensitivity in luminal B patients. This provides a rationale for the investigation of combination of eribulin with hormonal/anti-estrogen therapy for patients who present with luminal B subtype at baseline. Lending clinical support to this hypothesis, Kobayashi and colleagues (2016) identified an increase in time to treatment failure (TTF) when endocrine therapy was started immediately following eribulin treatment as compared to prior (mean: 1.4 months). While there was no significant difference in TTF for endocrine therapy in pre- and post-eribulin treated patients, the proportion of patients with longer TTF was significantly higher in patients who received eribulin prior to endocrine therapy (64% *vs* 24%; $P = 0.018$)[49]. This further emphasises the idea that eribulin could improve sensitivity to anti-estrogen therapy and potentially could be incorporated as a combination first-line therapy for ER+ breast cancer.

Eribulin has a potential role in overcoming resistance to endocrine therapy. In vitro study by Goto *et al*[15] showed that treatment with eribulin increased vascular remodelling and improved tumor hypoxia which enhanced the expression of epithelial and ER-related genes and proteins in hypoxia-resistant cell lines and tumor, leading to an enhanced anticancer effect of tamoxifen. While endocrine therapy-resistant breast cancer is relatively common, a proportion of HR-positive breast cancer eventually exhibit resistance to CDK4/6 inhibitors[50]. In a study by Pandey *et al*[51], treatment with eribulin followed by abemaciclib, a CDK4/6 inhibitor overcame resistance in CDK4/6 inhibitor-resistant cells. This is postulated to occur *via* synergistic pole-like kinase 1 inhibition in the G2/M phase by eribulin. The synergistic anti-tumor effect of eribulin in combination with abemacilib was confirmed in-vivo and certainly merits further investigation in clinical trials.

Eribulin in HER2-positive breast cancer

Eribulin has also been studied in HER2+ breast cancer. While prognosis has significantly improved in HER2+ breast cancer since the advent of anti-HER2 therapy and more recently, antibody drug conjugates such as TDM-1 and trastuzumab-deruxtecan, around 20% of patients still experience progressive disease[25,26]. Intolerance to taxanes which is often used in combination with anti-HER2 therapy is not uncommon and the recommended combination of trastuzumab with pertuzumab is often not available in many countries, making the availability of alternative agents valuable[52,53]. Given it is prudent to combine trastuzumab with pertuzumab and chemotherapy to avoid treatment resistance, a chemotherapeutic agent with proven efficacy in HER2+ breast cancer would be useful. Eribulin has been studied in the setting of HER2+ breast cancer in numerous clinical trials previously.

Several phase 2 trials have also examined the efficacy and safety of eribulin among HER2-positive breast cancers[54-56]. In a multicentre phase 2, single arm study conducted by Sakaguchi *et al*[55], the combination of trastuzumab and eribulin first-line produced a RR of 53.6%, CBR of 64.0% and median PFS of 11.3 months (344 d) in advanced or metastatic HER2+ breast cancer patients. 42.9% of patients experienced a grade 3/4 adverse event, with neutropenia being the most common (28.6%)[55]. The same combination was tested in advanced HER2+ breast cancer patients pre-treated with a median of 3 lines of previous treatment, including pertuzumab, lapatinib, and trastuzumab[54]. The results of this study, with an ORR of 41.7%, median OS and PFS of 8 and 5.4 months respectively, suggests that eribulin and trastuzumab combination could be considered for the treatment of pre-treated HER2-positive breast cancers[54]. Furthermore, eribulin has been studied as the first-line treatment in a triple combination therapy with trastuzumab and pertuzumab (ETP) in metastatic HER2+ breast cancer patients in a multicentre phase 2, single arm study[56]. Here, the ORR, CBR and median PFS were 80%, 84% and 23.1 months, clearly demonstrating an auspicious level of efficacy, comparable to the phase 3 CLEOPATRA trial (ORR 68.4%, OS 56.6 months, PFS 18.7 months, duration of response 20.2 months) which investigated the combination of trastuzumab, pertuzumab and docetaxel (DTP)[41,57]. Additionally, toxicity profile of the ETP regimen appeared favourable relative to the DTP regimen, with less severe neutropenia (32% *vs* 52.8%), and grade 3 peripheral neuropathy (0% *vs* 2.7% respectively)[41,57] - again paving the way for phase 3 trials. The application of eribulin combined with trastuzumab could offer alternative options to the current standard eviQ first-line of trastuzumab/pertuzumab, T-DM1, trastuzumab/capecitabine and DTP regimen, especially in cases where toxicity with capecitabine and paclitaxel are major issues[58]. Of note, allergy and intolerance and the development of acute and cumulative toxicities is common with taxanes[59-61]. Therefore, ETP could potentially be utilised as a first-line therapy or perhaps subsequent lines of therapy, for patients who have failed to respond to DTP and trastuzumab/capecitabine.

ERIBULIN AS AN EARLIER-LINE AGENT IN METASTATIC DISEASE

Recently, there has been increasing number of studies evaluating the use of eribulin as earlier-line therapy in the treatment of metastatic breast cancer. A prospective study investigating the efficacy of eribulin in first-/second-line compared to third-/fourth-line setting in women with advanced/metastatic breast cancer demonstrated that use in the former setting resulted in more superior TTF and OS compared to the latter setting (TTF: 135 d *vs* 119 d; OS: 555 d *vs* 383 d)[62]. These encouraging results of eribulin when used first-line concur with data from a recent multicentre, phase 2,

single-arm trial – MERIBEL study – which explored the role of eribulin as a first-line agent in patients with metastatic HER2-negative breast cancer who were pre-treated with taxanes in early-stage and had a DFI < 36 months[48]. In this study, the median investigator-assessed TTF, ORR and CBR were 4.1 months, 20.8% and 26.4% respectively – comparable to phase 2 studies of taxanes and anthracyclines in metastatic breast cancer. Additionally, the trial showed non-cross-resistance with eribulin and taxanes and viable responses regardless of ER status – shown by the non-significant difference in median TTF between TNBC and luminal phenotype (3.9 *vs* 6.2 months, HR 1.7, 95%CI: 0.9 to 3.4, $P = 0.111$) [48]. Furthermore, a phase 2 Japanese study demonstrated that eribulin in the first- or second-line setting in Japanese patients with metastatic breast cancer produced an ORR and CBR of to 43.8% and 56.3% respectively, and PFS of 8.3 months. However, the PFS in this study might be overestimated because radiographic evaluation was conducted after every 3 cycles of eribulin treatment, instead of every 2 cycles as performed in other first-line trials[63]. Additionally, the discrepancy in efficacy might also be accounted for by the fewer proportion of patients with TNBC subtypes compared to the study[48,63]. Another phase 2 study in Japan also evaluated the efficacy and safety of eribulin as the first-line treatment for HER2-negative metastatic breast cancer[64]. After receiving a median of 8 cycles of eribulin treatment, ORR and CBR were 54.3% and 62.9% respectively. The median PFS and OS was 5.8 (95%CI 4.8 to 8.1) and 35.9 months. A multicentre phase 2 study in the United States demonstrated the ORR, CBR, and PFS of 28.6% (95%CI 17.3-42.2), 51.8%, and 6.8 months (95%CI 4.4-7.6) after receiving a median of 7 cycles of eribulin therapy[65]. These findings suggest good antitumor activity of eribulin as first- or second-line treatment of locally advanced and metastatic breast cancer, warranting further exploration in phase 3 clinical trials.

First-line usage could be beneficial for a number of reasons. Firstly, it leaves the tumour in a less aggressive state post-therapy, considering that eribulin reverses the mesenchymal phenotype, may diminish stem-like cells and promote the luminal A phenotype, rendering the tumour more susceptible to subsequent lines of endocrine and chemotherapy. An augmented cytotoxic effect could also arise *via* the induction of vascular remodelling which allows higher drug concentrations to reach the tumour post-eribulin treatment. Enhanced infiltration into tumours of cytotoxic T-cells is also a possibility enhancing immunotherapy. Further phase 3 studies incorporating immunohistochemical analysis of serial biopsies and taking careful account of outcomes from later line therapies.

SAFETY OF ERIBULIN

In terms of safety, eribulin has demonstrated an acceptable level of toxicity in the treatment of patients with breast cancer. Safety data from the EMBRACE trial showed that serious adverse effects (AE) occurred at a similar proportion in the eribulin group compared with the control group – treatment of physician choice (96% received either capecitabine, vinorelbine or gemcitabine) – 25% *vs* 26% respectively[24]. Of these patients, 13% of eribulin-treated patients discontinued therapy relative to 15% of patients in the control group[24]. A closer look into specific AEs reveals that grade 3 and 4 were more common in the eribulin group including neutropenia (grade 3: 21% *vs* 14%; grade 4: 24% *vs* 7% respectively), leukopenia (grade 3: 12% *vs* 5%; grade 4: 2% *vs* 1% respectively) and peripheral neuropathy (grade 3: 8% *vs* 2%; grade 4: < 1% *vs* 0 respectively)[24]. A drawback with eribulin tends to be greater myelosuppression compared to other agents such as gemcitabine and vinorelbine, especially in heavily pre-treated patients[66,67]. leading to the need for either administration of granulocyte-colony stimulating factor or dose reduction from 1.4 mg/m^2 to 1.2 mg/m[63,68]. Interestingly, a retrospective analysis revealed that dose reduction was associated with better clinical outcomes compared to dose-interval prolongation, in cases where adjustments are necessary owing to toxicity[68]. Considering eribulin-induced peripheral neuropathy, similar dose reduction and/or duloxetine administration, and more recently, photobiomodulation may be employed to alleviate the effect[69,70]. Interestingly, eribulin has been shown to be less neurotoxic than ixabepilone in a randomised phase 2 study (incidence: 31% *vs* 44%)[71]. Of these patients who experienced peripheral neuropathy, 3.9% discontinued treatment with eribulin relative to 18% who were treated with ixabepilone[71]. Furthermore, quality of life assessment, assessed as a secondary end point in study 301 showed patients receiving eribulin treatment had better cognitive function ($P < 0.001$) than those receiving capecitabine[72]. However, patients receiving eribulin had inferior emotional function relative to those receiving capecitabine ($P = 0.033$)[72]. The difference in the effect on quality of life could be ascribed to the toxicity profile of the two drugs where eribulin had lower gastrointestinal toxicities such as nausea, vomiting and diarrhea while capecitabine had in general, less mucositis and alopecia[72].

Unlike capecitabine, special precaution must be exercised in patients on eribulin with mild to moderate hepatic impairment, as for taxanes and ixabepilone[73]. With regards to with renal impairment, pharmacokinetic studies suggest a dose reduction similar to those patients with hepatic impairment, tailored to the level of GFR[74]. As such, to avoid potential toxicity, clinicians may opt for a different agent for patients with comorbid hepatic and/or renal impairment, for instance, resorting to agents such as paclitaxel, doxorubicin and vinorelbine in patients with renal impairment. Considering the impact of age, pooled exploratory analysis of two single-arm phase 2 and one phase 3 trial in the metastatic setting revealed the incidence of adverse events, OS ($P = 0.82$), PFS ($P = 0.42$), ORR and CBR were completely independent of age[75]. In fact, an observational Italian study exploring the use of eribulin in elderly patients with metastatic breast cancer revealed that eribulin preserved quality of life regardless of geriatric status, except for a decline in instrumental activities of daily living and increase in geriatric depression[76].

FUTURE DIRECTIONS

Recent data from clinical trials emphasise that eribulin has potential as an earlier-line agent in the management of patients with metastatic breast cancer, especially in the area of TNBC where options are limited and prognosis is dismal [62,63]. This could have the added advantage of leaving tumours in a less aggressive and more treatment responsive state post-therapy – with a less mesenchymal phenotype, less immunosuppressive environment and better vascularisation – potentially rendering tumours more susceptible to further lines of therapy, translating to improved OS[14,16,17]. To confirm this, further clinical trials investigating eribulin as a first-/second- line agent in metastatic TNBC are warranted. One such a study is ongoing in the Dana Farber Cancer Institute in Boston, where eribulin is followed by doxorubicin and cyclophosphamide administration in patients with invasive HER2-negative inflammatory breast cancer in the neoadjuvant setting[77]. However, interestingly, recent data from early phase trials suffice to consider use of eribulin as a first-line and second-line agent in Japan and Europe respectively in the management of metastatic TNBC[78,79]. Observational study in Japan which investigated eribulin as first-/second line or later-line chemotherapy in HER2-negative, hormone resistant mBC demonstrated that median OS were 22.8 (17.3-31.0), 16.3 (12.4-19.9), and 12.6 (11.2-15.1) months respectively[78]. While median OS was superior in first-line compared to later-lines of therapy, it is statistically non-significant[78]. This could be attributed to the limited sample size in the study and the fact that the follow-up time was limited to 2 years. Furthermore, combination therapy involving eribulin and other agents such as immunotherapy, targeted therapy in mTNBC setting should further be explored. Such studies are currently ongoing – first is a phase 1b/2 study investigating the combination of rebastinib and eribulin or paclitaxel and second, a phase 3 trial exploring the combination of eribulin and balixafortide compared to eribulin monotherapy[80,81]. In the area of ER+ breast cancer, promising preclinical and early phase study discussed above suggests that future studies could explore pre-treating patients with metastatic ER+ breast cancer with eribulin prior to endocrine therapy. A potential study could investigate eribulin administration prior to current gold standard treatment with combination CDK4/6 inhibitors and aromatase inhibitors in patients with ER+, HER2-negative mBC. In the setting of HER2+ breast cancer, further phase 3 trials investigating the combination of eribulin, trastuzumab and pertuzumab ought to be conducted to confirm the promising efficacy and safety data from earlier phase trials.

CONCLUSION

Eribulin is a non-taxane chemotherapy agent which is utilised for the treatment of locally advanced or metastatic breast cancer patients who have progressed after 2-3 lines of taxane or anthracycline-based regimen. Eribulin's non-mitotic properties which include its anti-mesenchymal, immunomodulating and vascular remodelling features could make it a perfect candidate in becoming adjuncts to standard treatment regimen for breast cancer across different subtypes. In the era of targeted therapy, immunotherapy and antibody-drug conjugates, eribulin likely still has a role to play in earlier and later-line settings both as a single agent and in combination with other agents in patients with metastatic breast cancer across all subtypes of breast cancer, pending further phase 3 trials. Further studies exploring combination with targeted therapy and immunotherapy are warranted to further add to the available pharmacological armamentarium for metastatic breast cancer.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Association between acute peripancreatic fluid collections and early readmission in acute pancreatitis: A propensity-matched analysis

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Abstract

BACKGROUND

Patients with acute pancreatitis (AP) frequently experience hospital readmissions, posing a significant burden to healthcare systems. Acute peripancreatic fluid collection (APFC) may negatively impact the clinical course of AP. It could worsen symptoms and potentially lead to additional complications. However, clinical evidence regarding the specific association between APFC and early readmission in AP remains scarce. Understanding the link between APFC and readmission may help improve clinical care for AP patients and reduce healthcare costs.

AIM

To evaluate the association between APFC and 30-day readmission in patients with AP.

METHODS

This retrospective cohort study is based on the Nationwide Readmission Database for 2016-2019. Patients with a primary diagnosis of AP were identified. Participants were categorized into those with and without APFC. A 1:1 propensity score matching for age, gender, and Elixhauser comorbidities was performed. The primary outcome was early readmission rates. Secondary outcomes included the incidence of inpatient complications and healthcare utilization. Unadjusted analyses used Mann-Whitney *U* and χ^2 tests, while Cox regression models assessed 30-day readmission risks and reported them as adjusted hazard ratios (aHR). Kaplan-Meier curves and log-rank tests verified readmission risks.

RESULTS

A total of 673059 patients with the principal diagnosis of AP were included. Of these, 5.1% had APFC on initial admission. After propensity score matching, each cohort consisted of 33914 patients. Those with APFC showed a higher incidence of inpatient complications, including septic shock (3.1% *vs* 1.3%, $P < 0.001$), portal venous thrombosis (4.4% *vs* 0.8%, $P < 0.001$), and mechanical ventilation (1.8% *vs* 0.9%, $P < 0.001$). The length of stay (LOS) was longer for APFC patients [4 (3-7) *vs* 3 (2-5) days, $P < 0.001$], as were hospital charges ($29451 *vs* $24418, $P < 0.001$). For 30-day readmissions, APFC patients had a higher rate (15.7% *vs* 6.5%, $P < 0.001$) and a longer median readmission LOS (4 *vs* 3 days, $P < 0.001$). The APFC group also had higher readmission charges ($28282 *vs* $22865, $P < 0.001$). The presence of APFC increased the risk of readmission twofold (aHR 2.52, 95% confidence interval: 2.40-2.65, $P < 0.001$). The independent risk factors for 30-day readmission included female gender, Elixhauser Comorbidity Index ≥ 3, chronic pulmonary diseases, chronic renal disease, protein-calorie malnutrition, substance use disorder, depression, portal and splenic venous thrombosis, and certain endoscopic procedures.

CONCLUSION

Developing APFC during index hospitalization for AP is linked to higher readmission rates, more inpatient complications, longer LOS, and increased healthcare costs. Knowing predictors of readmission can help target high-risk patients, reducing healthcare burdens.

Key Words: Acute pancreatitis; Acute peripancreatic fluid collections; Readmission predictors; Inpatient complications; Healthcare utilization and costs

Core Tip: The specific association between acute peripancreatic fluid collection (APFC) and early readmission in patients with acute pancreatitis (AP) has not been well characterized. Using a propensity-matched cohort from the Nationwide Readmission Database, this is the first study to reveal that AP patients with APFC have a significantly higher risk of 30-day readmission compared to those without APFC. Patients with APFC also have a higher incidence of inpatient complications, longer hospital stays, and higher healthcare expenditures. Our findings underscore the need for targeted interventions and close monitoring of AP patients with APFC to reduce readmissions and healthcare costs.

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INTRODUCTION

Acute pancreatitis (AP) is an unpredictable and potentially lethal gastrointestinal disease[1]. The annual worldwide incidence of AP is 33.74 cases per 100000 person-years, and it is more than twice as high in some regions[2,3]. The epidemiological trends in AP are showing improvements, but the overall morbidity and mortality still remain high with an aging population[4,5]. It accounts for substantial healthcare utilization and expenditures in the United States, with hospitalization costs of over $30000 per person[6,7]. Hospital readmission is responsible for a considerable AP-related healthcare burden. In a recent nationwide study, Peery *et al*[8] revealed that 40036 patients had an early readmission documented out of 259284 index AP hospitalizations. In a narrative literature review, Bogan *et al*[9] described the overall AP-related readmission rate as ranging from 7% to 34%. Therefore, it is crucial to reduce readmission rates in AP[9]. It requires an adequate understanding of the risk factors associated with rehospitalization[9]. Previous research has identified a number of important risk factors, including recurrent AP, discharge to nonhome facilities, a higher Charlson Comorbidity Index, a longer hospital stay, smoldering symptoms, and/or local pancreatic complications[9-12]. Pertinently, early readmission can often be due to smoldering symptoms and the progression of local complications of AP[13]. These two factors are responsible for up to 38% of all readmissions in AP cases[9]. Therefore, it is imperative to investigate the specific effect of various AP-related local complications on readmission.

Acute peripancreatic fluid collection (APFC) is a homogeneous collection with fluid density that can form within or around the pancreas following acute interstitial edematous pancreatitis[14]. The revised Atlanta classification defines it as an early local complication that develops within four weeks with no associated peripancreatic necrosis[14]. A retrospective cohort study from Saudi Arabia revealed an APFC incidence of 48.3% in patients presenting with AP[15]. A prospective multicenter study from Korea also revealed an incidence of 42.7%[16]. It is known that significant morbidity may arise from APFC due to hemorrhage, biliary obstruction, gastric outlet obstruction, and secondary infection[17]. However, there is a paucity of population-based research investigating the relationship between APFC and 30-day readmission rates and inpatient outcomes in AP. In recent years, a number of endoscopic interventions have been introduced for pancreatic fluid collections with acceptable safety and efficacy[18-21]. Therefore, clinical evidence regarding APFC-related readmission rates and predictors may help in improving patient outcomes.

To our knowledge, this is the first cohort study conducted in the United States with the aim of evaluating 30-day readmission rates and predictors linked to APFC in patients with AP using a multicenter database. These predictors may help to identify high-risk patients, provide an opportunity to improve the quality of care and discharge planning, reduce morbidity, and save valuable hospital resources by reducing readmissions in AP. Our findings regarding APFC-related readmission risk may also help in refining the selection criteria for a timely treatment for peripancreatic fluid collections.

MATERIALS AND METHODS

Design and data source

We utilized data from the publicly available Nationwide Readmission Database (NRD) from 2016 to 2019[22]. NRD was developed by the Agency for Healthcare Research and Quality (AHRQ) as part of the Healthcare Cost and Utilization Project (HCUP)[22]. The database includes samples from 22 state inpatient registries, accounting for approximately 50% of the population and hospitalizations in the United States[22]. Complete information about sample procedures and NRD design can be accessed at: https://www.hcup-us.ahrq.gov/nrdoverview.jsp. NRD 2016 and above uses the International Classification of Diseases, Tenth Revision (ICD-10) codes to identify diagnoses and procedures. It also contains several hospital-specific variables and predefined comorbid conditions (Elixhauser comorbidities)[23]. The NRD uses unique identification numbers to follow the same patient through multiple hospital stays within the same state. However, it does not track patients across different states or over the transition to a new year. In line with previous research, individuals who had been discharged in December were not included in our analysis because their readmissions might have occurred in January of the subsequent year[24]. This retrospective cohort study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[25].

Study population

The ICD-10 codes were used to identify index admissions with a primary diagnosis of AP (I10_DX1) (Supplementary Table 1). These admissions were further classified into: (1) Patients with a secondary diagnosis of APFC on index admission; and (2) those without a secondary diagnosis of APFC on index admission using the ICD-10 code "K86.3" (I10_DX2-40). According to the revised Atlanta classification, a pancreatic pseudocyst takes at least four weeks to form[14]. Therefore, collections developed during index hospitalization should be reported as APFC. Hence, this code is thought to be more indicative of APFC than pancreatic pseudocyst. Participants were excluded if their age was < 18 years or they had concomitant comorbid conditions such as malignant neoplasm, lymphoma, end-stage renal disease, solid organ malignancies, paraplegia, or paresis. These were considered high-risk conditions that could confound the analysis. Patients were also excluded if they had elective or same-day readmissions. The final weighted analytical cohort had a total of 673059 and 67828 patients before and after propensity score matching, respectively.

Outcome measures

The major outcomes of interest included 30-day readmission rates, the incidence of inpatient complications, length of stay (LOS), hospital charges, and factors influencing readmission among AP patients with APFC compared to those without APFC.

Statistical analysis

Propensity score matching was used to create matched cohorts, which reduced the influence of comorbid imbalances between comparative cohorts. Each case was ascribed a propensity score based on a multivariable logistic regression model that considered the baseline demographics of the patient, any Elixhauser comorbidities, and the characteristics of the institution. We then utilized a 1:1 matching algorithm by general caliper matching (without replacement) using a caliper width equal to 0.2 of the standard deviation of the propensity score[26]. In unadjusted analyses, continuous variables were reported as medians with an interquartile range (IQR) and were compared using Mann-Whitney *U* tests. Categorical variables were presented as frequencies with percentages and were compared using χ^2 tests. The discharge weights provided by the HCUP were used to obtain national estimates. All *P* values were two-sided. A univariate Cox regression model was initially used for readmission risk to report hazard ratios (HR) with a 95% confidence interval (CI) in the matched cohort. A multivariate model was then prepared for final predictors, including variables with $P < 0.20$ from univariate analysis, and results were reported as adjusted hazard ratios (aHR). The Kaplan-Meier curve was generated to display the overall risk of readmission between cases and controls, and significance was assessed using the log-rank test. The Statistical Software for Data Science (STATA) (StataCorp LLC, College Station, TX, United States), version 16.0, was used for statistical analysis. The 'pmsampsize' command in STATA was utilized to calculate the minimum sample size to assess a risk ratio of at least 50% (HR 1.5) between cases and controls for readmission. This computation indicated that a minimum sample size of 800 in each arm was sufficient.

Ethical considerations

The NRD uses de-identification and anonymization strategies to protect the privacy of patients. The present study did not require institutional review board oversight as it contains de-identified, publicly available observations that cannot be connected to or identified with any specific person. The patient consent for participation and publication of these data was also waived. According to the HCUP Data Use Agreement, any individual table cell counts of ≤ 10 have been masked to ensure privacy and compliance.

RESULTS

Patient characteristics

Clinical characteristics of patients with a primary diagnosis of AP stratified by APFC on index admission are outlined (Table 1). In the unmatched cohort of 673059 patients, 5.1% had a secondary diagnosis of APFC. The propensity-matched cohort included 33914 in each arm with a satisfactory balance of comorbidities. In the matched cohort, the median (IQR) index LOS was longer among patients with APFC compared to those without APFC [4 (3-7) *vs* 3 (2-5) days, $P < 0.001$]. The median (IQR) index hospitalization cost was higher in the APFC cohort than the non-APFC cohort [$29451 ($17292-$56774) *vs* $24418 ($14865-$42640), $P < 0.001$]. The Elixhauser comorbidities of index AP hospitalizations before and after matching were also stratified by APFC (Table 2).

Clinical outcomes in index hospitalizations

In the matched cohort, there was a higher incidence of septic shock (3.1% *vs* 1.3%, $P < 0.001$), mechanical ventilation (1.8% *vs* 0.9%, $P < 0.001$), portal venous thrombosis (4.4% *vs* 0.8%, $P < 0.001$), splenic venous thrombosis (2.4% *vs* 0.5%, $P < 0.001$), intensive care unit (ICU) level care (1.5% *vs* 0.8%, $P < 0.001$), vasopressor use (0.4% *vs* 0.2%, $P < 0.001$), diarrhea (3.2% *vs* 2.6%, $P < 0.001$), and jaundice (3.0% *vs* 1.4%, $P < 0.001$) in patients with APFC compared to those without APFC (Table 3). Participants in both cohorts also showed a higher predilection for a number of endoscopic diagnostic and therapeutic procedures.

Acute peripancreatic fluid collections and early readmission

After propensity score matching, 30-day readmissions were higher among AP patients with APFC than non-APFC (15.7% *vs* 6.5%, $P < 0.001$). For patients who had an APFC on readmission, the median readmission LOS was longer than patients without an APFC [4 (IQR 3-7) *vs* 3 (IQR 2-5) days, $P < 0.001$]. The median readmission costs were also higher among patients who had APFCs on readmission compared to the non-APFC cohort [$28282 ($17012-$50543) *vs* $22865 ($14131-$39627), $P < 0.001$]. A plethora of causes were found to be responsible for hospital readmissions in both cohorts (Figure 1). Notably, 3.5% of patients who did not have APFC at their index admission were readmitted due to a new pseudocyst/APFC diagnosis. The presence of an APFC increased the risk of readmission twofold [aHR 2.52 (95%CI: 2.40-2.65), $P < 0.001$] (Figure 2).

Clinical predictors of early readmission

A complete univariate and multivariate analysis was conducted to find independent predictors of 30-day readmission after discharge with a primary diagnosis of AP with APFC (Supplementary Table 2). A number of variables were found to increase the risk of readmission, including female gender [aHR 0.93 (95%CI: 0.89-0.98) $P = 0.01$], Elixhauser Comorbidity Index ≥ 3 [aHR 1.55 (95%CI: 1.34-1.8), $P < 0.001$], chronic pulmonary diseases [aHR 1.15 (95%CI: 1.08-1.22), $P < 0.001$], chronic renal disease (ESRD not included) [aHR 1.17 (95%CI: 1.07-1.41), $P = 0.01$], protein-calorie malnutrition [aHR 1.19 (95%CI: 1.12-1.26), $P < 0.001$], alcohol abuse [aHR 1.17 95%CI: 1.11-1.23), $P < 0.001$], substance abuse [aHR 1.11 (95%CI: 1.03-1.2), $P = 0.005$], depression [aHR 1.11 (95%CI: 1.04-1.18), $P = 0.045$], portal venous thrombosis [aHR 1.65 (95%CI: 1.47-1.85), $P < 0.001$], and splenic venous thrombosis [aHR 1.57 (95%CI: 1.34-1.84), $P < 0.001$]. Several procedures

Table 1 Clinical characteristics of patients with primary diagnosis of acute pancreatitis, stratified by acute peripancreatic fluid collections on index admission, *n* (%)

Patient characteristics	Before matching			After matching		
	No acute peripancreatic fluid collections	Acute peripancreatic fluid collections	*P* value	No acute peripancreatic fluid collections	Acute peripancreatic fluid collections	*P* value
Total patients	638801	34258		33914	33914	
Age in years at admission, median (IQR)	52.0 (39.0, 63.0)	49.0 (38.0, 59.0)	< 0.001	51.0 (39.0, 61.0)	49.0 (38.0, 58.0)	< 0.001
Age groups (yr)			< 0.001			< 0.001
18-33	108146 (16.9)	5663 (16.5)		5716 (16.9)	5641 (16.6)	
34-49	181354 (28.4)	12056 (35.2)		10353 (30.5)	11962 (35.3)	
50-64	200484 (31.4)	11619 (33.9)		11169 (32.9)	11490 (33.9)	
65-79	107936 (16.9)	4118 (12.0)		4895 (14.4)	4033 (11.9)	
≥ 80	40881 (6.4)	802 (2.3)		1781 (5.3)	788 (2.3)	
Length of stay (days), median (IQR)	3.0 (2.0, 5.0)	4.0 (3.0, 7.0)	< 0.001	3.0 (2.0, 5.0)	4.0 (3.0, 7.0)	< 0.001
Total charges (USD), median (IQR)	24238.0 (14623.0, 42043.0)	29616.5 (17365.0, 57476.0)	< 0.001	24418.5 (14865.0, 42640.5)	29451.0 (17292.0, 56774.0)	< 0.001
30-day readmission	37949 (5.9)	5363 (15.7)		2215 (6.5)	5326 (15.7)	< 0.001
Elixhauser Comorbidity Index score			< 0.001			0.093
0	49536 (7.8)	1366 (4.0)		1430 (4.2)	1366 (4.0)	
1	97030 (15.2)	3875 (11.3)		3994 (11.8)	3875 (11.4)	
2	131055 (20.5)	6353 (18.5)		6472 (19.1)	6353 (18.7)	
≥ 3	361180 (56.5)	22664 (66.2)		22018 (64.9)	22320 (65.8)	
Primary payer			< 0.001			< 0.001
Medicare	195352 (32.1)	7702 (23.8)		9348 (29.1)	7572 (23.6)	
Medicaid	140070 (23.0)	9413 (29.1)		8332 (25.9)	9340 (29.2)	
Private	213873 (35.1)	11274 (34.8)		10763 (33.5)	11163 (34.8)	
Other	60026 (9.9)	3980 (12.3)		3697 (11.5)	3958 (12.4)	
Median household income national quartile for patient ZIP code			< 0.001			0.005
1st (0-25th)	203221 (32.2)	11120 (32.9)		11169 (33.3)	11002 (32.8)	
2nd (26th-50th)	177998 (28.2)	9619 (28.4)		9373 (28.0)	9535 (28.5)	
3rd (51st-75th)	148894 (23.6)	8093 (23.9)		7756 (23.2)	8020 (23.9)	
4th (76th-100th)	100757 (16.0)	5018 (14.8)		5199 (15.5)	4955 (14.8)	
Disposition of patient (uniform)			< 0.001			< 0.001
Routine	561425 (87.9)	28082 (82.0)		29204 (86.1)	27894 (82.3)	
Transfer to short-term hospital	4817 (0.8)	753 (2.2)		275 (0.8)	742 (2.2)	
Transfer other: SNF, ICF, another type of facility	17446 (2.7)	1392 (4.1)		1074 (3.2)	1320 (3.9)	
Home health care	27211 (4.3)	2600 (7.6)		1600 (4.7)	2540 (7.5)	

Against medical advice	25350 (4.0)	1209 (3.5)		1582 (4.7)	1207 (3.6)	
Died during hospitalization	2386 (0.4)	209 (0.6)	< 0.001	169 (0.5)	198 (0.6)	0.12

SNF: Skilled nursing facility; ICF: Intermediate care facility; IQR: Interquartile range.

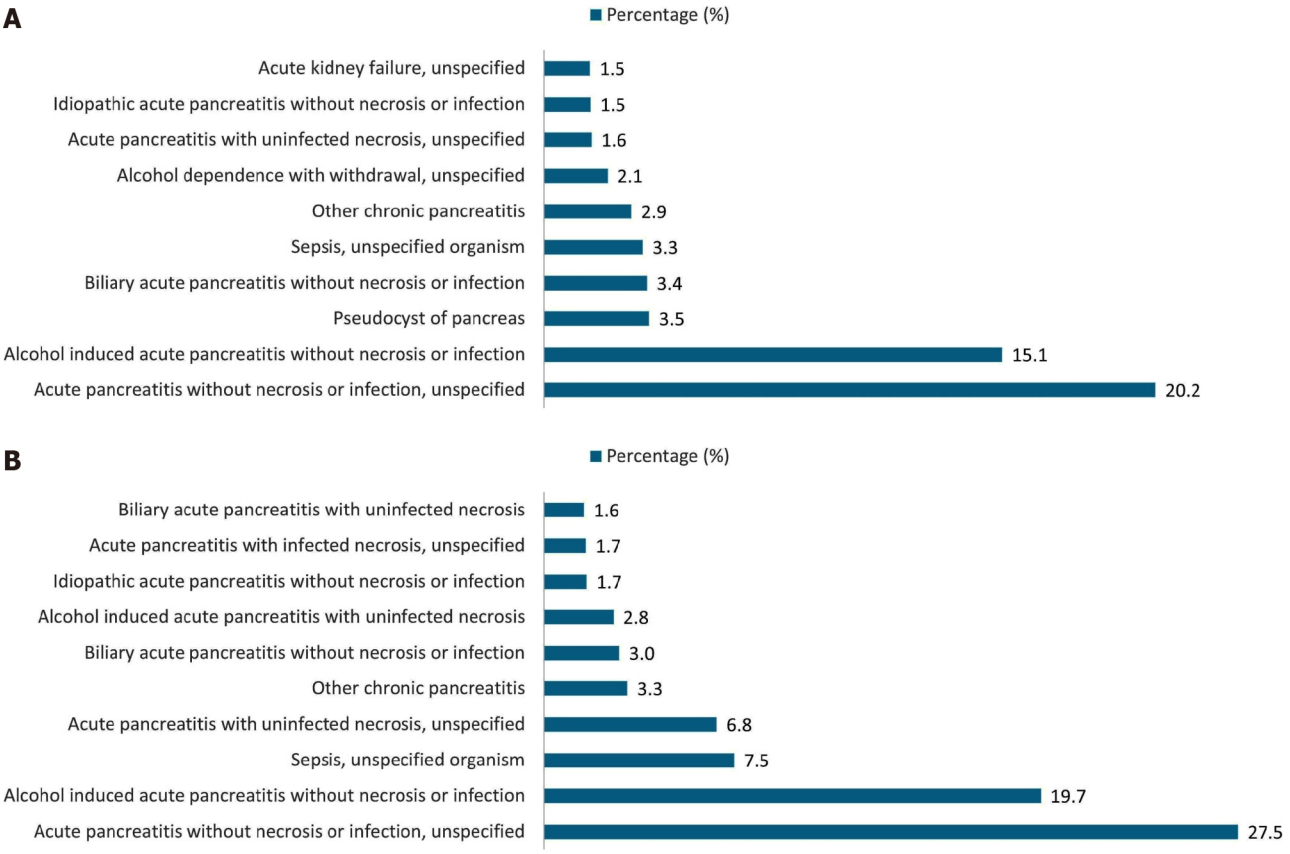

Figure 1 Absolute rates of cause-specific 30-day readmission stratified by acute peripancreatic fluid collections on index admission in the matched cohort. A: No acute peripancreatic fluid collection; B: Acute peripancreatic fluid collection.

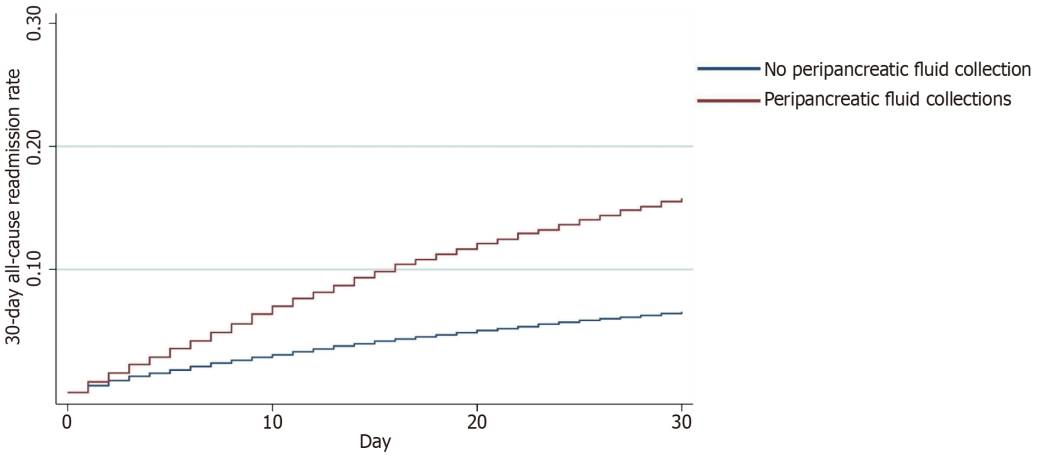

Figure 2 The 30-day readmission risk based on acute peripancreatic fluid collections present on readmission in patients with a primary diagnosis of acute pancreatitis in the matched cohort (log rank *P* < 0.01).

Table 2 Distribution of the Elixhauser comorbidities in patients with acute pancreatitis as a primary diagnosis during index hospitalizations, both before and after matching, stratified by acute peripancreatic fluid collections, *n* (%)

Factors	Before matching			After matching		
	No acute peripancreatic fluid collections	Acute peripancreatic fluid collections	*P* value	No acute peripancreatic fluid collections	Acute peripancreatic fluid collections	*P* value
Total patients	638801	34258		33914	33914	
Congestive heart failure	41809 (6.5)	1922 (5.6)	< 0.001	1804 (5.3)	1804 (5.3)	1.00
Cardiac arrhythmias	71251 (11.2)	3992 (11.7)	0.004	3745 (11.0)	3909 (11.5)	0.047
Valvular disease	13556 (2.1)	505 (1.5)	< 0.001	439 (1.3)	439 (1.3)	1.00
Pulmonary circulation	7130 (1.1)	482 (1.4)	< 0.001	380 (1.1)	380 (1.1)	1.00
Peripheral vascular disease	22966 (3.6)	1354 (4.0)	< 0.001	1248 (3.7)	1248 (3.7)	1.00
Uncomplicated hypertension	293986 (46.0)	16996 (49.6)	< 0.001	16804 (49.5)	16804 (49.5)	1.00
Chronic pulmonary diseases	99431 (15.6)	5533 (16.2)	0.004	5264 (15.5)	5440 (16.0)	0.064
Uncomplicated diabetes	90054 (14.1)	4365 (12.7)	< 0.001	4261 (12.6)	4261 (12.6)	1.00
Complicated diabetes	91044 (14.3)	4178 (12.2)	< 0.001	4049 (11.9)	4049 (11.9)	1.00
Hypothyroidism	58431 (9.1)	2132 (6.2)	< 0.001	2028 (6.0)	2028 (6.0)	1.00
Chronic renal disease	55947 (8.8)	2114 (6.2)	< 0.001	2458 (7.2)	2063 (6.1)	0.088
Liver disease	130401 (20.4)	8674 (25.3)	< 0.001	8303 (24.5)	8554 (25.2)	0.096
PUD excluding bleeding	10343 (1.6)	634 (1.9)	< 0.001	582 (1.7)	582 (1.7)	1.00
HIV/AIDS	2063 (0.3)	128 (0.4)	0.11	115 (0.3)	115 (0.3)	1.00
Rheumatoid arthritis/CVD	13799 (2.2)	569 (1.7)	< 0.001	504 (1.5)	504 (1.5)	1.00
Coagulopathy	42399 (6.6)	2778 (8.1)	< 0.001	2667 (7.9)	2667 (7.9)	1.00
Obesity	118599 (18.6)	4066 (11.9)	< 0.001	3946 (11.6)	3946 (11.6)	1.00
Weight loss	34338 (5.4)	6255 (18.3)	< 0.001	6053 (17.8)	6053 (17.8)	1.00
Fluid and electrolyte disorder	251252 (39.3)	16382 (47.8)	< 0.001	16174 (47.7)	16174 (47.7)	1.00
Blood loss anemia	1984 (0.3)	211 (0.6)	< 0.001	119 (0.4)	206 (0.6)	< 0.001
Iron-deficiency anemia	23358 (3.7)	2539 (7.4)	< 0.001	1557 (4.6)	2512 (7.4)	< 0.001
Alcohol abuse	198085 (31.0)	17070 (49.8)	< 0.001	16908 (49.9)	16908 (49.9)	1.00
Substance abuse	57163 (8.9)	4181 (12.2)	< 0.001	4074 (12.0)	4074 (12.0)	1.00
Tobacco use disorder	96674 (15.1)	4761 (13.9)	< 0.001	4616 (13.6)	4689 (13.8)	0.42
Smoking history	4722 (0.7)	276 (0.8)	0.16	199 (0.6)	274 (0.8)	< 0.001
Psychoses	7357 (1.2)	471 (1.4)	< 0.001	418 (1.2)	469 (1.4)	0.085
Depression	93044 (14.6)	5899 (17.2)	< 0.001	5445 (16.1)	5804 (17.1)	< 0.001
Complicated hypertension	72236 (11.3)	2941 (8.6)	< 0.001	3136 (9.2)	2836 (8.4)	< 0.001

PUD: Peptic ulcer disease; HIV: Human immunodeficiency virus; AIDS: Acquired immunodeficiency syndrome; CVD: Collagen vascular disorder.

were also identified as predictors, including endoscopic retrograde cholangiography (no intervention) [aHR 0.66 (95%CI: 0.52-0.85), P = 0.001], endoscopic dilation of the ampulla and biliary duct [aHR 0.65 (95%CI: 0.47-0.9), P = 0.01], and endoscopic removal of stone(s) from the biliary tract [aHR 0.69 (95%CI: 0.54-0.89), P = 0.005].

DISCUSSION

This population-based study shows that patients diagnosed with an APFC during their initial AP hospitalization have a higher 30-day readmission risk. Patients with APFC also have a higher incidence of inpatient complications, a longer LOS, and higher healthcare costs than those without APFC. A number of readmission predictors were identified to help stratify high-risk AP patients with APFC, which may aid in reducing healthcare burden.

Hospital readmission for AP has been extensively researched[9,27,28]. However, the specific association between APFC and early readmission has not been investigated. Our study revealed a significantly higher 30-day readmission risk among AP patients with APFC compared to those without APFC (aHR 2.52, P < 0.001). While there is a paucity of evidence on gender-specific outcomes of AP, readmission has often been associated with male gender[11,13,29]. However, a prospective study also identified female gender as a significant predictor of AP readmission (odds ratio 2.57, 95%CI: 1.13-5.81, P = 0.024)[30]. Our analysis also revealed female gender as a risk factor for 30-day readmission in AP patients with APFC. Consistent with previous research, an Elixhauser Comorbidity Index score of ≥ 3 was another independent predictor of 30-day readmission in our APFC cohort[9,29]. A retrospective study from the United States revealed chronic pulmonary disease as a readmission predictor after biliary AP (aHR 1.22, P < 0.001)[31]. Previous studies demonstrated that protein-energy malnutrition and chronic kidney disease may also predict readmission in patients with AP[27,32]. Similarly, early systemic anticoagulation in severe AP cases may help in reducing venous thrombosis, which may also help in decreasing readmission risk[33]. In our analysis, chronic pulmonary diseases, protein-calorie malnutrition, chronic kidney disease, and portal and splenic venous thrombosis were also identified as predictors of readmission. Therefore, it is important for clinicians to screen AP patients with APFC for these comorbidities to evaluate the readmission risk.

Alcohol-associated AP was the second most common etiology for 30-day readmissions in our study. It was also associated with worse outcomes in both the APFC and the non-APFC cohorts. Alcohol abuse has been linked in the past to increased rates of AP readmissions. In two retrospective studies from the United States, 30-day readmission rates for alcohol-related AP ranged from 12% to 70%[34,35]. Furthermore, alcoholic etiology is also independently associated with organ failure and pancreatic necrosis in index AP events[36]. Pertinently, Sorrento *et al*[37] conducted a retrospective study showing AP patients who received alcohol cessation counseling were half as likely to be readmitted after 30 days compared to those who did not get therapy (odds ratio 0.52, P = 0.046). Similarly, a post-hoc data analysis showed that 79% of patients with alcohol-related AP who received brief psychological intervention reported abstinence and no 30-day readmission for recurrent AP[38]. Moreover, the brief intervention effectively decreased gamma-glutamyl transferase levels, correlating this reduction with alcohol abstinence[38]. Notably, depression was also one of the predictors of AP readmission in our APFC cohort. Therefore, psychiatric evaluation and therapy may help to decrease readmissions in AP patients with APFC suspected to have depression or substance use disorder.

Hospital readmissions are a significant problem in the context of healthcare policy and reform[39,40]. The rates of readmission may indicate the quality of care offered by hospitals, which may be independent of patient-level factors[41]. As up to 50% of readmissions are potentially preventable, a decreased complexity of inpatient care may help improve the early readmission rate[42-44]. Our study revealed that the presence of APFC increases the risk of readmission up to twofold. In a retrospective study from the United States, AP patients with 30-day readmissions had a 4.5 times higher one-year mortality risk than those who were not readmitted (HR 4.5, 95%CI: 2.2-9.1)[45]. Therefore, the concurrent occurrence of APFC and any of the aforementioned predictors in index AP hospitalizations merits effective prognostication and clinical vigilance. AP patients with APFC may also require tailored clinical management[46]. Approximately 50% of APFCs produce minimal to no symptoms and undergo spontaneous resolution with supportive medical care[47]. However, persistent symptoms and APFC-related complications necessitate invasive treatments, including percutaneous, surgical, or endoscopic drainage procedures[48].

We also observed a higher rate of inpatient complications among AP patients with APFC compared to those without APFC. These included ICU admission, progression to septic shock, vasopressor use, mechanical ventilation, and portal and splenic venous thrombosis. In two retrospective studies from China, the systemic immune-inflammation index (SII) was considered a severity predictor and a marker of serious complications like acute kidney injury (AKI) in patients with severe AP[49,50]. In a retrospective study from Turkey, Solakoglu *et al*[51] revealed that the presence of APFC in AP patients was associated with higher values of SII and C-reactive protein. Therefore, the higher rate of inpatient complications may be explained by possible higher levels of SII in the APFC cohort compared to the non-APFC cohort. Sepsis, vasopressor use, and mechanical ventilation in AP patients may also predict other major complications, such as early AKI [52]. A prospective trial from Korea also underscored the clinical importance of close observation for late complications in patients with an early radiological identification of an APFC, especially in moderately severe and severe AP patients[53]. Therefore, APFC detection supplements the need for careful surveillance in moderate and severe AP.

In our study, AP patients with APFC showed higher odds of progression to septic shock compared to those without APFC. A prospective multicenter study from Germany showed colonization of peripancreatic fluid collections in 59% of cultures of the collections[54]. Notably, this study had no clear demarcation of the type of peripancreatic collections (50% of the patients were hospitalized for < 1 month), the positive cultures could have been related to pancreatic seeding of extrapancreatic infections, and it may be a simple colonization rather than a true infection[55]. APFCs have previously

Table 3 Clinical outcomes of patients with a primary diagnosis of acute pancreatitis during index hospitalizations, stratified by acute peripancreatic fluid collections on index admission, *n* (%)

Clinical outcomes	Before matching			After matching		
	No acute peripancreatic fluid collections	Acute peripancreatic fluid collections	*P* value	No acute peripancreatic fluid collections	Acute peripancreatic fluid collections	*P* value
Total patients	638801	34258		33914	33914	
Cholangitis	3814 (0.6)	164 (0.5)	0.005	190 (0.6)	160 (0.5)	0.11
Mechanical ventilation	3309 (0.5)	669 (2.0)	< 0.001	300 (0.9)	626 (1.8)	< 0.001
Nausea	13222 (2.1)	650 (1.9)	0.029	752 (2.2)	641 (1.9)	0.003
Diarrhea	15337 (2.4)	1089 (3.2)	< 0.001	898 (2.6)	1074 (3.2)	< 0.001
Septic shock	5693 (0.9)	1110 (3.2)	< 0.001	451 (1.3)	1057 (3.1)	< 0.001
Portal venous thrombosis	4079 (0.6)	1521 (4.4)	< 0.001	265 (0.8)	1487 (4.4)	< 0.001
Splenic venous thrombosis	3194 (0.5)	818 (2.39)	< 0.001	154 (0.5)	811 (2.4)	< 0.001
ICU level admission	3118 (0.5)	529 (1.5)	< 0.001	284 (0.8)	496 (1.5)	< 0.001
Vasopressor use	772 (0.1)	138 (0.4)	< 0.001	70 (0.2)	130 (0.4)	< 0.001
Acute kidney injury	68459 (10.7)	3843 (11.2)	0.004	4038 (11.9)	3744 (11.0)	< 0.001
New RRT during admission	8545 (1.3)	434 (1.3)	0.27	386 (1.1)	421 (1.2)	0.22
Abdominal pain	3070 (0.5)	124 (0.4)	0.002	159 (0.5)	120 (0.4)	0.019
Jaundice	8149 (1.3)	1024 (3.0)	< 0.001	461 (1.4)	1013 (3.0)	< 0.001
Obstruction of bile duct	35968 (5.6)	2340 (6.8)	< 0.001	2259 (6.7)	2319 (6.8)	0.36
Endoscopic retrograde cholangiography (no intervention)	19309 (3.0)	395 (1.2)	< 0.001	740 (2.2)	390 (1.1)	< 0.001
ERCP biliary with intervention	31525 (4.9)	1150 (3.4)	< 0.001	1339 (3.9)	1134 (3.3)	< 0.001
Endoscopic dilation of ampulla and biliary duct	9456 (1.5)	253 (0.7)	< 0.001	413 (1.2)	249 (0.7)	< 0.001
Endoscopic insertion of stent (tube) into bile duct	9784 (1.5)	751 (2.2)	< 0.001	495 (1.5)	743 (2.2)	< 0.001
Endoscopic removal of stone(s) from biliary tract	19161 (3.0)	388 (1.1)	< 0.001	759 (2.2)	384 (1.1)	< 0.001
Endoscopic biopsy of bile duct	2002 (0.3)	62 (0.2)	< 0.001	99 (0.3)	62 (0.2)	0.004
ERCP pancreatic with intervention	6282 (1.0)	1107 (3.2)	< 0.001	323 (1.0)	1094 (3.2)	< 0.001
Endoscopic insertion of stent (tube) into pancreatic duct	5263 (0.8)	1005 (2.9)	< 0.001	278 (0.8)	993 (2.9)	< 0.001
Endoscopic removal of stone(s) from pancreatic duct	1045 (0.2)	152 (0.4)	< 0.001	64 (0.2)	150 (0.4)	< 0.001
Endoscopic dilation of pancreatic duct	583 (0.1)	51 (0.1)	< 0.001	28 (0.1)	50 (0.1)	0.013

RRT: Renal replacement therapy; ERCP: Endoscopic retrograde cholangiopancreatography.

been considered low-risk entities for infections. However, our results are concerning due to the higher risk of septic shock among AP patients with APFC, possibly following infected APFCs or extrapancreatic infections. It shows the need for pertinent measures to avoid septic complications in these patients. Clinical practice guidelines from the American College of Gastroenterology, the International Association of Pancreatology (IAP), and the American Pancreatic Association (APA) require a confirmed pancreatic or extrapancreatic infection to start antibiotic treatment[56,57]. Therefore, clinicians should remain vigilant for concomitant infections in AP patients. Early diagnosis and treatment may help to avoid serious complications such as septic shock.

Inpatient complications may also occur following iatrogenic adverse events in index hospitalizations. Our data show that both cohorts underwent a variety of endoscopic diagnostic and therapeutic procedures. Moreover, published literature describes the risk of late complications weeks after the intervention, specifically for peripancreatic fluid collections[58,59]. In our analysis, several procedures were also identified as 30-day readmission predictors in AP patients with APFC, including endoscopic retrograde cholangiography (no intervention), endoscopic dilation of the ampulla and biliary duct, and endoscopic stone removal from the biliary tract. In a retrospective study from the United States, Kim *et al*[60] showed that surgical or percutaneous drainage of APFC and pancreatic pseudocysts may have a higher burden of illness and an increased local complication risk necessitating intervention compared to endoscopic drainage procedures. Therefore, it is important to opt for appropriate drainage procedures after careful patient selection[60]. Moreover, the American Society of Gastrointestinal Endoscopy recommends bacteremia risk assessment for endoscopic procedures such as drainage of peripancreatic collections[61]. Patients at risk of septic complications may receive antibiotic prophylaxis after this assessment[61]. Notably, the inpatient mortality was similar in both cohorts during index admissions (0.6% *vs* 0.5%, $P = 0.12$). However, AP patients with APFC may require targeted clinical treatment due to their higher risk of complications.

The APFC cohort showed higher hospital resource utilization compared to the non-APFC cohort in our analysis. In index hospitalizations, the LOS was longer (4 *vs* 3 days, $P < 0.001$) and costlier ($29451 *vs* $24418, $P < 0.001$). These trends could be attributed to the higher incidence of inpatient complications in our APFC cohort. Furthermore, these patients were more frequently discharged to short-term hospitals, skilled nursing facilities, and home health care. This trend may have also contributed to additional healthcare costs in the APFC cohort. The readmissions also revealed higher costs in the APFC cohort than the non-APFC cohort ($28282 *vs* $22865, $P < 0.001$). The median cost of readmissions was also higher compared to index hospitalizations. In a recent international survey, Nagy *et al*[62] revealed the efficacy of the AP discharge protocol in significantly reducing median LOS and recurrent AP-related readmission rates. Our findings could enable pancreatologists to devise novel discharge protocols that include index admission APFC and assess their impact in future research.

This retrospective cohort study has several strengths. It has a large sample size, sufficient statistical power to detect meaningful differences in outcomes, and the generalizability of the findings. Our sample population from the NRD also permits the evaluation of real-world healthcare utilization trends, including readmission rates and healthcare costs. Moreover, we used propensity-matching techniques in our analysis. In retrospective studies, these techniques can minimize confounding variables and increase the validity of the findings. Therefore, this study has pertinent implications for highlighting the clinical association between APFC and readmission risk in patients with AP. It also provides crucial insights into healthcare outcomes and utilization patterns in these patients.

Limitations

There are certain limitations to our study. One major limitation is the lack of data regarding the severity of illnesses and laboratory evaluations in the NRD. Furthermore, coding accuracy in administrative databases may vary, potentially leading to errors in identifying outcomes of interest or misclassification bias. There is also potential for selection bias in propensity score matching, which balances patient characteristics between those with and without APFC. During data extraction for our analysis, efforts were made to include only patients with an APFC diagnosis and exclude those with pancreatic necrosis (K8501, K8502, K8511, K8512, K8521, K8522, K8531, K8532, K8581, and K8582). However, there is a possibility that some misclassification may have occurred. Finally, retrospective studies cannot establish causality as they are observational and cannot account for unmeasured confounding variables.

CONCLUSION

This study reveals a correlation between the development of an APFC during index AP hospitalization and higher rates of readmission, increased inpatient complications, longer LOS, and higher healthcare costs. The readmission predictors included female gender, Elixhauser Comorbidity Index ≥ 3, chronic pulmonary diseases, chronic renal disease, protein-calorie malnutrition, alcohol abuse, substance abuse, depression, portal and splenic venous thrombosis, and certain procedures. The readmission rate for AP patients with APFC may be reduced by vigilant surveillance of these predictors, efficient infection screening, and safe interventions. Psychological evaluation and counseling strategies can also help AP patients with psychiatric comorbidities. Our analysis may enable pancreatologists and gastroenterologists to improve patient outcomes by including APFC as a factor in AP discharge protocols.

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FOOTNOTES

Author contributions: Ali H, Inayat F, Rasheed W, Afzal A, Chaudhry A, Patel P, and Rehman AU concepted and designed the study, participated in the acquisition of data, interpretation of results, writing of the original draft, and critical revisions of the important intellectual content of the final manuscript; Anwar MS, Nawaz G, Afzal MS, Sohail AH, Subramanium S, Dahiya DS, Budh D, and Mohan BP contributed to the analysis and interpretation of results and drafting of the manuscript; Adler DG reviewed, revised, and improved the manuscript by suggesting pertinent modifications; and all authors critically assessed, edited, and approved the final manuscript and are accountable for all aspects of the work.

Institutional review board statement: The data of patients was not acquired from any specific institution but rather open-access United States National Readmission Database (NRD) database. The NRD contains de-identified information, protecting the privacy of patients, physicians, and hospitals. Therefore, it was deemed exempt from the institutional review board (IRB).

Informed consent statement: Participants were not required to give informed consent for this retrospective cohort study since the analysis of baseline characteristics used anonymized clinical data.

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Ashok Kumar Nanda, Bijnya Birajita Panda, Asish Swain, Logesh Balakrishnan

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Abstract

BACKGROUND

Knowledge about refractive stabilization and the accuracy of postoperative refractive error measurements are crucial for improved patient outcomes after phacoemulsification. Existing guidelines typically recommend waiting 4-6 wk before prescribing corrective lenses. Our research focused on identifying factors that influence refractive errors in the early stages of post-cataract surgery, thus contributing to the existing literature on this topic.

AIM

To investigate the time required for refraction stability after uneventful phacoemulsification surgery.

METHODS

We compared the variation and statistical significance of the difference in spherical, cylindrical components, and the spherical equivalent between the 1- and 6-wk follow-up period in a group of 257 eyes that underwent uneventful phacoemulsification with foldable intraocular lens implantation, all performed by a single experienced surgeon. The Wilcoxon-Signed Rank Test was utilized to assess the magnitude of the change and determine its statistical significance. The refractive stability was defined as the point at which the change in spherical equivalent was within ± 0.5 dioptres for two consecutive visits.

RESULTS
The average age of the patients was 64.9 ± 8.9 yr. The differences observed in both the visits in spherical power (0.1 ± 0.2), cylinder power (0.3 ± 0.4), and spherical equivalent (0.2 ± 0.2) were minimal and not statistically significant. The majority of eyes (93.4%) achieved refractive stability within 6 wk after the surgery. The cylindrical power differed between age groups at the 6th wk post-operative and the difference was statistically significant (*P* value 0.013). There were no significant differences in refractive stability when considering sex and axial length.

CONCLUSION
Phacoemulsification with foldable intraocular lens implantation results in no significant changes in refraction for the majority of cases during the 6-wk follow-up period. Therefore, a spectacle prescription can be given at the completion of 1 wk.

Key Words: Cataract surgery; Phacoemulsification; Refraction stability; Visual acuity; Spherical equivalent

Core Tip: Achieving refractive stability in Indian eyes after uneventful phacoemulsification involves meticulous preoperative planning, including accurate biometry and corneal assessment, tailored intraocular lens selection, addressing astigmatism, patient education, and diligent postoperative follow-up. These strategies, combined with adapting to new technologies and personalized care, can significantly improve the satisfaction and visual outcomes for patients. Spectacles can be prescribed after 1 wk of completion. This study is the inaugural research focused on Indian eyes, offering an extensive analysis of the factors affecting the time needed to achieve stable vision across different patient groups.

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INTRODUCTION

Early visual recovery is a known advantage of Phacoemulsification. Determining the appropriate timing for spectacle prescription after cataract surgery is crucial in providing patients with the best possible visual outcomes and ensuring their post-operative satisfaction. The timeframe for stabilization of refraction varies among individuals and can range from a few weeks to several months[1]. Factors affecting stabilization include the healing process, corneal changes, and adjustments in the eye's focusing power secondary to stability of effective lens position. Research has shown that utilizing smaller incisions during intraocular lens (IOL) insertion leads to accelerated refractive stability and less corneal astigmatism[2-6]. This not only shortens the time required for stabilization but also enhances the clarity of uncorrected vision. Moreover, knowledge about refraction stability and precise post-operative refractive error measurements lead to better outcomes for patients undergoing sequential cataract surgeries, particularly those seeking monovision[7]. Mounting evidence indicates that refractive stabilization happens swiftly after phacoemulsification[1-7]. However, despite this, numerous guidelines continue to recommend waiting for 4-6 wk before prescribing corrective lenses. In light of this, our study aimed to complement the current literature on refractive stabilization and examine various factors that addresses refractive errors in the early stages following routine cataract surgeries.

MATERIALS AND METHODS

Methods

This was a retrospective analysis of 300 case records of patients who underwent surgery by a single surgeon between October 2022 to March 2023. Case records of 254 subjects with 257 eyes which satisfied the inclusion criteria were taken up for the study to analyze the data. Age distribution was divided into three subgroups: < 55 years, 55-65 years, and > 65 years. The axial length (AL) was subdivided into three groups comprising group 1 (< 22 mm), group 2 (22-25 mm), and group 3 (> 25 mm).

Inclusion criteria: All patients who underwent uneventful phacoemulsification underwent uncomplicated phacoemulsification cataract surgery with foldable posterior chamber IOL implantation by the same surgeon, completed both 1st wk and the 6th wk follow up and were evaluated by the same optometrist. All patients were evaluated for dry eyes before surgery and those who had Non-invasive tear break up time (NIBUT) ≥ 10 seconds were included.

Exclusion criteria: The study excluded patients who had evidence of dry eye with a NIBUT score less than 10, a prior history of glaucoma, corneal pathology, retinal pathology, traumatic and complicated cataracts that could potentially lead to a poor visual outcome postoperatively. Additionally, patients with comorbid systemic conditions, eventful postoperative periods and non-compliance to follow up were excluded. Preoperative biometry was performed by IOL Master 700 with Barret's Universal II formula.

Surgical procedure: All surgeries were performed under topical anaesthesia. A clear corneal incision of size 2.2 mm was given on the steep axis and routine steps of phacoemulsification were performed. At the end, hydrophobic, acrylic IOL were placed in the capsular bag. Postoperatively, patients received a topical antibiotics-steroids combination for 2 wk followed by topical non-steroidal anti-inflammatory agents for 4 wk along with lubricants three times daily.

Follow-up: Patients were evaluated after completion of the 1 wk and 6 wk following surgery. Keratometry readings and subjective refraction were performed by a single experienced optometrist. From the refraction measurements, the spherical, cylindrical power and the calculated spherical equivalent [SE = sph + (0.5 × cyl)] were analysed for variation. The axis values were not considered as the meridian position of postoperative astigmatism has a minimal change of effect compared to its magnitude**.**

Statistical analyses

Descriptive statistics were presented with frequency (percentage) and mean ± SD for the categorical and continuous factor, respectively. Median (interquartile range) was presented for the continuous variable while the data follows non-normal distribution. The normality of the data was assessed by using Shapiro-Wilk test. BCVA by Snellen's chart was converted to Log MAR. Paired *t*-test/Wilcoxon Signed Rank test were used to find out the significant difference between time points. Mann Whitney *U* test was used to find out the significant changes in refraction between males and females. Kruskal -Wallis test was used to find out the significant changes in the refractive error between age group and AL. Box plot graph was performed to visualize the distribution of refractive error for the post-operative time intervals. *P* value < 0.05 was considered as statistically significant. All the analysis was carried out by using statistical software STATA (14, TX, United States)

RESULTS

The study included a total of 254 subjects (257 eyes) who underwent 2.2 mm phacoemulsification with foldable in-the-bag IOL implantation, all performed by a single experienced surgeon. Three cases were bilateral. Demographic details presented in Table 1 revealed an almost equal male-to-female ratio of 1:1.01, with approximately 49.6% males and 50.4% females. The mean age was 64.9 ± 8.9 years, ranging from 34 years to 90 years. The AL subgroups comprised 55 eyes (21.4%) in group 1, group 2 included 197 eyes (76.6%) and group 3 included 5 eyes (2%). Females were more in number in both group 1 and group 3. The mean IOL power used in the study was 21.6 ± 2.3 dioptres (D) with a range between 12.5-27 D. When the ocular biometric parameters were compared at the two follow ups, it was found that there was no statistically significant difference between the keratometry 1 (K1) and keratometry 2 (K2) readings at both the 1st wk and 6th wk postoperatively in all the groups. Out of the 257 operated cases, 240 (93.4%) cases experienced either no change in spherical equivalent or a change within ± 0.5 D. In the remaining 17 (6.6%) cases, changes in cylindrical power were seen in 15 cases, spherical power in 2 cases, and both powers in none of the cases. The mean spherical error value of 0.04 ± 0.4 D (P = 0.851) at the 1-wk follow-up and 0.01 ± 0.3 D at the 6-wk follow-up, with a difference of 0.1 (0.2) between both time points. The mean cylindrical error at the 1-wk follow-up was -0.3 ± 0.7 D, which decreased to -0.2 ± 0.6 D at the 6-wk follow-up, showing a difference of 0.3 (0.40). However, these changes were not statistically significant (P > 0.05). Similarly, the mean refractive spherical equivalent error was -0.05 ± 0.4 D at the 1-wk follow-up and -0.07 ± 0.4 D at the 6-wk follow-up, with a mean difference of 0.2 (0.2) between the two time points as shown in Table 2. No statistically significant differences were found in any of the three groups between the 1st- and 6th-wk follow-up values. The refraction stability among the age groups, sex and AL's at two follow up visits of 1 wk and 6 wk has been tabulated in Tables 3-5 respectively.

DISCUSSION

Phacoemulsification techniques and foldable IOLs have become the prevailing standard of care in today's eye care setting. These modern approaches have led to smaller incision sizes, resulting in reduced induced astigmatism, which do stabilize more quickly resulting in early visual recovery. As cataract surgery has become more common among younger individuals, who are still in their pre-retirement years, and considering the growing reliance on near vision in the digital era, it becomes essential to optimize vision as soon as possible for the patients' benefit. The objective of this research was to gain a deeper understanding of the existing guidelines for spectacle prescription after cataract surgery, with the ultimate goal of enhancing the quality of life for patients during the post-operative phase.

Numerous publications have focused on determining the period required for refraction stabilization after phacoemulsification, aiming to find the most optimal time for prescribing corrective glasses to patients[8,9]. One such investigation was carried out by Berk *et al*[9], who studied 1838 eyes that underwent phacoemulsification manually and with a

Table 1 Demographic details

Parameters	Axial length, *n* (%)			Overall	*P* value[1]
	Hypermetropia	Emmetropia	Myopia		
Age in yr					0.337
< 55	8 (14.5)	21 (10.8)	2 (40)	31 (12.2)	
55–65	22 (40)	83 (42.8)	2 (40)	107 (42.1)	
> 65	25 (45.5)	90 (46.4)	1 (20)	116 (45.7)	
Sex					0.036[a]
Male	20 (36.4)	105 (54.1)	1 (20)	126 (49.6)	
Female	35 (63.6)	89 (45.9)	4 (80)	128 (50.4)	
Eye					0.861
Right eye	29 (52.7)	101 (51.3)	2 (40)	132 (51.4)	
Left eye	26 (47.3)	96 (48.7)	3 (60)	125 (48.6)	
K1-reading (1 wk)[1]					
mean ± SD	43.8 ± 1.7				
Range	(39-47.5)				
K1-reading (6 wk)[1]					
mean ± SD	43.8 ± 1.7				
Range	(39-47.8)				
K2-reading (1 wk)[1]					
mean ± SD	44.5 ± 1.7				
Range	(39.5-49.5)				
K2-reading (6 wk)[1]					
mean ± SD	44.6 ± 1.7				
Range	(40-49)				

[a]*P* value < 0.05, Chi-square/Fisher's exact test.
[1]Paired *t*-test/Wilcoxon Sign rank test.
K: Keratometry.

femtosecond laser[10]. Both methods indicated that visual acuity and refraction stabilized 3 wk after the procedure. In a separate study by Sugar *et al*[10], refraction stabilization (spherical equivalent, cylinder, and cylinder axis) was achieved within 1 wk[4]. This result was also supported by de Juan *et al*[4], who found that refraction stabilized after 1 wk of phacoemulsification surgery[4]. The present study also supports the fact that the refractive stability is achieved at the end of 1 wk of an uneventful phacoemulsification in Indian eyes.

The present study shows that refractive stability is independent of the demographic parameters such as age and sex similar to the study by Mrugacz *et al*[11] where the findings were not statistically significant[11]. Comparing the AL as a variable, it was found that the stabilization of refraction in the 3rd wk was achieved in 91% of the emmetropic, 77% of the myopic, and 46% of the hypermetropic patients, respectively. Similarly in the present study, 175/197 (88.8%) of patients in group 2 (AL = 22-25 mm), 48/55 (77.3%) in patients in group 1 (AL ≤ 22 mm) and 3/5 (60%) of patients in group 3 (AL ≥ 25 mm) experienced refractive stability within 0.5 D at the end 1 wk after the procedure. The maximum observed difference in spherical equivalent between the 1st wk and the 6 wk was 1.5 D. Stabilization of refraction at completion of 6 wk was achieved in a little higher percentage in group 2 eyes (89.8%), constant in group 3 eyes but a significantly higher percentage in 92.7% group 1 eyes.

There was no statistically significant difference observed in the mean keratometry corneal astigmatism between 1st wk and 6th wk after the surgery. The average astigmatism at 1 wk postoperatively was 0.3 ± 0.7 D, and at 6 wk postoperatively was 0.2 ± 0.6 D. Similarly, the 1st wk K1 value was 43.8 ± 1.7 D, and at 6 wk there was no change in the values, whereas 1st wk mean K2 value was 44.5 ± 1.7 D (39.5-49.5) and at 6 wk postoperatively, it was 44.6 ± 1.7 D (40–49). However, there were no statistically significant differences among all these values ($P > 0.05$ for all variables, Paired *t* test/Wilcoxon Sign rank). It suggests that refractive stability is independent of the corneal keratometry values throughout the observational period which has not been studied in any of the published literature.

Table 2 Refractive stability at two follow up visits of 1 wk and 6 wk

Parameters	Post-operative visits			*P* value[1]
	1st wk	6th wk	Difference	
Sphere				0.851
mean ± SD	0.04 ± 0.4	0.01 ± 0.3	0.1 ± 0.2	
Median (IQR)	0 (0-0)	0 (0-0)	0 (0-0)	
Range	-1.5 to 1	-0.75 to 1.5	0 to 1.5	
Cylinder				0.695
mean ± SD	-0.3 ± 0.7	-0.2 ± 0.6	0.3 ± 0.4	
Median (IQR)	-0.5 (-0.75 to 0.5)	-0.5 (-0.75 to 0.5)	0.25 (0 to 0.25)	
Range	-3.5 to 1.5	-1.5 to 1.5	0 to 2.75	
Spherical equivalent				0.277
mean ± SD	-0.05 ± 0.4	-0.07 ± 0.4	0.2 ± 0.2	
Median (IQR)	0 (-0.25 to 0)	0 (-0.25 to 0)	0.12 (0 to 0.25)	
Range	-1.75 to 1.37	-1 to 1.75	0 to 1.5	

[1]Wilcoxon Sign rank test to compare refractive error between 1st wk and 6th wk post-operative.
IQR: Interquartile range.

Table 3 Refractive stability by age distribution

Parameters	1-wk post-operative			6-wk post-operative			*P* value[1]	*P* value[2]	*P* value[3]
	Age in yr			Age in yr					
	< 55	55-65	> 65	< 55	55-65	> 65			
Sphere							0.392	0.451	> 0.99
mean ± SD	-0.05 ± 0.4	0.1 ± 0.4	0.01 ± 0.4	-0.12 ± 0.3	0.03 ± 0.4	0.04 ± 0.3			
Median (IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (-0.5 to 0)	0 (0-0)	0 (0-0)			
Range	-0.75 to 1	-0.8 to 1	-1.5 to 1	-0.75 to 0.75	-0.75 to 1.5	-0.5 to 1			
Cylinder							0.769	0.408	0.717
mean ± SD	-0.6 ± 0.1	-0.2 ± 0.7	-0.3 ± 0.7	-0.5 ± 0.3	-0.1 ± 0.6	-0.4 ± 0.7			
Median (IQR)	-0.5 (-0.75 to -0.5)	-0.5 (-0.5 to 0.5)	-0.5 (-0.75 to 0.5)	-0.5 (-0.5 to 0.5)	-0.5 (-0.5 to 0.5)	-0.5 (-0.75 to 0.5)			
Range	-0.75 to 0.5	-3.5 to 1	-1.5 to 1.5	-1 to 0.5	-0.75 to 1.25	-1.5 to 1.5			
Spherical equivalent							0.173	0.894	0.249
mean ± SD	-0.1 ± 0.4	-0.01 ± 0.4	-0.1 ± 0.4	-0.2 ± 0.3	-0.01 ± 0.4	-0.1 ± 0.3			
Median (IQR)	0 (-0.37 to 0)	0 (-0.25 –0.25)	0 (-0.25 –0)	0 (-0.31– 0)	0 (-0.25 –0.25)	0 (-0.25 –0)			
Range	-0.87 to 1	-1.75 to 1	-1.5 to 1.37	-1 to 0.5	-1 to 1.75	-1 to 0.87			
P value[4]	0.358			0.287			-		
P value[5]	0.052			0.013					
P value[6]	0.246			0.057					

[1]Wilcoxon Sign rank test to compare the patients with age < 55 yr between 1st wk and 6th wk post-operative.
[2]Wilcoxon Sign rank test to compare the patients with age 55 yr to 65 yr between 1st wk and 6th wk post-operative.
[3]Wilcoxon Sign rank test to compare the patients with age > 65 yr between 1st wk and 6th wk post-operative. Boldface indicates statistical significance.
[4]Kruskal-Wallis test for comparing age and spherical power.

[5]Kruskal-Wallis test for comparing age and cylindrical power.
[6]Kruskal-Wallis test for comparing age and spherical equivalent.
IQR: Interquartile range.

Table 4 Refractive stability by sex wise distribution

Parameters	1 wk postoperative			6 wk postoperative			*P* value[1]	*P* value[2]
	Male	Female	*P* value[3]	Male	Female	*P* value[3]		
Sphere			0.523			0.281	0.698	0.933
mean ± SD	0.06 ± 0.4	0.03 ± 0.4		0.05 ± 0.4	-0.03 ± 0.3			
Median (IQR)	0 (0-0)	0 (0-0)		0 (0-0)	0 (0-0)			
Range	-1.5 to 1	-0.75 to 1		-0.5 to 1.5	-0.75 to 1			
Cylinder			0.979			0.585	0.788	0.783
mean ± SD	-0.3 ± 0.6	-0.3 ± 0.7		-0.3 ± 0.6	-0.2 ± 0.6			
Median (IQR)	-0.5 (-0.8 to 0.5)	-0.5 (-0.8 to 0.5)		-0.5 (-1.5 to 1)	-0.5 (-0.5 to 0.5)			
Range	-1.5 to 1.25	-3.5 to 1.5		-1.5 to 1	-1 to 1.5			
Spherical equivalent			0.651			0.724	0.419	0.464
mean ± SD	-0.04 ± 0.4	-0.1 ± 0.4		-0.1 ± 0.4	-0.1 ± 0.4			
Median (IQR)	0 (-0.25 to 0)	0 (-0.25 to 0)		0 (-0.25 to 0)	0 (-0.25 to 0)			
Range	-1.5 to 1.12	-1.75 to 1.37		-0.87 to 1.75	-1 to 1			

[1]Wilcoxon Sign rank test to compare the male between 1st wk and 6th wk post-operative.
[2]Wilcoxon Sign rank test to compare the female between 1st wk and 6th wk post-operative.
[3]Mann Whitney *U* test.
IQR: Interquartile range.

Table 5 Refractive stability by axial length wise distribution

Parameters	1 wk post operative			6 wk postoperative			*P* value[1]	*P* value[2]	*P* value[3]
	Axial length in mm			Axial length in mm					
	< 22	22-25	> 25	< 22	22-25	> 25			
Sphere							> 0.99	0.989	0.317
mean ± SD	0.1 ± 0.4	0.1 ± 0.4	-0.2 ± 0.4	0.04 ± 0.3	0.01 ± 0.3	-0.2 ± 0.3			
Median (IQR)	0 (0-0)	0 (0-0)	0 (-0.75 to 0)	0 (0-0)	0 (0-0)	0 (-0.5 to 0)			
Range	-0.75 to 1	-1.5 to 1	-0.75 to 0	-0.75 to 1	-0.75 to 1.5	-0.5 to 0			
Cylinder							0.765	0.783	0.782
mean ± SD	-0.1 ± 0.7	-0.3 ± 0.6	-1.6 ± 1.7	-0.2 ± 0.6	-0.2 ± 0.7	-0.8 ± 0.4			
Median (IQR)	-0.5 (-0.6 to 0.5)	-0.5 (-0.75 to 0.1)	-0.75 (-3.5 to -0.5)	-0.5 (-0.5 to 0.5)	-0.5 (-0.75 to 0.5)	-0.75 (-1.2 to -0.5)			
Range	-1.5 to 1.25	-1.5 to 1.5	-3.5 to 0.5	-1.25 to 0.75	-1.5 to 1. 5	-1.25 to 1.5			
Spherical equivalent							0.982	0.19	0.477
mean ± SD	-0.01 ± 0.4	-0.1 ± 0.4	-0.6 ± 0.7	-0.04 ± 0.3	-0.1 ± 0.4	-0.4 ± 0.3			
Median (IQR)	0 (-0.25 to 0.25)	0 (-0.25 to 0)	-0.37 (-1 to 0)	0 (-0.25 to 0.25)	0 (-0.25 to 0)	-0.37 (-0.62 to 0)			
Range	-1 to 1	-1.5 to 1.37	-1.75 to 0	-1 to 1	-1 to 1.75	-0.75 to 0			
P value[4]	0.559			0.657			-		

P value[5]	0.123	0.226
P value[6]	0.091	0.164

[1]Wilcoxon Sign rank test to compare the patients with axial length < 22 mm between 1st wk and 6th wk post-operative.
[2]Wilcoxon Sign rank test to compare the patients with axial length 22 to 25 mm between 1st wk and 6th wk post-operative.
[3]Wilcoxon Sign rank test to compare the patients with axial length > 25 mm between 1st wk and 6th wk post-operative.
[4]Kruskal-Wallis test for comparing axial length and spherical power.
[5]Kruskal-Wallis test for comparing axial length and cylindrical power.
[6]Kruskal-Wallis test for comparing axial length and spherical equivalent.
IQR: Interquartile range.

A study by Landers *et al*[12] tried to find out whether any refractive shift occurs with different varieties of IOL, however they could not find any statistically significant difference when IOL with rigid haptics or with pliable haptics were used[12]. In the present study however, only one type of foldable IOLs with pliable haptics were used and therefore there was no chance of bias due to variable lens models with different types of haptics, which may impact the lens stabilization period.

In the present study, out of 17 eyes which had a significant refractive change only 2 eyes had change in sphere more than 0.5 D which can be due to anterior or posterior displacement of the IOL bag complex due to capsular contraction. The rest of the 15 eyes out of 257 eyes which is about 6% eyes had a change in astigmatism more than 0.5 D between their 1-wk and 6-wk values. The possible reasons may be increase in phaco energy used for emulsification which could have modulated the wound more to cause a delayed change in corneal astigmatism or wound stretch caused by insertion of thicker IOL or IOL with high dioptric value. Since we have not analysed the refraction stability considering these variables, it is not possible to conclude the reason for the delayed change of astigmatism.

The strength of our study is that a single experienced optometrist performed the subjective manifest refraction at both the postoperative visits, the 1st wk and end of 6 wk which gives more precise results than an auto refractometer as is also supported by Kozlov *et al*[13]. Secondly, an optical biometer was used to perform the ocular biometric measurements which can perform better than an ultrasound biometer. The high postoperative emmetropic accuracy in our study was also due to the use of Barret's universal II IOL power calculator and using the steep axis for incision.

The limitation of the study is the retrospective nature of the study due to which a better standardization could not be done. Moreover, there were no control points between the 1st wk and 6 wk after surgery. Even though the shorter and longer eyes took longer than the normal length eyes for refraction stability, it was not statistically significant. One limitation of our study is that if we give early spectacle dispensing, the subjective satisfaction of patients after 6-8 wk of spectacle dispensing needs to have been studied. A change in spherical or cylindrical value of more than 0.5 D will surely affect the subjective acceptance of the patients and with increase in digital platform use, the eye strain would be much more. So, in 6.6% of our patients, an early dispensing of spectacle would have led to a refractive instability and would have made a necessity to change their spectacle power after the last follow up.

Therefore, a prospective cohort study including a greater number of short and long eyes, subjective satisfaction questionnaire outcomes and a long term follow up may be needed in future to evaluate the AL dependency and the time frame for refractive stability.

CONCLUSION

Refraction after cataract surgery stabilizes at the end of the 1st wk in a majority of cases, especially in eyes in between 22 to 25 mm AL and therefore is a safe time for prescribing corrective glasses to all these patients irrespective of age and sex.

FOOTNOTES

Author contributions: Nanda AK contributed to the formulation of the study and data collection; Panda BB contributed to data analysis and interpretation, manuscript preparation and editing; Swain A contributed to data collection; Balakrishnan L contributed to data statistical analysis.

Institutional review board statement: This study was reviewed and approved by Ethics Committee of Kar Vision Eye Hospital, No. 23-06.

Informed consent statement: Patients were not required to give informed consent to the study because the study was retrospective, and analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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ORIGINAL ARTICLE

Observational Study

Hepatic grooves: An observational study at laparoscopic surgery

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Abstract

BACKGROUND

In traditional descriptions, the upper surface of the liver is smooth and convex, but deep depressions are variants that are present in 5%-40% of patients. We sought to determine the relationship between surface depressions and the diaphragm.

AIM

To use exploratory laparoscopy to determine the relationship between surface depressions and the diaphragm.

METHODS

An observational study was performed in all patients undergoing laparoscopic upper gastro-intestinal operations between January 1, 2023 and January 20, 2024. A thirty-degree laparoscope was used to inspect the liver and diaphragm. When surface depressions were present, we recorded patient demographics, presence of diaphragmatic bands, rib protrusions and/or any other source of compression during inspection.

RESULTS
Of 394 patients, 343 had normal surface anatomy, and 51 (12.9%) had prominent surface depressions on the liver. There was no significant relationship between the presence of surface depressions and gender nor the presence of rib projections. However, there was significant association between the presence of surface depressions and diaphragmatic muscular bands ($P < 0.001$).

CONCLUSION
With these data, the diaphragmatic-band theory has gained increased importance over other theories for surface depressions. Further studies are warranted using cross sectional imaging to confirm relationships with intersectional planes as well as beta-catenin assays in the affected liver parenchyma.

Key Words: Liver; Variant; Vein; Hepatic; Surgery

Core Tip: The upper surface of the liver is usually smooth and convex, but deep depressions are present in 5%-40% of patients. Laparoscopic surgery provides an opportunity to examine the relationship between surface depressions and the diaphragm. This study showed that 51 (12.9%) of 394 patients had prominent surface depressions on the liver. There was significant association between the presence of surface depressions and diaphragmatic muscular bands, giving credence to the diaphragmatic-band theory for surface depressions.

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INTRODUCTION

In conventional anatomic descriptions, the liver has a smooth, rounded convex upper surface contacting the diaphragm. However, there are deep depressions on the diaphragmatic surface of the liver in 5%[1] to 40%[2] of unselected patients. Some authorities theorized that these deep depressions may be the result of post-mortem compression from the ribs[3,4] or the diaphragm[4-6]. These theories were summarily dismissed because evidence of compression could not be proven in many cases.

However, when laparoscopic surgery became the standard of care for many operations in the late 20th Century, this presented an additional tool to examine the compression theory in living humans. In this paper, we examined the relationship of the liver and diaphragm in patients undergoing laparoscopic upper abdominal surgery.

MATERIALS AND METHODS

This study was carried out at a tertiary referral hospital in Trinidad & Tobago after permission was secured from the local institutional review board. All participants provided written informed consent prior to enrollment. Two independent observers were present in the operating room during all laparoscopic upper gastro-intestinal operations performed between January 1, 2023 and January 20, 2024. After insertion of the visual trocar, a thirty-degree laparoscope was directed to the upper abdomen to visually inspect the liver and diaphragm. This was strictly an observational study and no change in operative treatment was imposed by the study methodology.

When surface depressions were present, we recorded the patient demographics, presence of diaphragmatic bands, rib protrusions and/or any other source of compression during inspection.

Inclusion and exclusion criteria

All patients who underwent laparoscopic upper abdominal operations during the study period were potential candidates. We excluded patients who had dense adhesions in the upper abdomen because these patients would require adhesiolysis to view the liver. Patients with diaphragmatic pathologies that could alter the liver surface, such as diaphragmatic hernias and penetrating trauma, were excluded. We also excluded patients with liver pathologies that may have affected the liver surface, including cirrhosis, liver metastases, and primary liver neoplasms.

Definitions

Diaphragmatic bands were defined as visible, well-defined muscular bundles that connected the central tendon of the diaphragm to the inner aspect of the lower thoracic cage[7]. When present, we identified the band by using the name of

Table 1 Distribution of operations

Operations	*n*
Cholecystectomy	259
Liver resection	15
Pancreaticoduodenectomy	3
Gastrectomy	4
Distal pancreatectomy	14
Colectomy	59
Adrenalectomy	2
Small bowel resection	8
Adhesiolysis	12
Ventral hernia repair	18

the corresponding hepatic segment. For example, a band that was present to the immediate left of the gallbladder fossa was named a "segment 4b band." Any localized projection extending ≥ 5 mm into the peritoneum and associated with a visible rib was considered rib protrusion.

Statistical analyses

The data were divided into two groups: Patients with surface depressions and those with conventional surface anatomy. We compared the presence of diaphragmatic bands and rib projections between the two groups. We used the Statistical Package for the Social Sciences Version 16 to perform statistical analyses, with significance assigned to a P value < 0.05. The data in each group were compared using the Chi Square test for categorical variables between the groups.

RESULTS

Over the study period, 190 men and 204 women underwent varied upper abdominal operations using the laparoscopic approach, as outlined in Table 1. Of the total 394 patients, 343 (87.1%) had normal surface anatomy (Figure 1A), and 51 (12.9%) had prominent surface depressions on the liver (Figure 1B).

Surface depressions were present in 30 (11.5%) males and 21 (16%) females, with no significant gender predilection (P = 0.1977). The mean age of patients with surface depressions was 47.73 years (range 26-70; median 48; SD ± 9.04).

Rib projections were present in 28 (7.1%) patients. This included 22 males and 6 females, with a mean age of 49.6 years (range 30-70; median 48; SD ± 9.16). When rib projections were present, they were associated with floating ribs (Figure 1C). Only 3 of the patients with rib projections also had surface depressions (P = 0.7153). And, when present their location did not correspond to the location of the surface depression. Most of the rib projections were present at the lateral costal margin corresponding to hepatic segments V and VI.

There were 51 patients with surface depressions. This included 32 males and 19 females at a mean age of years (range 26-70; median 49; SD ± 9.53). Table 2 demonstrates that there was significant association between the presence of surface depressions and diaphragmatic muscular bands (Figure 1B). Of 51 patients with surface depressions, there were 40 (78.4%) with co-existent diaphragmatic bands (P < 0.001). Additionally, when present there was a spatial relationship in all cases.

DISCUSSION

There is documentation that the prevalence of surface depression in cadaveric studies in the Caribbean diaspora ranges from 12% to 15%[8]. In this study, we were able to demonstrate that the prevalence of surface depressions in living persons (12.9%) was comparable to that documented in cadaveric studies.

We could not demonstrate a reliable association with rib projections in this study. When rib projections were present, there was no spatial relationship with the surface depressions. Most rib projections appeared at the lateral aspect of the chest, over hepatic segments 5/6. This, we believe, is sufficient to discount the theory of rib compression. Some proposed that these are post-mortem changes that are due to the ribs continuously compressing a single area on the diaphragmatic surface after death, creating a corresponding depression[3,4]. It is true that proponents of this theory make the argument that this accounts for the increased prevalence on cadaveric studies[4,9]. However, our study demonstrates that these surface depressions are visible in living patients and that there is no association with projecting ribs.

There was a significant association with diaphragmatic muscular bands. Interestingly, this relationship was not borne out when we performed cadaveric studies in our population[8] - probably because the muscular bands would lose muscle

Table 2 Relationship of potential compression in surface depressions

Parameter	Conventional surface anatomy (343)	Surface depressions (51)	*P* value
Rib protrusion	25	3	0.7153
No rib protrusion	318	48	
Diaphragmatic bands	153	40	< 0.0001
No bands	190	11	
Male (*n* = 264)	233	30 (11.5%)	0.1977
Female (*n* = 131)	110	21 (16%)	

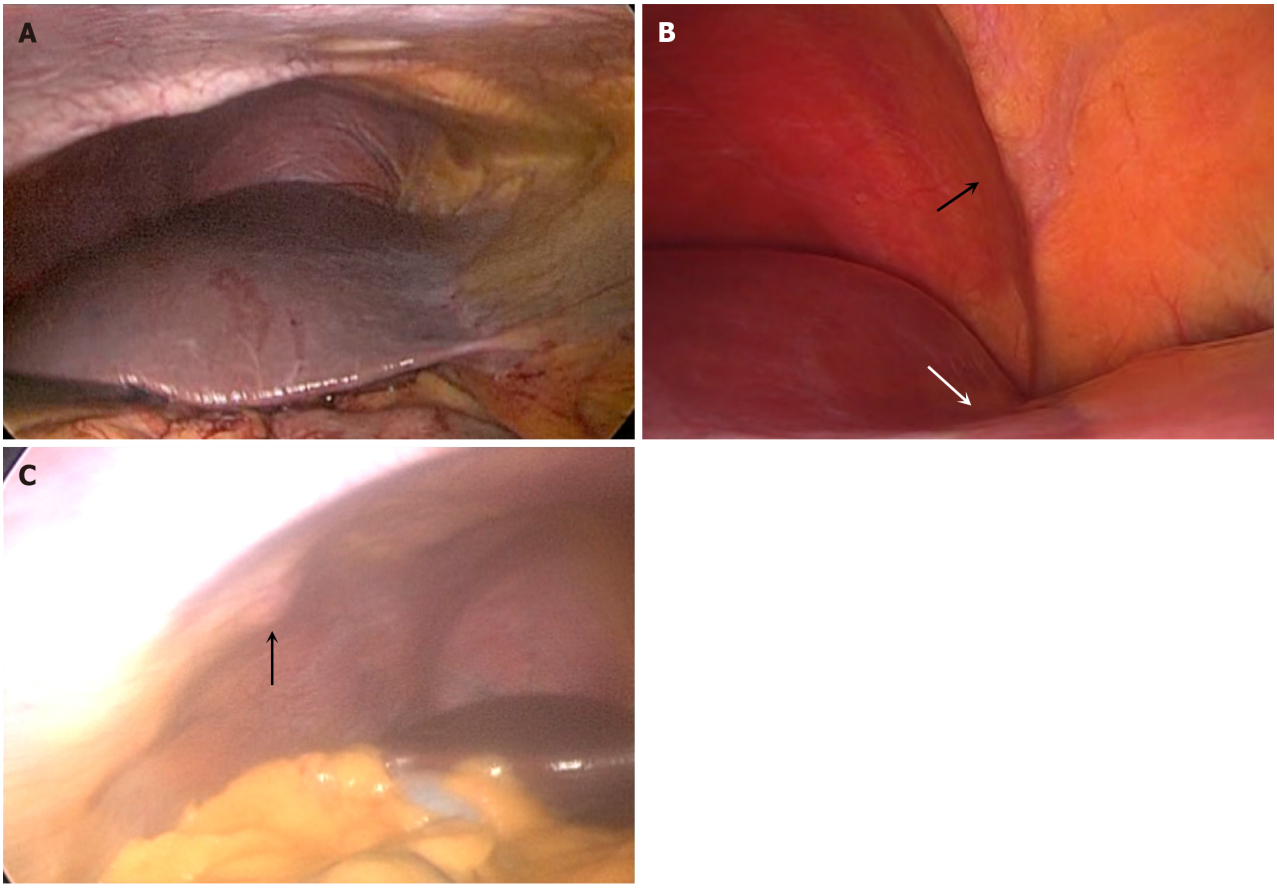

Figure 1 Laparoscopic view. A: Laparoscopic view of the sub-phrenic space. There is normal anatomy present with a smooth, rounded liver surface contacting a normal diaphragm; B: Laparoscopic view of the right sub-phrenic space. There is a well-defined diaphragmatic band (black arrow) that corresponds to a depression (white arrow) on the surface of segment VIII of the liver; C: Laparoscopic view of the sub-phrenic space, with a rib projection (arrow) present. Note the normal smooth appearance of the liver surface.

tone in the post-mortem state.

In light of this new data, we believe that the diaphragmatic band theory should be reconsidered as one of the main causes of surface depressions. Originally proposed by Macchi *et al*[2], the diaphragmatic band theory suggested that there are "weak zones" on the liver surfaced susceptible to compression. Newell and Morgan-Jones[4] also noted that the orientation of the bands was related to the surface depressions – similar to the orientation in our study.

There is existing data from animal studies demonstrating that hepatocytes are sensitive to changes in concentration of beta-catenin, resulting in altered liver size and shape[10,11]. Further data in humans demonstrated that there is upregulation of beta-catenin when there is hepatic venous congestion in right heart failure[12]. This may give some insight into the relationship with diaphragmatic bands. It is well known that the liver is supplied in sections[13], and that there is relatively less vascularity at intersectional planes[14]. This forms the basis of hepatic segmentectomy[15]. It also stands to reason that, with less vascularity, there would be down-regulation of beta-catenin levels at these watershed areas. We propose that this, combined with the presence of diaphragmatic bands, could explain the presence of surface depressions. This could also explain the observations by Ono *et al*[16] and later by Macchi *et al*[2] that surface depressions were closely related to inter-sectional planes. This could be the basis of further study by attempting to correlate the occurrence of

surface depressions with intersectional planes and also with measurements of beta-catenin levels in the corresponding liver parenchyma. These measurements could not be made with the existing study model.

CONCLUSION

The findings of this study show a significant relationship between diaphragmatic bands and liver surface depressions. With this data, the diaphragmatic-band theory has gained increased importance over other theories for surface depressions. Further studies are warranted using cross sectional imaging to confirm relationships with intersectional planes as well as beta-catenin assays in the affected liver parenchyma.

FOOTNOTES

Author contributions: Cawich SO, Gardner MT, Kedambady RS, Craigie M, Thomas D, Mohammed F and Johnson S designed the research; Cawich SO, Gardner MT and Kedambady RS, performed the research; Kedambady RS, Mohammed F and Johnson S contributed analytic tools; Kedambady RS, Thomas D and Johnson S analyzed the data; Cawich SO, Gardner MT, Kedambady RS, Craigie M and Thomas D wrote the paper; All authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Expression levels of K_{ATP} channel subunits and morphological changes in the mouse liver after exposure to radiation

Ming Zhou, Tao-Sheng Li, Hiroshi Abe, Hideo Akashi, Ryoji Suzuki, Yoshio Bando

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Abstract

BACKGROUND

ATP sensitive K^+ (K_{ATP}) channels are ubiquitously distributed in various of cells and tissues, including the liver. They play a role in the pathogenesis of myocardial and liver ischemia.

AIM

To evaluate the radiation-induced changes in the expression of K_{ATP} channel subunits in the mouse liver to understand the potential role of K_{ATP} channels in radiation injury.

METHODS

Adult C57BL/6 mice were randomly exposed to γ-rays at 0 Gy (control, n = 2), 0.2 Gy (n = 6), 1 Gy (n = 6), or 5 Gy (n = 6). The livers were removed 3 and 24 h after radiation exposure. Hematoxylin and eosin staining was used for morphological observation; immunohistochemical staining was applied to determine the expression of K_{ATP} channel subunits in the liver tissue.

RESULTS

Compared with the control group, the livers exposed to 0.2 Gy γ-ray showed an initial increase in the expression of Kir6.1 at 3 h, followed by recovery at 24 h after exposure. Exposure to a high dose of 5.0 Gy resulted in decreased expression of Kir6.1 and increased expression of SUR2B at 24 h. However, the expression of

Kir6.2, SUR1, or SUR2A had no remarkable changes at 3 and 24 h after exposure to any of these doses.

CONCLUSION

The expression levels of Kir6.1 and SUR2B in mouse liver changed differently in response to different radiation doses, suggesting a potential role for them in radiation-induced liver injury.

Key Words: Radiation exposure; ATP-sensitive K^+ channel; Mouse; Liver

Core Tip: ATP-sensitive K^+ (K_{ATP}) channels are ubiquitously distributed in various cell types and tissues, including the liver; however, their role in the development of radiation-induced liver damage remains unknown. In the current study, the expression of K_{ATP} channel subunits in the liver tissue changed dose-dependently in response to radiation exposure, suggesting their potential role in radiation-induced liver injury.

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INTRODUCTION

Radiation-induced liver injury is becoming increasingly familiar with the advancements in technology and increased exposure to low levels of radiation during medical procedures and treatment[1]. Ionizing radiation can induce functional and structural changes in the liver, leading to radiation-induced liver diseases[2]. Using radiation therapy for the treatment of hepatic tumors is rapidly increasing; the application of radiation treatment for tumors in the liver, right lower lung, distal esophagus, and whole abdomen is on the increase, resulting in cell damage to the non-tumor compartments of the liver[3,4]. Clinically, radiation-induced liver diseases have drawn particular attention from physicians recently as the main challenge in delivering curative tumors doses[5]. Novel approaches to prevent radiation-induced liver damage during radiation therapy are urgently needed.

ATP-sensitive K^+ (K_{ATP}) channels are observed in various cells and tissues[6], including pancreatic β cells[7], skeletal and cardiac muscles[8-11], and the brain[12-14], kidneys[15,16], and liver[17,18]. These channels may close or open depending on the concentration of intracellular ATP, playing a protective role during ischemia[19] by coupling changed energy metabolism *via* membrane potential[20]. K_{ATP} channels are formed by two kinds of subunits, pore-forming subunits (Kir6.1 or Kir6.2) and regulatory subunits, such as sulfonylurea receptors (SUR1, SUR2A, and SUR2B), with a hetero-octamer structure of four pore-forming subunits and four regulatory subunits[21,22]. Different subunit compositions show different physiological and pharmacological functions[23,24]. K_{ATP} channels in the hypothalamus are involved in glucose homeostasis by controlling glucagon and catecholamines[25]. The liver has diverse functions, such as producing bile, metabolism of ingested nutrients, eliminating waste products, and synthesizing protein; it is directly involved in glucose metabolism[4], which influences the change in intracellular ATP concentrations to which K_{ATP} channels are sensitive. K_{ATP} channels regulate the proliferation of primary rat hepatocytes[26]. A previous study revealed that K_{ATP} channel subunits are widely localized in hepatocytes, hepatic stellate cells, and Kupffer cells[18].

Radiation exposure is generally classified as low (< 0.5 Gy), moderate (0.5-5 Gy), or high (> 5 Gy)[27]. Radiation causes ultrastructural and biochemical changes in hepatocytes, which are known to depend on lipid metabolism[28]. Radiation exposure is one of the causes of hepatic fibrosis after liver injury recovery[29-31]. However, there is no particular information on the role of K_{ATP} channel subunits in radiation-induced liver damage. This study investigated the expression of K_{ATP} channel subunits in the liver after different irradiation doses.

MATERIALS AND METHODS

Animals

Twenty adult (8-12 wk old) male C57BL/6 mice (CLEA Japan, Inc.) were applied for this study. The study was approved by the Nagasaki University Japan's Institutional Animal Care and Use Committee and conducted following the Committee's protocols. All animal procedures followed the institutional and national guidelines. The mice were kept under constant environmental conditions in group cages with a condition of 12-h light/dark cycle and given food and water *ad libitum*. The mice were randomly separated into four groups: (1) Control, without γ-ray irradiation (n = 2); (2) lower dose (0.2 Gy) exposure (n = 6); (3) medium dose (1 Gy) exposure (n = 6); and (4) high dose (5 Gy) exposure (n = 6).

Radiation exposure

The mice were placed in a special cage for radiation exposure to assess radiation-induced liver injury corresponding to dose dependency. PS-3100SB (Pony Industry Co., Ltd., Osaka, Japan), a γ-ray irradiation system with a Cs source was used. The mice were exposed to a dose of 0, 0.2, 1, or 5 Gy/min. The mice were not anesthetized before irradiation.

Preparation of samples

After radiation exposure, the mice were sacrificed for experiments at 3 and 24 h later. Mice were euthanized *via* general anesthesia by intraperitoneal injection of pentobarbital (160 mg/kg), followed by perfusion with cold Zamboni's fixatives through the heart. The liver was quickly collected and further fixed for 6 h in the same fixative, and then put into 30% sucrose over 12 h until it sank down to the bottom. Cryosections were cut (8 to10 μm thick) with a Leica CM1950 cryostat, thaw-mounted on MAS-coated glass slides (Matsunami Industries, Kishiwada, Japan), and kept at -20 ℃ until use.

Histological and immunohistochemical staining

After air-drying for 30 min, several sections were stained with Mayer hematoxylin and eosin (H&E) for morphological observations. The others sections were pre-incubated with 5% normal donkey serum for 30 min, and then incubated with K_{ATP} channel subunit antibodies, which were goat anti-human Kir6.1 and Kir6.2 (Santa Cruz Biotechnology), and rabbit anti-rat SUR1, SUR2A, and SUR2B[18] at 1:500 dilution. After washing in PBS three times, 5 min each, the sections were incubated with biotinylated rabbit anti-goat IgG (BA5000; Vector Laboratories, Inc., Burlingame, CA, United States) or biotinylated goat anti-rabbit IgG (BA1000; Vector Laboratories, Inc.) at 1:200 each for 30 min, and subsequently with the ABC complex (Vectastain ABC Kit; Vector Laboratories, Inc.) according to the manufacturer's instructions. Immunoreactions were carried out with 3,3'-diaminobenzedine in the presence of 0.003% H_2O_2, and counterstained with Mayer's hematoxylin.

To show how the pore-forming subunit Kir6.1 co-localizes with the regulatory subunit SUR2B in the liver after radiation, sections were treated with primary goat anti-human Kir6.1 and rabbit anti-rat SUR2B antibodies, each diluted 1:500, for one night (12 h) at room temperature. After being washed three times in PBS for 15 min, the sections were incubated with a mixture of secondary antibodies labeled with Alexa 488 and Alexa 594 (A21432 and A21207; Molecular Probes, Inc., Eugene, OR), each diluted 1:200. Fluorescence images were acquired using an Olympus microscope (BX51; Tokyo, Japan).

RESULTS

Dose dependency of radiation-induced injury in mouse liver

Compared with the control (Figure 1), 0.2 Gy induced hepatocyte atrophy and sinusoid enlargement 3 h after exposure (Figure 2A), which recovered 24 h after irradiation (Figure 2B). Degeneration of hepatocytes, constriction of the hepatocyte cord, and enlarged sinusoids were observed in the 1.0 Gy and 5.0 Gy dose groups 3 h after exposure (Figure 2C and D), with the effects worsening 24 h after γ-ray irradiation (Figure 2E and F).

Radiation-induced changes in expression of K_{ATP} channel subunits

Immunohistochemical staining revealed that immunoreactivity for Kir6.1 was widely observed in hepatocytes in the control group (Figure 3A). The intensity of immunoreactivity was higher in the central vein and lower in the distal area in the cell membrane and cytoplasm of hepatocytes. Under high magnification, punctate immunoreactive products of Kir6.1 were observed in the cytoplasm of hepatocytes (Figure 3B). Compared with the control group, the expression of Kir6.1 was enhanced in the liver 3 h after exposure (Figure 4A), which almost recovered 24 h after exposure (Figure 4B) to 0.2 Gy. In the livers exposed to the medium (1 Gy) and high (5 Gy) doses of γ-ray, the expression level of Kir6.1 was decreased at 3 h (Figure 4C and D) and significantly reduced at 24 h after irradiation (Figure 4E and F).

No remarkable changes were observed in the expression levels of Kir6.2 (Figure 5), SUR1 (Figure 6), or SUR2A (Figure 7) in hepatocytes at 3 and 24 h after irradiation at all doses.

In contrast, compared with the control group (Figure 8A and B), SUR2B expression showed no apparent changes at 3 and/or 24 h after exposure to 0.2 and 1 Gy (Figure 9A-D), whereas SUR2B expression clearly increased 3 h after exposure to 5.0 Gy (Figure 9C), and more highly increased after exposure to 5.0 Gy (Figure 9E), and more highly increased 24 h after exposure to 5.0 Gy (Figure 9F).

Immunofluorescence double staining for Kir6.1 and SUR2B after 1 Gy γ-ray irradiation showed only partial co-localizion of Kir6.1 with SUR2B (Figure 10A-C). However, at 24 h after mice were exposed to 5 Gy, despite a lower expression level of Kir6.1 compared to the lower dose group, almost all Kir6.1 was co-localized with SUR2B, described as punctate immunoreactivity (Figure 10D-F).

DISCUSSION

Radiation effects were considered mainly due to nuclear DNA damage and their management by repair mechanisms[32]. The liver has numerous functions including bile production, ingested nutrient metabolism, waste product elimination, glycogen storage, and protein synthesis[4]. Additionally, it is radiosensitive, particularly in young animals[33]. Liver cancer, with high mortality, is one of the most common solid tumors in the world[34]. Although surgical resection is the

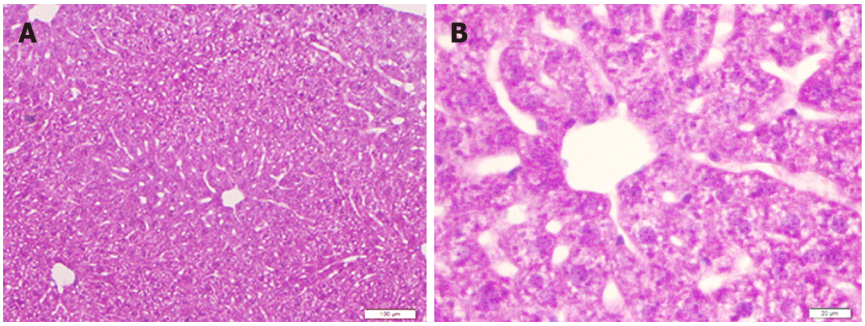

Figure 1 Liver sections of mice in the control group. A: Low magnification; B: High magnification. H&E staining. Bars: 100 μm (A); 20 μm (B).

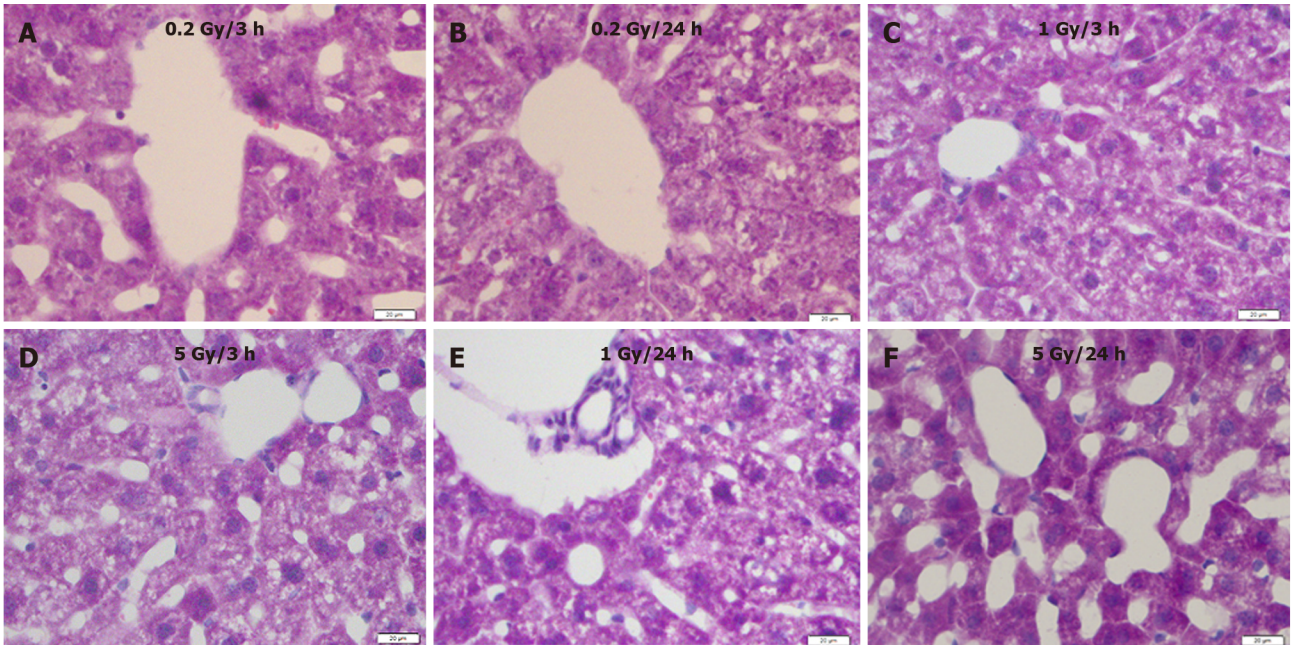

Figure 2 Mouse liver sections showing morphological changes with different radiation doses at different time points. A: 3 h after exposure to low dose (0.2 Gy); B: 24 h after exposure to low dose (0.2 Gy); C: 3 h after exposure to medium dose (1 Gy); D: 3 h after exposure to high dose (5 Gy); E: 24 h after exposure to medium dose (1 Gy); F: 24 h after exposure to high dose (5 Gy). H&E staining. Bars: 20 μm.

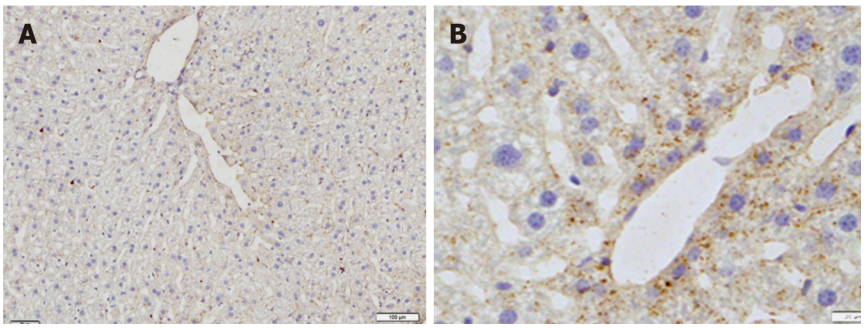

Figure 3 Immunostaining for Kir6.1 in the liver of mice in the control group. A: Low magnification; B: High magnification. Bars: 100 μm (A); 20 μm (B).

first choice of treatment for hepatocellular carcinoma, radiation therapy is an actively used and essential treatment modality for locally advanced hepatocellular carcinoma or tumors in the upper abdomen, right lower lung, distal section of the esophagus, or whole body[4,5,35]. Liver injury induced by radiation is a significant complication of radiotherapy for liver cancer or other upper abdominal malignant tumors that have poor pharmacological therapeutic options because normal tissues exposed to radiation during radiotherapy or radioscopy will suffer injury and metabolic alterations[36]. A significant challenge in radiotherapy is to promote the tolerance of normal cells by protecting their transformation into malignant cells, thus increasing patients' quality of life[37].

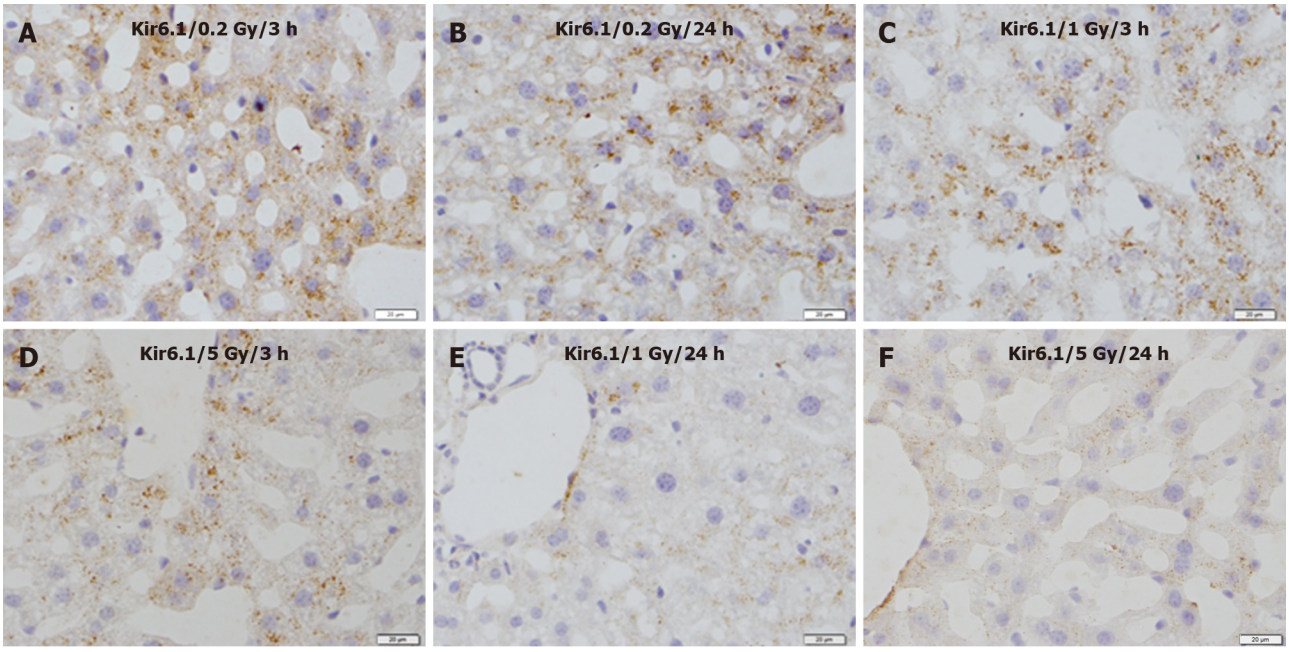

Figure 4 Immunostaining for Kir6.1 in the liver of mice after γ-ray irradiation with different doses. A: 3 h after exposure to low dose (0.2 Gy); B: 24 h after exposure to low dose (0.2 Gy); C: 3 h after exposure to medium dose (1 Gy); D: 3 h after exposure to high dose (5 Gy); E: 24 h after exposure to medium dose (1 Gy); F: 24 h after exposure to high dose (5 Gy). Bars: 20 μm.

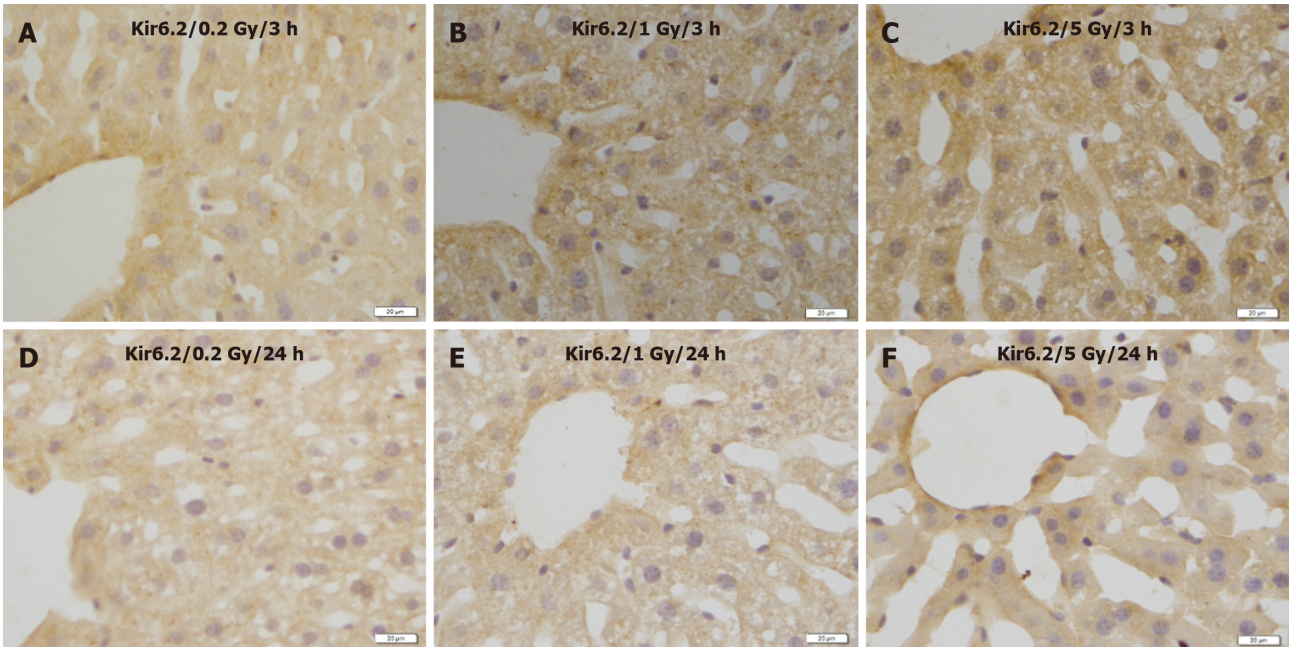

Figure 5 Immunostaining for Kir6.2 in the liver of mice after γ-ray irradiation with different doses. Representative images show no remarkable change induced by irradiation. A: 3 h after exposure to low dose (0.2 Gy); B: 3 h after exposure to medium dose (1 Gy) ; C: 3 h after exposure to high dose (5 Gy); D: 24 h after exposure to low dose (0.2 Gy); E: 24 h after exposure to medium dose (1 Gy); F: 24 h after exposure to high dose (5 Gy). Bars: 20 μm.

Ionizing radiation induces oxidative stress and is pivotal in the pathogenesis of cellular damage induced by radiation; dietary antioxidants were suggested to protect against irradiation-induced subsequent tissue damage[37].

Oxidative stress from generating reactive oxygen species (ROS) results in an imbalance in cells' pro-oxidant/antioxidant status[38]. Cell survival and proliferation capacities are highly dependent on the activation and regulation through molecular components of organelles, especially mitochondria, which are pivotal in maintaining cellular homeostasis and genomic stability after irradiation[32].

Mitochondria ensure general cellular metabolism and high-energy provision *via* ATP and oxidative phosphorylation to maintain cell survival and homeostasis. During oxidative phosphorylation, a few electrons may react with oxygen, forming ROS and oxidative stress, thus constituting non-negligible targets for irradiation[32]. Excessive production of ROS results in mitochondria dysfunction; a series of pathological changes can be induced by radiation *via* direct energy

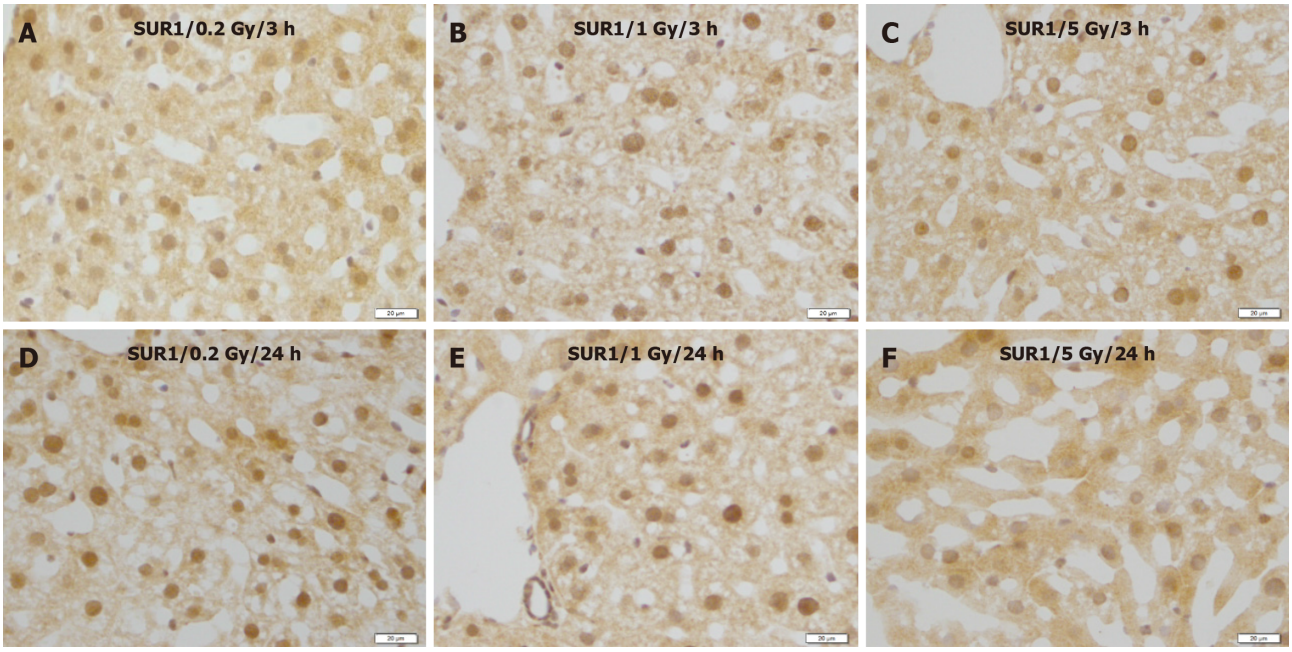

Figure 6 Immunostaining for SUR1 in the liver of mice after γ-ray irradiation with different doses. These images show no remarkable changes in different time courses. A: 3 h after exposure to low dose (0.2 Gy); B: 3 h after exposure to medium dose (1 Gy); C: 3 h after exposure to high dose (5 Gy); D: 24 h after exposure to low dose (0.2 Gy); E: 24 h after exposure to medium dose (1 Gy); F: 24 h after exposure to high dose (5 Gy). Bars: 20 μm.

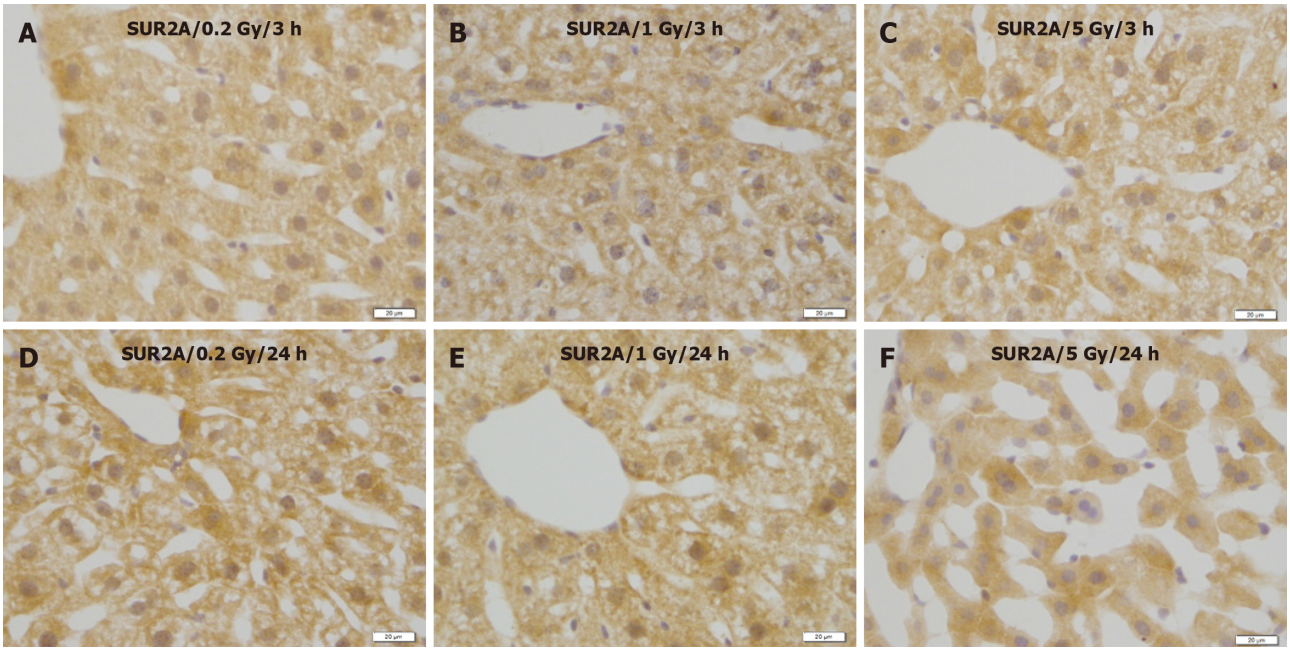

Figure 7 Immunostaining for SUR2A in the liver of mice after γ-ray irradiation with different doses. A: 3 h after exposure to low dose (0.2 Gy); B: 3 h after exposure to medium dose (1 Gy); C: 3 h after exposure to high dose (5 Gy); D: 24 h after exposure to low dose (0.2 Gy); E: 24 h after exposure to medium dose (1 Gy); F: 24 h after exposure to high dose (5 Gy). Bars: 20 μm.

deposition or reactive free radical generation[36]. Mitochondria are involved in oxidative stress-induced apoptotic cell death[39].

Upregulation of antioxidant enzymes was mimicked by treatment with the sulfonylureas tolbutamide and gliclazide (K_{ATP} channel blockers and inhibitors); the loss of K_{ATP} channel activity conferred resistance to radiation[39], suggesting a probable role for K_{ATP} channels in radiation-induced injury.

K_{ATP} channels are composed of two subunits: Kir6.x and SURs. Different combinations of these subunits are expressed in different cells and tissues and exhibit different physical characteristics[40]. K_{ATP} channels are involved in various physiological conditions, including hyperglycemia, hypoglycemia, ischemia, and hypoxia[19,40]. Kir6.1 and SUR2B are important for cellular energy and stability in the cell membrane and the mitochondrion. Mitochondria generate energy for the cell to maintaining cellular homeostasis, genomic stability, and sensitivity to irradiation[32]. Cellular oxidative

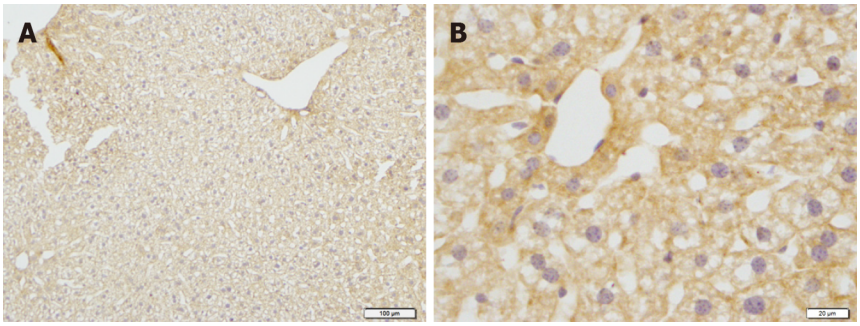

Figure 8 Immunostaining for SUR2B in the liver of mice in the control groups. A: Low magnification image; B: High magnification image. Bars: 100 μm (A); 20 μm (B).

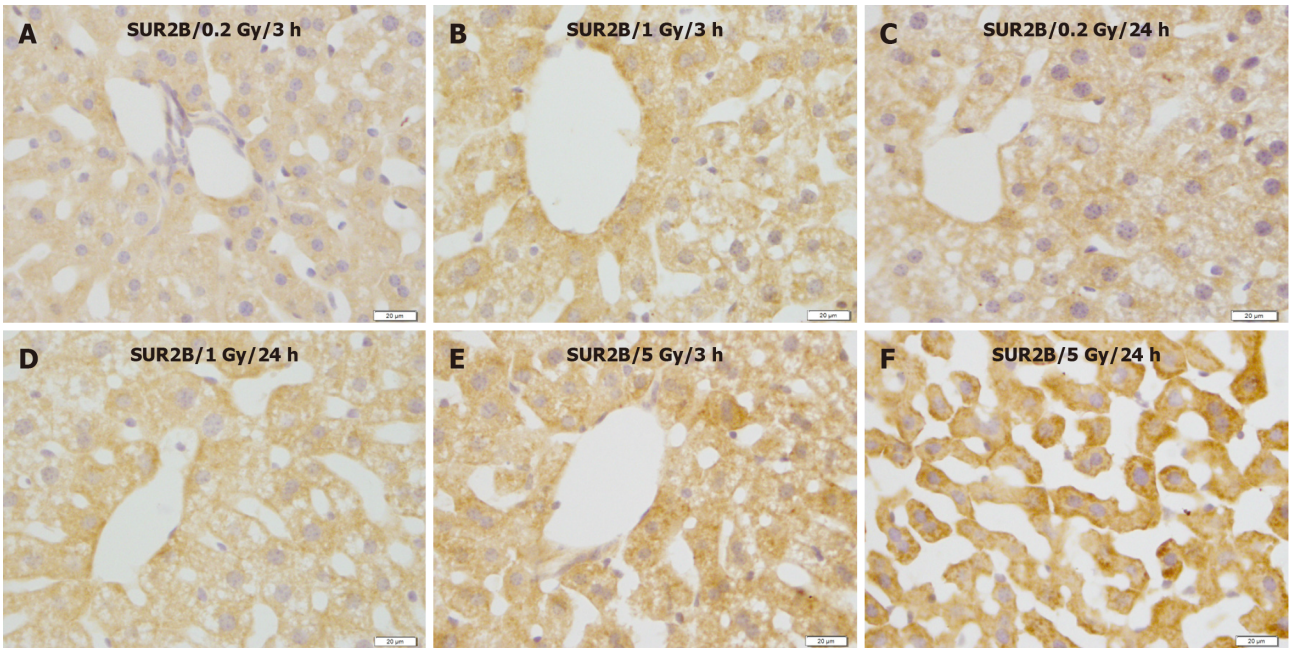

Figure 9 Immunostaining for SUR2B in the liver of mice after γ-ray irradiation with different doses. SUR2B expression showed no obvious change 3 and 24 h after exposure to 0.2 and 1.0 Gy, but increased remarkably 24 h after exposure to 5 Gy. A: 3 h after exposure to low dose (0.2 Gy); B: 3 h after exposure to medium dose (1 Gy); C: 24 h after exposure to low dose (0.2 Gy); D: 24 h after exposure to medium dose (1 Gy); E: 3 h after exposure to high dose (5 Gy); F: 24 h after exposure to high dose (5 Gy). Bars: 20 μm.

stress can affect the function of potassium channels, influencing the vasomotor function in multiple disease states[41]. We have previously reported that the mitochondrial K_{ATP} channel subunits Kir6.1 and SUR2B protect heart tissues damaged by ischemia, hypoxia, or other conditions [10,11]. A recent study demonstrated that Kir6.1 and SUR2B are essential for functional brain hyperemic responses and vascular smooth muscle cell differentiation[42]. In this study, high levels of γ-ray irradiation in mice induced liver injury (Figure 2) and modulated K_{ATP} channel subunits expression, resulting in decreased Kir6.1 (Figure 4) and increased SUR2B (Figure 9) levels. These phenomena were documented using double fluorescence immunostaining (Figure 10). Although Kir6.1 had a lower expression level, the two subunits were more co-localized. High expression levels of SUR2B regulate the K_{ATP} channel in the mouse liver during high-dose irradiation. Thus, redox modulation of potassium channel activity induced by radiation is a crucial mechanism for regulating smooth muscle membrane potential, ultimately influencing hepatocyte injury.

Resent research has revealed that opening the K_{ATP} channels may increase liver tolerance to ischemia/reperfusion injury and reduce the systemic inflammatory responses[43]. The K_{ATP} channel enhances liver regeneration after partial hepatectomy[17]. However, another study showed that glibenclamide, a K_{ATP} channel blocker, prevents acute radiation-induced liver injury[44]. The effects of K_{ATP} channels in different organs and tissues are not always evident; for example, the K_{ATP} channel blocker glibenclamide ameliorated the ischemia-reperfusion injury in the rat testis, whereas 5-hydroxy-decanoate does not; K_{ATP} openers mediate pharmacological post-conditioning in the heart or brain, but cannot reduce ischemia-reperfusion injury in the kidneys, intestine, and lungs[45].

Thus, the expression levels of the K_{ATP} channel subunits in the liver and their potential roles in radiation-induced liver injury still need to be investigated.

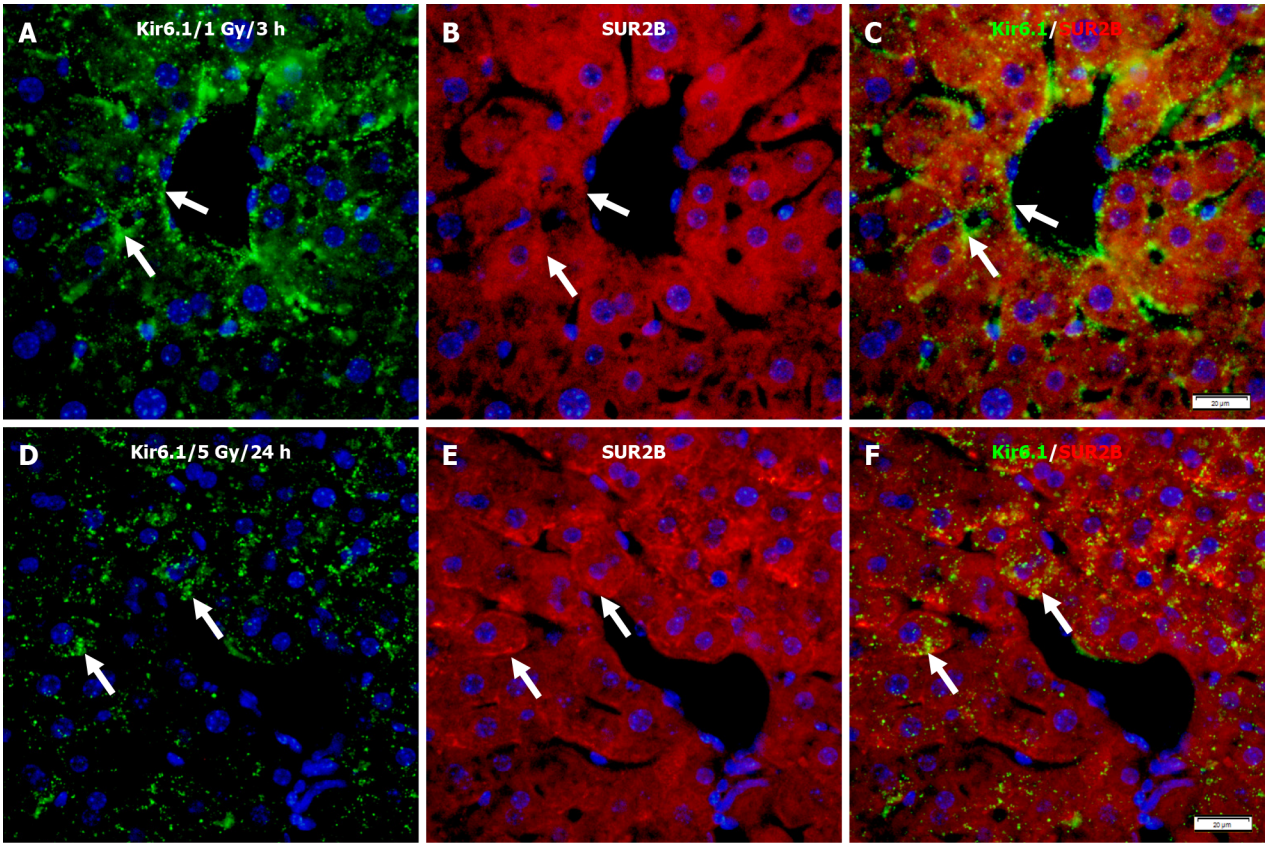

Figure 10 Immunofluorescence double staining for Kir6.1 and SUR2B in the liver of mice after irradiation with different doses. A-C: Representative images show the expression of Kir6.1 alone (green, A), SUR2B alone (red, B), and their co-localization (white arrows, C) 3 h after 1 Gy exposure; D-F: Representative images show the expression of Kir6.1 alone (green, D), SUR2B alone (red, E), and their co-localization (F) 24 h after 5 Gy exposure (white arrows, F). Bars: 20 μm.

This study observed that high levels of γ-ray exposure induced liver injury in mice and modulated K_{ATP} channel subunit expression, resulting in decreased Kir6.1 and increased SUR2B levels. These findings suggest that K_{ATP} channels affect radiation-induced liver injury, and may serve as parameters for the evaluation of radiation injury. Further investigation is required to elucidate the precise roles of K_{ATP} channels in radiation-induced liver injury.

CONCLUSION

The expression of the K_{ATP} channel subunits Kir6.1 and SUR2B in the liver changed differently in response to different radiation doses, suggesting that they play a potential role during radiation-induced liver injury.

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FOOTNOTES

Author contributions: Zhou M performed the main experiments, collected the data, and wrote the manuscript; Akashi H and Suzuki R participated in the treatment of animals; Li TS designed the program and experiment; Li TS, Abe H, and Bando Y supervised the investigation and approved the manuscript for publication. This study was a collaboration between Zhou M and Li TS. Both authors applied for and obtained the funds for this research project, and have made indispensable contributions to completion of this research and manuscript preparation as the co-corresponding authors. This collaboration between Zhou M and Li TS is crucial for the publication of this manuscript.

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SYSTEMATIC REVIEWS

Experimental models of high-risk bowel anastomosis in rats: A systematic review

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Abstract

BACKGROUND

Anastomotic leaks remain one of the most dreaded complications in gastrointestinal surgery causing significant morbidity, that negatively affect the patients' quality of life. Experimental studies play an important role in understanding the pathophysiological background of anastomotic healing and there are still many fields that require further investigation. Knowledge drawn from these studies can lead to interventions or techniques that can reduce the risk of anastomotic leak in patients with high-risk features. Despite the advances in experimental protocols and techniques, designing a high-quality study is still challenging for the investigators as there is a plethora of different models used.

AIM

To review current state of the art for experimental protocols in high-risk anastomosis in rats.

METHODS
This systematic review was performed according to The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. To identify eligible studies, a comprehensive literature search was performed in the electronic databases PubMed (MEDLINE) and Scopus, covering the period from conception until 18 October 2023.

RESULTS
From our search strategy 102 studies were included and were categorized based on the mechanism used to create a high-risk anastomosis. Methods of assessing anastomotic healing were extracted and were individually appraised.

CONCLUSION
Anastomotic healing studies have evolved over the last decades, but the findings are yet to be translated into human studies. There is a need for high-quality, well-designed studies that will help to the better understanding of the pathophysiology of anastomotic healing and the effects of various interventions.

Key Words: High-risk anastomosis; Rats; Experimental models; Bowel; Colon; Anastomotic leak; Colon resection; Inflammatory bowel disease; Intra-abdominal sepsis; Bursting pressure

Core Tip: Anastomotic leakage (AL) is a fatal complication after colorectal surgery, with high morbidity and mortality rates. AL rate is increased under emergency conditions. This review can be used as a tool to standardize and refine future research leading to studies that can be translated to human research regarding bowel anastomoses under complicated conditions.

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INTRODUCTION

Colon diseases are among the most common disorders encountered by general surgeons, since more than 600000 surgical procedures are performed in the United States annually for management of colon-related disorders[1]. The most common indication for colorectal surgery is colon cancer, while other indications for resection include diverticular disease, ischemic colitis, stoma reversal or inflammatory bowel disease[2].

Despite the increased safety of colorectal surgery due to minimally invasive surgery and perioperative management advances, anastomotic leakage (AL) remains a fatal complication of colonic anastomosis, leading to increased morbidity and mortality rates, permanent stoma impaired oncological outcomes and poorer quality of life postoperatively[3,4]. AL rate varies by the level of anastomosis and is approximately 1%–3% for ileocolic anastomosis, 6%–12% for left colon anastomosis, and 3%–19% for colorectal anastomosis[5]. In general, risk factors associated with increased AL rate can be classified to preoperative, intraoperative and postoperative factors[6]. Among them, colonic resection and anastomosis in the emergency setting has been proven to be an independent risk factor for anastomotic dehiscence, as well as death after AL[7,8]. A recent prospective multi-centre study by the American Association for the Surgery of Trauma showed that anastomotic failure rate after emergent bowel resection and colo-colonic anastomosis had a failure rate of 23%, while in patients managed with an open abdomen the same rate was approximately 22%[9].

The traditional surgical dictum suggested that in emergency colectomy due to obstruction or peritonitis of large bowel origin, construction of a colostomy was imperative independently of the severity of peritonitis or the patient's condition [10]. Stomas are also associated with numerous early and late complications, as well as impaired Quality of Life and reduced rate of closure, when performed in the emergency setting[11]. However, recently emerged evidence propose the safety of anastomosis with diverting stoma under circumstances in cases of feculent or purulent peritonitis[12]. Decision regarding anastomosis in the setting of large bowel obstruction is mostly determined by the cause and the site of the obstruction[13]. In addition to peritonitis and obstruction, a series of other local and systemic conditions impair wound healing and render the construction of anastomosis perilous[14].

Animal experimental models constitute the basis of experimental study of colorectal anastomosis healing and permit monitoring of anastomotic healing with use of functional tests, clinical scores, molecular examination and histopathological examination[15]. Pommergaard *et al*[16], in their systematic review, evaluated the different experimental animal models that have been used for study of colorectal AL. Animal models reported in the literature include mice, pigs, rats, dogs and rabbits, with mice and pigs being the proposed by the authors experimental models for mimicking AL[16]. In addition, numerous studies have investigated the role of different potential therapeutic agents in healing of anastomosis in experimental models both under normal and pathological conditions, such as inflammation, peritonitis, obstruction,

ischemia or jaundice[17]. However, pathophysiological mechanisms behind the formation of high-risk anastomoses for research aims have been scarcely evaluated and reported in the literature.

The aim of the present systematic review was to identify and classify types of experimental anastomosis that mimic high-risk colonic anastomosis in humans, in order to provide a guide for formation of standardized and easily reproducible experimental colonic anastomosis models and a translational basis for future clinical trials. The authors aspire that this review will be used as a guidance to facilitate future experimental studies, as it will give a comprehensive overview of current state of the art concerning the experimental protocols in high-risk anastomosis in rats. Experimental models to induce high-risk anastomosis are well described in the literature and will not be in the focus of the current review. On the other hand, an appraisal on the different methods of anastomotic quality assessment is lacking in the literature and will be attempted. This will hopefully help clarify questions that emerge during experimental protocol designing.

MATERIALS AND METHODS

Protocol and registration

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, after approval of the study protocol by all authors[18]. A completed PRISMA checklist has been submitted.

Search strategy

To identify eligible studies, a comprehensive literature search (last search date as of 18/10/2023) was performed in the electronic databases PubMed (MEDLINE) and Scopus, covering the period from conception until 18 October 2023 (Supplementary material). Exported results were imported into Rayyan (Qatar Computing Research Institute, Doha, Qatar), and deduplication was performed[19]. Titles and abstracts were initially screened by two independent researchers (Georgios Ntampakis and Elissavet Anestiadou), and irrelevant articles were excluded. Potentially eligible full-text articles were screened for inclusion, according to the inclusion criteria and disagreements were resolved through discussion with participation of a third researcher (Orestis Ioannidis). In addition, a manual search was performed using the snowball methodology to identify and include any relevant studies in the list of references of the included articles.

Inclusion and exclusion criteria

Specific inclusion criteria were established prior to literature search: (1) Rats as experimental models; (2) English language; and (3) anastomotic healing as primary outcome. Studies were excluded if: (1) Were *in vitro*, human studies or studies in experimental models other than rats; (2) the definition of each outcome was not clearly stated; (3) incomplete information was reported; (4) they were kin studies; and (5) they were studies with no free access or no available full text.

Data extraction and synthesis

Two researchers (Georgios Ntampakis and Elissavet Anestiadou) reviewed all eligible studies. The following data were extracted and recorded: (1) First author; (2) year of publication and country of origin; (3) total number of animals used; (4) animal model (type, age, sex); (5) type of anastomosis; (6) type of intervention; (7) sampling day; (8) tests used for anastomosis assessment; and (9) characteristics of high-risk anastomosis.

Data analysis

Datasets were stratified based on the mechanism and type of high-risk anastomosis. High heterogeneity regarding anastomosis types, intervention types, method of assessment and reported outcomes of interest between the included studies rendered the conduction of a meta-analysis non-applicable. As a result, qualitative analysis of the outcomes was conducted. The provided results were assembled to identify strengths, weaknesses, and trends of each intervention.

RESULTS

As a result of our search strategy 102 studies were included in our systematic review and were categorized based on the mechanism used to create a high-risk anastomosis. The different categories of high-risk anastomosis were ischaemia, colitis, malnutrition, peritonitis, obstruction, radiotherapy, ischaemia/reperfusion injury, chemotherapy and immunosuppression. A flow diagram is shown in Figure 1.

Ischaemia

Ischaemia models were some of the commonest high risk anastomosis models found. In total 15 different studies were included. Ischaemia was induced with ligation or electrocauterization of a wide segment of rat's left colon just above the pubic symphysis. The different kinds of intervention attempted are shown in Table 1[20-40].

Various tests were used to assess the quality of the anastomosis, such as bursting pressure (BPR), Hydroxyproline levels (HP), different cytokines, oxidative stress markers, macroscopic and microscopic assessment using different protocols.

Table 1 Ischaemia models of high-risk anastomosis

Ref.	Year	Sample size	Intervention	Test	Sampling day
Uzun *et al*[20]	2008	56	Sildenafil citrate	NOx, thiobarbituric acid reactive substances, glutathione	3/7
Karliczek *et al*[21]	2009	34	Visible light spectrometry evaluation	BPR *in situ*, tensile strength	3/7
Coneely *et al*[22]	2010	50	Ketotifen	BPR *ex situ*, HP, IL-6, VEGF	4
Karataş *et al*[23]	2010	24	Amelogenin	BPR *ex situ*, HP	4
Kaya *et al*[24]	2010	48	Tadalafil	BPR *ex situ*, HP, histology (as per Ehrlich *et al*[25])	4
Adas *et al*[26]	2011	40	Bm-MSCs 1 × 10^6	BPR *in situ*, HP	4/7
Karatepe *et al*[27]	2011	40	Adrenomedulin	BPR *ex situ*, HP, spectrophotometry, MDA, NOx, TNF-a, IL-6, VEGF, histology	3/7
Kennelly *et al*[28]	2011	30	Electrical field stimulation	BPR *ex situ*, HP, IL-6, VEGF	4
Sümer *et al*[29]	2011	30	Pentoxifylline and vinpocetine	BPR *in situ*, HP, histology (as per García *et al*[30])	5
Yoo *et al*[31]	2012	60	AdMSC 1 × 10^6	BPR *ex situ*, weight loss, macroscopic (adhesions as per van der Ham and Kort[32], strictures, ulcers), histology (as per Phillips *et al*[33]), wound infection, ileus, mortality	7
Wu *et al*[34]	2013	24	Triptolide	Histology, calprotectin, MPO, INF-g, IL-4, IL-17, TGF-b	56
Portilla-de Buen *et al*[35]	2014	180	Fibrin glue	BPR *ex situ*, macroscopic, microscopic, HP	5
Boersema *et al*[36]	2016	40	Hyperbaric oxygen	Macroscopic (as per Zühlke *et al*[37]), histology (as per Phillips *et al*[33]), serum creatinine	3/7
Ruiz-Luque *et al*[38]	2019	93	Alprostadil	BPR *in situ*, macroscopic (as per Knightly *et al*[39]), histology (as per García *et al*[30])	8
Kayapinar *et al*[40]	2021	60	CORM-2	BPR *ex situ*, HP, glutathione, MDA, histology (as per Ehrlich *et al*[25])	3/7

BmMSC: Bone marrow derived mesenchymal stem cells; AdMSC: Adipose tissue derived mesenchymal stem cells; CORM-2: Carbon monoxide releasing molecule-2; NOx: Nitric oxide; BPR: Bursting pressure; HP: Hydroxyproline; IL: Interleukin; VEGF: Vascular endothelial growth factor; MDA: Malondialdehyde; TNF-a: Tumor necrosis factor–alpha; MPO: Myeloperoxidase; INF-g: Interferon-gamma; TGF-b: Tumor growth factor-beta.

Colitis

In total, 6 different studies of anastomosis in colitis environment were included. In these models, Dextran sodium sulphate or 2,4,6-Trinitrobenzene sulfonic acid were used to induce acute colitis. In one study, intra-jejunal injection of iodoacetamide had been used. The different kinds of interventions can be found in Table 2[41-50]. The tests used to assess the quality of the anastomosis were: Anastomotic BPR, HP, different cytokines, oxidative stress markers, macroscopic and microscopic assessment using different protocols.

Malnutrition

In total, 6 different studies of anastomosis in malnourished rat were included. Starvation for 7-15 d or 50% food restriction for 28 d were the different methods used to induce malnutrition. The different kinds of interventions attempted, can be found in Table 3[51-58]. The tests used to assess the quality of the anastomosis and animal status were BPR, tensile strength, body weight changes, nutrition markers, macroscopic and microscopic assessment using different protocols.

Obstruction

In total, 6 different studies of anastomosis in rats with obstruction were included. Obstruction methods were silk ligation of the distal colon or use of a silicone ring to mimic decrease in bowel diameter. There was also a study with obstructive jaundice, where the distal common bile duct was tied. Krarup *et al*[59] introduced a new method of inducing bowel obstruction by laparoscopic clip application to the colon. The different kinds of interventions attempted, can be found in Table 4[60-65]. The tests used to assess the quality of the anastomosis were BPR, tensile strength, hydroxyproline, body weight changes, macroscopic and microscopic assessment using different protocols.

Peritonitis

In total, 29 different studies of anastomosis in rats with peritonitis were included. The different techniques to induce acute peritonitis were caecal ligation and puncture which was the commonest technique used, incomplete anastomosis, as

Table 2 Colitis models of high-risk anastomosis

Ref.	Year	Colitis method	Sample size	Intervention	Test	Sampling day
Kirkil *et al*[41]	2008	Intra-jejunal injection of iodoacetamide	28	Endothelin receptor blockade by bosentan	BPR *in situ*, macroscopic (Mannheim index[42]), HP, histology (as per Mei *et al*[43])	4
Rijcken *et al*[44]	2010	7 d DSS 5%	Not mentioned	rhIGF-1	BPR *in situ*, microscopic (as per Phillips *et al*[33], Ki-67), HP, MPO	1/3/7
Myrelid *et al*[45]	2015	5 d DSS 3%	140	IP prednisolone, AZA, infliximab	BPR *ex situ*, bowel WT/length, histology, zymography	3
Alvarenga *et al*[46]	2019	2,4,6-TNBS	66	AdMSC 2 × 10^6	Histology, IL-10, IL-17, IFN-g, TGF-b, TNF-a, MMP-2, MMP-9	7
Reischl *et al*[47]	2021	ANXA-1 k/o mice and 7 d DSS 2%-3%-5%	Not mentioned	Ac2-26-nanoparticles	Fluorescence imaging of MMPs, histology (as per Phillips *et al*[33]), whole transcriptome RNA sequencing and analysis	3/7
Weber *et al*[48]	2023	7 d DSS 2%	84	Prednisolone	BPR *in situ*, macroscopic (adhesions scoring), histology (as per Philips *et al*[33])	3/7
Ntampakis *et al*[49]	2023	7 d DSS 5%	24	AdMSC 5 × 10^6	BPR *ex situ*, macroscopic (as per Bosmans *et al*[50]), HP, IL-6, TNF-a, VEGF	7

DSS: Dextran sodium sulphate; TNBS: 2,4,6-trinitrobenzene sulfonic acid; rhIGF-1: Recombinant human insulin-like growth factor-1; IP: Intra-peritoneal; AZA: Azathioprine; AdMSC: Adipose tissue derived mesenchymal stem cells; BPR: Bursting pressure; HP: Hydroxyproline; IL: Interleukin; VEGF: Vascular endothelial growth factor; TNF-a: Tumor necrosis factor-alpha; MPO: Myeloperoxidase; INF-g: Interferon-gamma; TGF-b: Tumor Growth Factor–beta; MMP: Metalloproteases.

Table 3 Malnutrition models of high-risk anastomosis

Ref.	Year	Colitis method	Sample size	Intervention	Test	Sampling day
McCauley *et al*[51]	1991	3,5% agar diet for 7 d	30	BCAA	Body WT, BPR *ex situ*, tensile strength, protein content, HP	-
Karahasanoglu *et al*[52]	1998	Low-protein diet for 10 d	40	Growth hormone	Body WT, BPR *in situ*, hydroxyproline	4
Salman *et al*[53]	2008	15 d	72	*Cholerella sp. microalgae*	Body WT, macroscopic (adhesions as per Ham *et al*[32]), BPR *in situ*, HP, histology (as per de Roy van Zuidewijn *et al*[54]), albumin, prealbumin, transferrin	3/5/7/9/11/13/15
Gonçalves *et al*[55]	2009	50% food restriction for 21 d	80	Pre-op nutrition	Tensile strength, histology	5
Gündoğdu *et al*[56]	2015	50% food restriction for 26 d	18	Pre-op nutrition	Body WT, BPR *in situ*, HP	4
Vizzotto Junior *et al*[57]	2015	Paired feeding	160	Omega-3	Body WT, tensile strength, histology	5
Danielski *et al*[58]	2016	50% food restriction for 26 d	45	Vitamin C	Body WT, macroscopic (as per Knightly *et al*[39]), histology, HP, MPO, TNF-a, nitrite/nitrate, oxidative damage	7

WT: Weight; BPR: Bursting pressure; HP: Hydroxyproline; TNF-a: Tumor necrosis factor–alpha; MPO: Myeloperoxidase; BCAA: Branch chained amino acids.

well as faecal inoculation in the abdominal cavity. In one of the studies, Vaneerdeweg *et al*[66] used barium sulphate and gelatine sponges with faeces to mimic bacterial and chemical peritonitis. The different kinds of interventions attempted, can be found in Table 5[66-100]. The tests used to assess the quality of the anastomosis were BPR, tensile strength, hydroxyproline, cytokines, tPA activity, oxidative stress markers, body weight changes, macroscopic and microscopic assessment using different protocols.

Table 4 Obstruction models of high-risk anastomosis

Ref.	Year	Obstruction method	Sample size	Intervention	Test	Sampling day
Törnqvist *et al*[60]	1990	Silicone ring 6.5 mm	54	Diverting colostomy	Tensile strength, weight, HP	2/7
Aguilar-Nascimento *et al*[61]	1997	Silk ligature	108	Nutritional solutions lavage of the colon	Macroscopic, histology	3/6
Erbil *et al*[62]	2000	Silk ligature	144	Nutritional solutions lavage of the colon	BPR *ex situ*, HP, macroscopic	3/6
Cağlikülekçi *et al*[63]	2002	Common bile duct ligation	40	rGH	BPR *ex situ*, macroscopic, HP, macroscopic, histology (as per Greenhalgh *et al*[64])	7
Lelyanov *et al*[65]	2004	Silk ligature	60	Sodium hypochlorite and ozone therapy	BPR *ex situ*, survival, macroscopic, histology	1/3/6/9/12
Krarup *et al*[59]	2017	Laparoscopic clip application	32	MMP inhibition	Body WT, HP, BPR *in situ*	3

HP: Hydroxyproline; WT: Weight; BPR: Bursting pressure; MMP: Metalloproteases; rGH: Recombinant growth hormone.

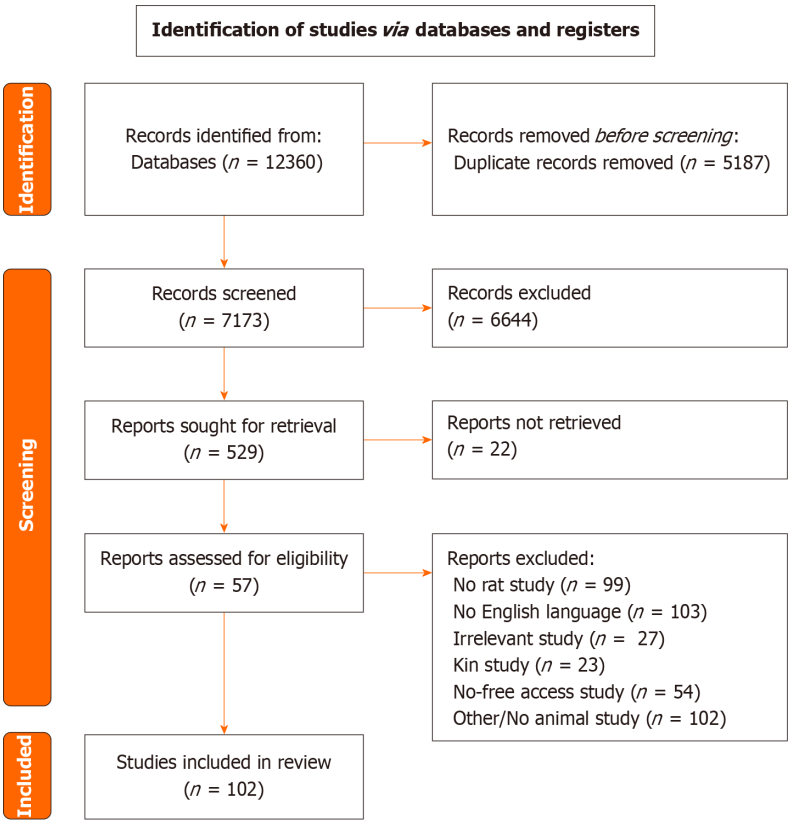

Figure 1 The preferred reporting items for systematic reviews and meta-analyses flow diagram.

Radiotherapy

In total, 8 different studies of anastomosis after radiotherapy in rats were included. In each protocol different doses of radiation were used depending on the nature of the experiment. The different kinds of interventions attempted, can be found in Table 6[101-109]. The tests used to assess the quality of the anastomosis were BPR, hydroxyproline, oxidative stress markers, body weight changes, macroscopic and microscopic assessment using different protocols. Van de Putte *et al*[107] used positron emission tomography/computedtomography to investigate the tropism of the AdMSCs to the anastomosis along with colonoscopy for direct assessment of anastomotic site and histological examination

Ischaemia/reperfusion injury

In total, 10 different studies of anastomosis after ischaemia injury in rats were included. In all protocols superior mesenteric artery was clamped for 30-45 mins. In each protocol different doses of radiation were used depending on the

Table 5 Peritonitis models of high-risk anastomosis

Ref.	Year	Peritonitis method	Sample size	Intervention	Test	Sampling day
Vaneerdeweg *et al* [66]	2000	Gelatin capsule with faeces and barium sulphate	40	Gentamicin Sponges	BPR *in situ*, mortality, weight loss	4
Reijnen *et al*[67]	2002	Caecal ligation/perforation	198	Hyaluronan-based agents	tPA activity	1/3/7
Aydin *et al*[68]	2006	Caecal ligation/perforation	24	Laparostomy with Bogota bag	BPR *in situ*, HP, adhesions (as per Zühlke *et al*[37])	5
Li *et al*[69]	2006	Enterotomy	360	Fibrin glue and growth hormone	BPR *in situ*, HP, tensile strength, histology	1/3/5
Buyne *et al*[70]	2009	Faecal inoculation	148	Recombinant tPA	BPR *in situ*, tensile strength, macroscopic, HP	3/7
Kayaoglu *et al*[71]	2009	Caecal ligation/perforation	80	N-butyl-2-cyanoacrylate	BPR *ex situ*, macroscopic (as per Knightly *et al*[39]), histology	3/7
Pantelis *et al*[72]	2010	Caecal ligation/perforation or incomplete anastomosis	206	Collagen matrix coagulation factors I and IIa (Tachosil)	BPR *ex situ*, histology (as per Biert *et al*[73], Verhofstad *et al* [74], Attard *et al*[75]), collagen type I-II, HP	2/5/14
Rocha *et al*[76]	2010	Caecal ligation/perforation or incomplete anastomosis	45	Hyperbaric oxygen therapy	Total energy rupture test (tensile strength)	4
Silva *et al*[77]	2012	Caecal ligation/perforation or incomplete anastomosis	40	Bromopride	Macroscopic (Nair *et al*[78]), tensile strength (Versa test), histology, quantitative collagen analysis, HP	3/7
Holmer *et al*[79]	2014	Faecal inoculation	72	Collagen fleece coating	BPR *in situ*, histology, collagen I, III, VEGF, MMP-13	1/3/7
Camargo *et al*[80]	2013	Faecal inoculation	40	Peritoneal lavage with bupivacaine	Tensile strength, survival	5
Arikanoglu *et al* [81]	2013	Colon injury	21	Antibacterial suture	BPR *in situ*, HP, histology	10
Donmez *et al*[82]	2013	Colon injury	40	Glutamine and GH	BPR *ex situ*, HP	5
Senol *et al*[83]	2013	*E. Coli* inoculation	40	Fibrin glue	BPR *ex situ*, histology (as per Ehrlich-Hunt[25]), HP	10
Silva *et al*[84]	2014	Caecal ligation/perforation	80	Bromopride	MMP-1a, MMP-8, MMP-13, IL-1b, IL-6, IL-10, TNF-a, IFN-g	3/7
Erginel *et al*[85]	2014	Caecal ligation/perforation	40	IP O3	BPR *ex situ*, histology (as per Verhofstadt *et al*[74] and Philips *et al*[33]), HP	7
Pommergaard *et al* [86]	2014	Incomplete anastomosis	80	Tachosil coating	Tensile strength, clinical assessment	7
Ercan *et al*[87]	2015	Caecal ligation/perforation	40	IP L-Carnitine	BPR *ex situ*, histology (as per Ehrlich- Hunt[25]), HP	5
Cakir *et al*[88]	2015	Incomplete anastomosis	64	Sildenafil	BPR *ex situ*, histology (as per Phillips *et al*[33]), HP, MDA, GSH	3/7
Suárez-Grau *et al* [89]	2016	Incomplete anastomosis	56	Fibrinogen - thrombin collagen patch	Histology (as per Biert scheme [73]), macroscopic (adhesions), survival	15/30
Sozutek *et al*[90]	2016	Caecal ligation/perforation	50	PRP	BPR *in situ*, Body WT, HP, histology (as per Verhofstadt *et al*[74])	7
Ersoy *et al*[91]	2016	Caecal ligation/perforation	60	Melatonin	BPR *ex situ*, HP, histology, IL-6, IL-10, INF-γ, CRP	7
Çakır *et al*[92]	2016	Caecal ligation/perforation	18	IP O3	BPR *ex situ*, TNF-a, IL-1β, MDA, MPO, histology	22
Sukho *et al*[93]	2018	Incomplete anastomosis	60	AdMSC	BPR *in situ*, macroscopic (as	3/7

					per Verco *et al*[94] and Zühlke *et al*[37]), histology	
Lorenzi *et al*[95]	2017	Caecal ligation/perforation	40	Omiganan	BPR *in situ*, histology (as per García *et al*[30]), HP	7
Yıldırım *et al*[96]	2021	Caecal puncture	21	Growth factor collagen (FGF-C), abx collagen (AB-C)	BPR *ex situ*, HP, macroscopic (as per Bosmans *et al*[50]), histology (as per Ehrlich *et al* [25])	7
Nakamura *et al*[97]	2021	Incomplete anastomosis	60	HSMM	BPR *ex situ*, macroscopic (as per Ham *et al*[32]), histology	3/5/7/14/28
Aksu *et al*[98]	2021	Colon injury	21	Chlorhexidine gluconate and metronidazole-soaked sponges	BPR *in situ*, hydroxyproline, histology	10
Yilmaz *et al*[99]	2021	Caecal ligation/perforation	32	Polyurethane membrane	BPR *ex situ*, HP, NOx, IL-6, TNF-a, tPA, macroscopic (as per Mazuji *et al*[100]), histology	5

BPR: Bursting pressure; WT: Weight; tPA: Tissue plasminogen activator; HP: Hydroxyproline; VEGF: Vascular endothelial growth factor; MMP: Metalloproteases; IL: Interleukin; MDA: MDA: Malondialdehyde; GH: Growth hormone; TNF-a: Tumor necrosis factor-alpha; IFN-g: Interferon-gamma; IP: Intra-peritoneal; GSH: Glutathione; MPO: Myeloperoxidase; AdMSC: Adipose tissue derived mesenchymal stem cells; HSMM: Human skeletal muscle myoblast; NOx: Nitric oxide.

Table 6 Radiation models of high-risk anastomosis

Study	Year	Radiation dose	Sample size	Intervention	Test	Sampling day
Liu *et al*[101]	2001	10 Gy	74	Lactobacillus plantarum 299v	Body weight, WBC, mucosal MPO, HP, nucleotide, DNA and RNA content, colonic bacterial microflora, bacterial translocation, histology	4/7/11
Kerem *et al* [102]	2006	500 cGy	84	Soluble Fiber	Macroscopic (adhesions as per van der Ham and Kort[32]), BPR *in situ*, HP, histology (as per de Roy van Zuidewijn *et al*[54]), MMP-2 activity	3/7
Ozdemir *et al* [103]	2013	800 rad	30	Amifostine	BPR *ex situ*, HP, histology	5
Seker *et al*[104]	2014	485 cGy	60	Pycnogenol	BPR *ex situ*, HP, MDA, histology (as per Houdart *et al*[105])	3/7
Simões Neto *et al*[106]	2013	660 cGy	30	Fraction electron beam	BPR (not specified), histology	7
Van de Putte *et al*[107]	2017	27Gy	48	AdMSC	18F-FDG-PET/CT, colonoscopy, histology	32
Taşdöven *et al* [108]	2019	6Gy	48	Ozon PR	BPR *in situ*, histology (as per Houdart *et al*[105]), HP, MDA, MPO	3/7
Yilmaz *et al* [109]	2022	20Gy	32	Ozon PR	BPR *in situ*, macroscopic (as per Knightly *et al*[39]), HP, MPO, histology (as per de Roy van Zuidewijn *et al*[54])	5

AdMSC: Adipose tissue derived mesenchymal stem cells; PR: Per rectum; MPO: Myeloperoxidase; HP: Hydroxyproline; MMP: Metalloproteases; BPR: Bursting pressure; MDA: Malondialdehyde.

nature of the experiment. The different kinds of interventions attempted, can be found in Table 7[110-124]. The tests used to assess the quality of the anastomosis were BPR, hydroxyproline, oxidative stress markers, cytokines, macroscopic and microscopic assessment using different protocols.

Chemotherapy

In total, 9 different studies of anastomosis after different schemes of chemotherapy in rats were included. Chemotherapy includes cytotoxic agents against cancer cells and was administered IV or with intra-peritoneal infusion. The different kinds of interventions attempted, can be found in Table 8[125-133]. The tests used to assess the quality of the anastomosis were BPR, tensile strength hydroxyproline, oxidative stress markers, cytokines, macroscopic and microscopic assessment using different protocols.

Table 7 Ischaemia/reperfusion models of high-risk anastomosis

Ref.	Year	I/R method	Sample size	Intervention	Test	Sampling day
Terzi *et al*[110]	2001	SMA clampling 30 min	65	Allopurinol	BPR *in situ*, macroscopic (adhesions as per Knightly *et al*[39]), histology (as per de Roy van Zuidewijn *et al*[54])	3/7
Tireli *et al*[111]	2003	SMA clampling 30 min	20	Pentoxifiline	BPR *ex situ*, HP	7
Miranda *et al*[112]	2010	SMA clamping 45 min	45	Methylene blue	BPR *ex situ*, macroscopic, histology	7
Celik *et al*[113]	2013	SMA clamping 45 min	24	Modelukast	BPR *ex situ*, HP, MPO, MDA, caspase-3 activity, catalase, NOx, glutathione, SOD, TNF-a, IL-6, ALT, AST	5
Akarsu *et al*[114]	2017	SMA clamping 10 min	40	Simvastatin	BPR *ex situ*, HP	8
Özkan *et al*[115]	2018	SMA clamping 30 min	30	Melatonin	BPR *ex situ*, HP, histology (as per Nursal *et al*[116]), SOD, glutathione	7
Özçay *et al*[117]	2018	SMA clamping 45 min	40	GH	Macroscopic (as per Galili *et al*[118]), BPR *ex situ*, histology (as per Greenhalgh *et al*[64])	7
Sayin *et al*[119]	2020	SMA clamping 45 min	40	IP montelukast	BPR *ex situ*, macroscopic score (as per Knightly *et al*[39]), HP, histology (fibrosis)	7
Eryilmaz *et al*[120]	2020	SMA clamping 45 min	30	Hydrogen rich saline	BPR *in situ*, histology (as per Park *et al*[121] and Chiu *et al*[122]), TNF-a, IL-6, MDA, MPO	5
Akıncı *et al*[123]	2022	SMA clamping 30 min	36	Genistein	BPR *ex situ*, HP, SOD, glutathione, histology (as per Piroglu *et al*[124])	5

SMA: Superior mesenteric artery; GH: Growth hormone; IP: Intra-peritoneal; SOD: Superoxide dismutase; BPR: Bursting pressure; HP: Hydroxyproline; MPO: Myeloperoxidase; MDA: Malondialdehyde; IL: Interleukin; TNF-a: Tumor necrosis factor-alpha; I/R method: Ischaemia/reperfusion method.

Immunosuppression

In total, 6 different studies of anastomosis after different schemes of chemotherapy in rats were included. Either steroids or other immunosuppression drugs were used, depending on the protocol. The different kinds of interventions attempted, can be found in Table 9[134-140]. The tests used to assess the quality of the anastomosis were BPR, tensile strength hydroxyproline, oxidative stress markers, cytokines, macroscopic and microscopic assessment using different protocols.

DISCUSSION

Bursting pressure

One of the most common tests used to assess the quality of anastomosis is anastomotic BPR. In the literature, there are 2 ways of performing BPR test, either *in situ* (*in vivo*) or *ex situ* (*in vitro*). *In vivo*, a catheter is inserted into the rat's rectum, dyed water is infused, and the manometer is attached more proximal to the anastomosis, recording the maximum pressure in which the anastomosis bursts. *In vitro*, the anastomosis is dissected away from the rat, is tied distally, and is attached to a three-way system with the manometer on one side, and the syringe with the dyed water on the other. Water is infused in the bowel segment, and maximum pressure in which the anastomosis bursts is recorded. Curran *et al*[141] compared the two techniques in canine small bowel and they concluded that the *in vitro* technique had similar results compared to the *in vivo* one, but it was easier to perform as the researchers do not have to carry out intensive bowel dissection. In one of our team's previous studies we described the *in vitro* technique in detail[49]. Some technical pearls of the *in vitro* technique are that it is slightly more time consuming, and it requires careful and meticulous dissection as the adhesions formed around the anastomosis might be the factor that keeps the anastomosis patent, and extensive dissection might result in anastomotic dehiscence, rendering the specimen invalid.

Along with BPR, Sakallioglu *et al*[136] also documented the bursting site of the anastomosed bowel, and they found out that it is usually around the anastomosis and not on the anastomosis itself.

Tensile strength

Tensile strength is traditionally used along with BPR to assess the strength of the anastomosis. The technique includes dissecting the anastomosis out and attaching it to a device which allows application of traction force to one end of the anastomosis and recording of the force applied to the apparatus on the other end. The force at which the anastomosis is disrupted is then recorded. Either a simple commercial dynamometer[142] or more precise and expensive solutions can

Table 8 Chemotherapy models of high-risk anastomosis

Ref.	Year	Chemo agent	Sample size	Intervention	Test	Sampling day
Nayci *et al*[125]	2003	IP 5-FU	40	Electromagnetic field	Tensile strength, HP	7
Cetinkaya *et al*[126]	2005	IP mitomycin-C	81	GM-CSF	BPR *ex situ*, HP, histology (as per Ehrlich *et al*[25])	3
Kanellos *et al*[127]	2006	IP 5-FU and LEV	60	Fibrin glue	BPR *ex situ*, HP, macroscopic (adhesions as per van der Ham and Kort[32]), histology (as per Phillips *et al*[33])	8
Yildiz *et al*[128]	2013	5-FU and 20 Gy	60	HBOT	BPR *ex situ*, Weight, HP, histology (Fibrosis)	5
Arapoglou *et al*[129]	2017	Irinotecan	40	Iloprost	BPR *ex situ*, macroscopic (as per van der Ham and Kort[32]), histology (as per Phillips *et al*[33]), HP	8
Akyuz *et al*[130]	2018	5-FU IV	32	Melatonin	BPR *ex situ*, HP, histology, TNF-a, IL-1β	7
Ocak *et al*[131]	2019	HIPEC with CIS	30	PRP	BPR *ex situ*, HP, histology (as per Verhofstad *et al*[74])	7
Gorur *et al*[132]	2020	IP 5-FU	40	PRP	Body weight, BPR *in situ*, HP, histology (as per Verhofstad *et al*[74])	7
Buk *et al*[133]	2020	IP OX	30	PRP	BPR *ex situ*, histology (as per Verhofstad *et al*[74]), HP	7

IP: Intra-peritoneal; 5-FU: 5-Fluorouracil; LEV: Leucovorin; HIPEC: Hyperthermic intra-peritoneal chemotherapy; CIS: Cis-platina; OX: Oxaliplatin; GM-CSF: Granulocyte–macrophage colony stimulation factors; HBOT: Hyperbaric oxygen treatment; PRP: Platelet rich plasma; BPR: Bursting pressure; HP: Hydroxyproline; IL: Interleukin; TNF-a: Tumor necrosis factor-alpha.

Table 9 Immunosuppression models of high-risk anastomosis

Ref.	Year	Immunosuppression method	Sample size	Intervention	Test	Sampling day
Dinc *et al*[134]	2002	Methylprednizolone	80	GM-CSF	BPR *ex situ*, HP, histology (as per Ehrlich *et al*[25])	3
Colak *et al*[135]	2003	Dexamethasone	24	Trapidil	BPR *ex situ*, HP, histology (as per Ehrlich *et al*[25]), NOx, MDA	7
Sakallioglu *et al*[136]	2004	Dexamethasone	60	eGF	BPR *ex situ*, bursting site, HP, histology	7
Inglin *et al*[137]	2008	MMF	63	IGF-I	BPR *ex situ*, histology, Ki-67	2/4/6
Netta *et al*[138]	2014	Methylprednizolone	50	SCFA	BPR *ex situ*, CRP, IL-6, TNF-a	4
Karakaya *et al*[139]	2021	Everolimus	60	AdMSC	Macroscopic (as per Houston and Rotstein[140]), BPR *ex situ*, HP, histology	4/7

MMF: Mycophenolate mofetil; GM-CSF: Granulocyte-macrophage colony stimulation factors; eGF: Epidermal growth factor; IGF-I: Insulin-like growth factor; SCFA: Short chain fatty acids; AdMSC: Adipose tissue derived mesenchymal stem cells; BPR: Bursting pressure; HP: Hydroxyproline; NOx: Nitric oxide; MDA: Malondialdehyde; IL: Interleukin; TNF-a: Tumor necrosis factor-alpha.

be used[77].

Ikeuchi *et al*[143] reported that there is no correlation between anastomotic bursting strength and tensile strength of anastomosis, and both tests should be used in assessing the anastomotic quality. They also suggest that minimum strength in which the anastomosis starts to rupture and maximum strength in which the anastomosis is completely ruptured should be documented.

Macroscopic assessment

Macroscopic assessment consists of both clinical observations of the rats, as well as macroscopic assessment of the anastomosis using different scales to grade it, depending on what parameters need to be observed.

Clinical parameters used, especially in malnutrition models, are weight changes of the rats, and survival curves. The general welfare of the animals, while easily appreciated by direct observation, can be considered but is not easily

countable. Examples of clinical parameters are reduced mobility, fur erection, neglection of hygiene and reduced food intake. Specifically in colitis protocols, bloody diarrhea, and rectal mucosa erythema can be easily observed and colonoscopy can be used to verify intestinal inflammation before starting the experimental process[46,49].

Van de Putte *et al*[107] used colonoscopy to directly assess anastomotic healing internally.

Macroscopic assessment scores and what they assess can be found in Table 10. Of note, although the score by van der Ham and Kort[32] is one of the widest used for adhesion formation, van der Ham cite Houston and Rotstein[140] as the original creators of the same scoring system.

All the adhesions scoring systems are similar, and all of them assess the existence or severity of the adhesions using different criteria. The score that we considered to be more complete for the assessment of a bowel anastomosis in rats is the one created by Bosmans *et al*[50] in an international consensus statement. This score takes into account the presence of adhesions, abscesses, anastomotic dehiscence underneath adhesions, as well as faecal peritonitis/death of the animas.

Histologic assessment

One of the most interesting findings of the current review are the different methods used in research to assess the quality of the anastomosis. As described in Table 11[144], all models have common characteristics, such as the presence of inflammatory cells in bowel tissues, fibroblastic activity, neovascularization, and collagen deposition which play a pivotal role in anastomotic healing. The oldest and most widely used model with the above characteristics is this of Ehrlich *et al*[25] in 1973 and later on the same model as modified by Phillips *et al*[33]. in 1992, both of which set the basis for histological assessment of anastomotic healing.

The appreciation of healing layer by layer is added by de Roy van Zuidewijn *et al*[54] in 1992 who added the elements of re-epithelialization, muscularis propria damage and regeneration and the presence of necrosis. Their model, despite providing information on the layer-by-layer healing and inflammatory changes of the tissues, does not address the deposition of collagen or fibroblastic activity. Other models, proposed by different teams provide a more complete approach to anastomotic healing by adding the element of connective tissue regeneration as well[73-75].

As opposed to the models described above that have a semi-quantitative approach, Park *et al*[121] and Chiu *et al*[122] suggested a more qualitative approach, with grading of mucosal layer healing. This model is used to evaluate the anastomosis after ischemia reperfusion type injury[121,122].

Miltschitzky *et al*[15] have a very different approach in their histology grading system which includes neovascularization, fibroblastic activity and collagen formation, inflammatory cell infiltration and a more extensive layer by layer evaluation of the anastomosis in semi-quantitative way[15]. In the authors' view this represents the most holistic approach to anastomotic healing, is easy to use and is applicable in different types of anastomosis research.

In a few cases, as shown in Tables 1-9, researchers did mention histological evaluation in their research but they did not use any of the histological grading models described above.

Collagen formation/degradation assessment

Hydroxyproline has proven to be one of the commonest markers used in experimental protocols of anastomotic healing. With hydroxyproline we indirectly assess collagen content of anastomosis and appreciate anastomotic healing, with higher values of hydroxyproline suggesting enhanced anastomotic healing.

Matrix Metalloproteases (MMPs) and their inhibitors (TIMPs) play an important role in wound healing as they play an important role in collagen degradation, neovascularization and are regulated by chemokines and cytokines[145].

The selection and investigation of different kinds of MMPs and TIMPs depends on the nature of the research and their objectives. In the current study we identified a few authors investigating the role of different MMPs (MMP-1, MMP-8, MMP-13) that belong to the groups of collagenases which degrade triple helical collagen and MMP-2, MMP-9 gelatinases which are involved in the processes of angiogenesis and collagenesis[46,79,84,145]. Abnormal expression of MMPs can impair anastomotic healing and can be used as biomarkers in anastomosis research to evaluate the efficacy of different interventions or the effect of a condition/factor on an anastomosis.

Cytokine studies

Different kinds of cytokines can be used in research of high-risk anastomotic healing as they provide us with valuable information on the biological processes during anastomotic healing or in response to an intervention to a high-risk anastomosis. Cytokines can be measured either with ELISA or polymerase chain reaction according to local laboratory protocols.

Some of the cytokines used in research are interleukin (IL)-1b, IL-4, IL-6, IL-17, interferon gamma, tumor necrosis factor α, as pro-inflammatory cytokines to assess the severity of inflammatory response to the anastomosis after applying the stimulus and appreciating their fluctuation after the intervention[46,49]. On the other hand, increased expression of IL-10 and tumor growth factor–beta (TGF-b) which are known as anti-inflammatory cytokines can be used as a marker of effectiveness of experimental intervention. TGF-b as reported by Alvarenga *et al*[46] also seems to regulate the expression of certain MMPs leading to fibrosis[46].

Vascular endothelial factor is one of the cytokines that can be used to assess the anastomosis for neo-vascularization. Increased values of vascular endothelial growth factor (VEGF) will suggest increased vascularization in the anastomosis which is important for anastomotic healing. In early stages of anastomotic healing VEGF might not be significantly increased but our group showed tendency to increase in post-operative day 7 in anastomoses treated with Adipose tissue derived stem cells[49].

A meticulous study design combining the appropriate MMPs and cytokines can extract valuable information about anastomotic healing and the various signaling pathways by which the inflammatory response is regulated.

Table 10 Macroscopic assessment of the anastomosis

Macroscopic assessment	Wound healing
van der Ham and Kort[32] score	Adhesions
Zühlke *et al*[37] score for adhesions	Adhesions
Mannheim index[42]	Peritonitis presence and severity
Knightly *et al*[39] score for adhesions	Adhesions
Bosmans *et al*[50] score	Anastomotic complication score
Nair *et al*[78] score	Adhesions
Verco *et al*[94] score	Abscess formation
Mazuji *et al*[100] score	Adhesions
Galili *et al*[118] score	Adhesions
Houston and Rotstein[140] score	Adhesions

Table 11 Histologic assessment of the anastomosis

Ref.	Histologic assessment
Ehrlich *et al*[25]	Erythrocytes, polymorphonucleated cells, mononuclear cells, fibroblasts, collagen fibers, fibrin
Houdart *et al*[105] and Hutschenreiter *et al*[144] as modified by García *et al*[30]	Mucosal anastomotic re-epithelialization, neovascularization, fibroblasts, fibrosis, muscle layer destruction, neutrophil infiltration, lymphocyte infiltration, histiocyte infiltration, giant cell infiltration
Ehrlich *et al*[25] as modified by Phillips *et al*[33]	Inflammatory cell infiltration, blood vessel in growth, fibroblast ingrowth, collagen deposition
Houdart *et al*[105]	Granulocyte infiltration, mononuclear cell infiltration, fibroblastic proliferation, focal necrosis, exudate formation
Mucosal damage index by Mei *et al*[43]	0: Normal mucosa, no damage on mucosal surface; 1: Mild hyperemia and edema, no erosion or ulcer on mucosal surface; 2: Moderate hyperemia and edema with erosion on mucosal surface; 3: Severe hyperemia and edema with necrosis and ulcer on mucosal surface, the major ulcerative area < 1 cm; 4: Severe hyperemia and edema with necrosis and ulcer on mucosal surface, the major ulcerative area > 1 cm
de Roy van Zuidewijn *et al*[54]	Re-epithelialization, regeneration of muscularis propria, mucosal muscularis propria damage, necrosis, inflammatory exudate, granulation tissue, granulocytes, macrophages, fibroblasts, granulation tissue
Greenhalgh *et al*[64]	Epithelization, cellular infiltration, fibroblastic proliferation, collagen deposition, neovascularization
Biert *et al*[73]	Necrosis, polymorphonuclear cells, lymphocytes, macrophages, edema, epithelium, submucosal - muscular continuity, neovascularization, fibrosis
Attard *et al*[75]	Mucosal continuity, muscular continuity, re-epithelialization, granulation tissue, polymorphonuclear cells, lymphocytes, macrophages, fibroblasts
Verhofstad *et al*[74]	Necrosis, polymorphonuclear cells, lymphocytes, macrophages, edema, mucosal continuity, submucosal – muscular continuity
Nursal *et al*[116]	Fibroblast infiltration, capillary formation, re-epithelialization, granulocyte infiltration, mononuclear cell infiltration
Park *et al*[121] and Chiu *et al*[122]	Grade 1: Normal mucosa; grade 2: The subepithelial space at the tip of the villus; grade 3: Increase in subepithelial space; grade 4: Overlapping and spills of the floor of the villus; grade 5: Disintegration of the lamina propria; grade 6: Crypt layer injury; grade 7: Transmucosal infarction and grade 8: Transmural infarction
Piroglu *et al*[124]	Inflammatory cell infiltration/concentration, neovascularization, fibroblastic activity, collagen fibers
Miltschitzky *et al*[15]	Blood vessel ingrowth, fibroblasts, collagen formation, inflammatory cell infiltration, first layer in which continuity has been restored, number of healed layers, epithelium closed, crypt architecture restored, overall healing quality

Oxidative stress

Another state of matter that can be used in research of an anastomotic healing is oxidative stress. Authors, as shown in Tables 1-8 used markers that indicate either oxidative stress damage, such as free radicals (NOx), myeloperoxidase (MPO) and Malondialdehyde, or antioxidant markers such as superoxide dismutase and glutathione. Neutrophils contain MPO and increased levels of this marker also suggests increased neutrophilic infiltration to the tissues[34].

CONCLUSION

Our review demonstrated the evolution of different high-risk anastomosis protocols in rats as well as the different techniques used to assess anastomotic healing. We emphasize the importance of systematization of research, by standardizing experimental protocols and designing high quality studies that will give us more information on the complex pathophysiological pathways of anastomotic healing. Understanding these pathways will allow us to create interventions that will attenuate the inflammation, decrease anastomotic related complications, and negate the need for diverting stomas in surgical patients.

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