Emerging molecular mechanisms and genetic targets for developing novel therapeutic strategies for treating bladder diseases

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Abstract

Bladder diseases affect millions of patients worldwide and compromise their quality of life with a substantial economic impact. The not well understood aetiologies of bladder diseases limit the current diagnosis and therapeutic options to primarily symptomatic treatment. In addition, bladder targeted drug delivery is challenging due to its unique anatomical features and its natural physiological function of urine storage and frequent voiding. Therefore, current treatment options often fail to provide a highly effective, precisely targeted and long-lasting treatment. Thus, comprehensive studies are needed to provide a better understanding of the molecular mechanisms underpinning bladder diseases to identify novel gene therapeutic targets and biomarkers for treating bladder diseases and develop novel treatments such as gene loaded nanoparticles. This review examined the recent development on the discovery of molecular mechanisms of bladder diseases and discussed recently proposed new treatments, including novel bladder targeted gene therapies using nanoparticle-based formulations and probiotics adjuvant therapies.

Key words: Bladder cancer, overactive bladder, interstitial cystitis, nanoparticles, probiotics, gene therapy

1. Introduction

Bladder disease is a generic term that comprises several lower urinary tract disorders and abnormalities, which severely affect many individuals (GuhaSarkar and Banerjee, 2010). Bladder cancer (BC), interstitial cystitis (IC) and over-active bladder syndrome (OAB) vary from mild to severe medical bladder diseases, which usually result in long-lasting complications (van de Merwe *et al.*, 2008; GuhaSarkar and Banerjee, 2010; Tran *et al.*, 2021).

Targeting bladder diseases using systemic drug delivery has been proven inefficient due to insufficient drug available at the site of action explained by the physiological conditions of the bladder and the poorly vascularised urothelium (GuhaSarkar and Banerjee, 2010; Kolawole *et al.*, 2017). Additionally, due to the high prevalence of bladder diseases recurrences, continuous systemic drug delivery would imbalance the level of microbiomes and result in advanced complications. Hence, intravesical drug delivery (IDD) approaches were developed to ensure the direct instillation of the drug into the bladder with maintaining high local drug concentration and lower systemic side effects (GuhaSarkar and Banerjee, 2010; Kolawole *et al.*, 2017).

However, IDD suffered a group of limitations that could be related to the low permeability of the urethral layer and the need for frequent instillations due to the voiding process, which washes out the drug solutions immediately (GuhaSarkar and Banerjee, 2010; Nirmal *et al.*, 2012). Therefore, IDD and nanotechnology were used simultaneously as an integrated approach to deliver the drug to the bladder and increase its residence time at the site of action. Several nanocarriers such as liposomes, polymeric nanoparticles and protein nanoparticles were found efficient in improving drug delivery while maintaining a sustained drug release (GuhaSarkar and Banerjee, 2010; Kolawole *et al.*, 2017; Kumar and Das, 2017).

However, due to the complexity of some bladder diseases and their inadequate response to drug therapy, there is still a considerable need to develop novel therapeutic strategies and adjuvant therapies to improve the therapeutic outcome (Zhang *et al.*, 2013; Martínez-Fernández *et al.*, 2015). Therefore, developing novel strategies requires a deep understanding of the underpinning molecular mechanisms, gene targets and biomarkers associated with the bladder disease development. Since genes are responsible for the diversity of organs functions, several studies have focused on probing the relationship between specific genes expression and bladder disorders epidemiology and pathogenesis (Ohnishi *et al.*, 2003; Zhang *et al.*, 2013; Tseng *et al.*, 2016; Yu *et al.*, 2019). These studies have paved the way for a group of studies focused on modulating the expression rates of these genes as a novel therapy for bladder disorders with inadequate response to drug therapy (Minami *et al.*, 2017; Li, Xie and Zhang, 2019).

As an alternative treatment strategy, some studies looked into more natural therapies that could offer preventative and/or therapeutic options for various bladder diseases without using synthetic pharmaceuticals. As an example, many studies have highlighted positive outcomes of probiotic treatment not only in the intestine where they help to maintain and stabilise a healthy microbiome, but also in other organs, where they have shown to strengthen immune function and protect epithelium layer from pathogen invasion (Lu *et al.*, 2021). Until recently it was believed that urine is sterile, thus urinary tract does not have its own healthy microbiome (Thomas-White *et al.*, 2016). A decade ago 16S rRNA sequencing and expanded quantitative urine culture techniques confirmed a wide range of bacteria present in healthy female urine (Siddiqui *et al.*, 2011; Wolfe *et al.*, 2012). Since then, many studies have investigated probiotics as alternative to antibiotic prophylaxis, adjuvant therapy to conventional treatment options and preventative measure from certain disease recurrence (Hoesl and Altwein, 2005).

This review observes current research that focuses on establishing safe and effective therapies that could have matching or exceeding therapeutic effects when compared to conventional treatment options. For instance, gene-based drug delivery can offer a novel and precise tool to modify cancer related genetic alterations by ceasing progression of the disease and restoring normal cell growth (Zacchè, Srikrishna and Cardozo, 2015). In addition, the use of nanotechnology can tailor the active-agent delivery properties, by enabling direct tissue targeting, enhancing drug permeation, establishing a sustained release of the encapsulated material, and preventing active agent from potential degradation (Armendáriz-Barragán *et al.*, 2016; Nakamura *et al.*, 2016). Additionally, supplementation of probiotics was shown to stimulate immune system and potentially halt tumour progression (Sivan *et al.*, 2015; Cai *et al.*, 2016). Introducing these properties into current practise would enable more efficient, precise and safe treatment options, eliminating current limitations, such as short drug retention times in the bladder, low drug bioavailability, harsh side effects and more (GuhaSarkar and Banerjee, 2010).

This review discusses the limitations of the available therapeutic options and provides a detailed analysis of the most recently identified molecular mechanisms and gene targets associated with bladder disorders. Novel therapeutic approaches including the development of novel gene silencing therapeutic strategies and the use of the probiotic supplementation as a complimentary treatment strategy were discussed.

2. Urinary bladder physiology

The human urinary bladder is a tetrahedron shaped organ, that lies in an extraperitoneal position within the pelvis (Mangera, Osman and Chapple, 2013). The inner layer of bladder –

mucosa – is comprised of transitional epithelium (often referred to as urothelium), basement membrane and sub-urothelium (**Figure 1A**) (Livingston, 2016). This layer is important for transducing physical and chemical stimuli, as well as functions as a barrier from pathogens and various molecules from entering deeper tissues in bladder wall (Livingston, 2016). The joining tissue between inner mucosa layers and outer muscular layers is called sub-urothelium, comprised of interstitial cells and afferent nerves (Livingston, 2016; Tanabalan and Ballaro, 2019). Mucosa and sub-urothelium layers are covered by smooth muscle called detrusor, which is protected by external serosa (Mangera, Osman and Chapple, 2013; Janssen, Schalken and Heesakkers, 2017). Detrusor muscle layer consists of interlacing randomly orientated muscle fibres, only organising into distinct layers – longitudinal and circumferential – near the internal urethra (Mangera, Osman and Chapple, 2013). The organised muscle layers help form the bladder sphincter, which facilitates passage of urine through urethra, while maintaining urinary continence and allowing volitional voiding. Smooth muscles located in the bladder provides elasticity to the bladder wall, ensuring that urine can be stored during filling process (Janssen, Schalken and Heesakkers, 2017).

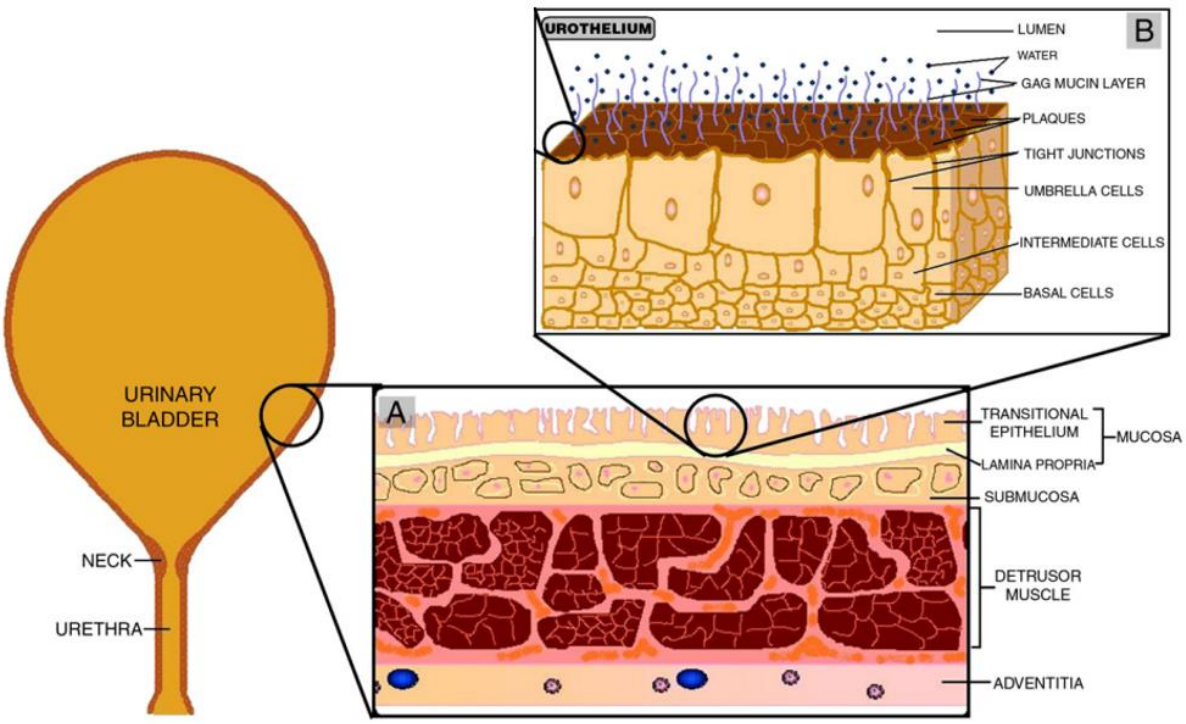

Figure 1. Detailed structure of (A) layers forming urinary bladder wall: mucosa, comprised of urothelium tissue, submucosa, and muscle layer; (B) different cell layers of the urothelium tissue: GAG and plaque, comprised of uroplakins, layer covering the outer layer of urothelium, with umbrella, intermediate and basal cells located in the inner layers of urothelium. Adapted and reprinted with permission from GuhaSarkar and Banerjee, 2010 (GuhaSarkar and Banerjee, 2010).

The urinary tract is lined with urothelium layer, which is a stratified epithelium tissue comprised of single layer of umbrella cells, one to multiple layers of intermediate cells, and single layer of basal cells (**Figure 1B**) (Dalghi *et al.*, 2020). Urothelium is a permeability barrier, that accommodates the urine flow and volume, while controlling metabolic product exchange between urine and blood (Tamadonfar *et al.*, 2020). The outermost layer of umbrella cells, which forms a barrier comprised of apical membrane, umbrella cell tight junctions and glycocalyx, plays a crucial role in protecting deeper layers of urothelium from pathogens (Dalghi *et al.*, 2020).

Urothelium surface is lined with gel-like mucin layer, which is comprised of sulfonated glycosaminoglycans (GAGs) and glycoproteins, that adhere to the glycocalyx of urothelium (Kamhi *et al.*, 2013; Birder, 2014). GAGs are long, linear and highly negatively-charged polysaccharides, that bind water molecules resulting in well-hydrated bladder surface (Gomelsky and Dmochowski, 2012). In addition to permeability barrier function, GAG layer also coats urothelium in non-adhesive surface to prevent bacterial adherence (Gomelsky and Dmochowski, 2012).

Small family of transmembrane proteins called uroplakins act as an equally important barrier of permeability of the bladder wall (Dalghi *et al.*, 2020). Uroplakins form urothelial plaque, which covers the umbrella cells and is constantly recycled (Jackson *et al.*, 2020). This plaque confers transcellular resistance, therefore controlling water and urine absorption from the urine (Grabnar, Bogataj and Mrhar, 2003; Jackson *et al.*, 2020).

GAG and uroplakin layer injury can lead to increased urothelium permeability, allowing toxic agents, urine and bacteria to reach deeper bladder tissues (Birder, 2005; Klingler, 2016). This can cause chronic bladder epithelial damage, that could then further escalate into chronic inflammatory bladder diseases, such as recurring urinary tract infections, IC or OAB (Birder, 2005; Cicione *et al.*, 2014).

3. Overview of drug delivery strategies for treating bladder diseases

Conventional oral treatment of bladder diseases is often considered to be challenging and inefficient (GuhaSarkar and Banerjee, 2010). This is because systemic drug exposure via oral route reduces drug concentration at the desired target site, as drugs are affected by first pass metabolism and enzymatic degradation (Crane, Isharwal and Zhu, 2018; Yoon *et al.*, 2020). To ensure that drug concentration reaches therapeutic levels drugs are often administrated orally in higher doses, which in turn increases the risk of side effects and off-site targeting (Zacchè, Srikrishna and Cardozo, 2015; Crane, Isharwal and Zhu, 2018). However, this type of treatment is the most cost-effective, as well as has the good patients compliance (Zacchè, Srikrishna and Cardozo, 2015). Targeted drug delivery directly to the bladder has been recognised as a promising alternative, due to easy access when using conventional catheter and convenient organ shape for liquid storage **(Figure 2)** (Crane, Isharwal and Zhu, 2018). However, this type of localised treatment also has its own limitations, which are related to low

permeability of the urothelium and the dynamic drug dose dilution and clearance due to urine filling and bladder emptying (GuhaSarkar and Banerjee, 2010).

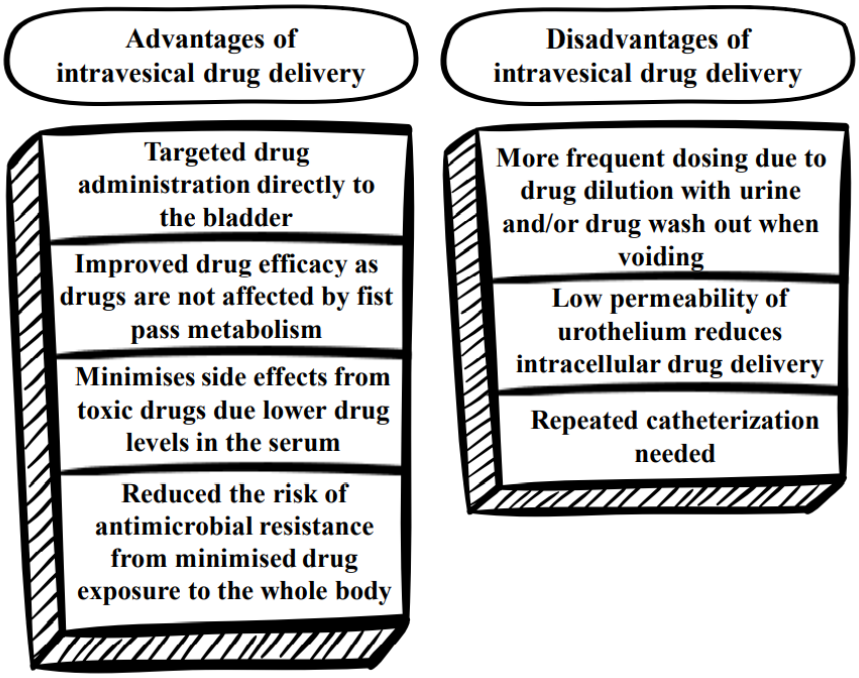

Figure 2. Advantages and disadvantages of intravesical drug delivery to the bladder.

Bladder surface lined with uroplakin and GAG layers cause its non-adhesive properties, therefore creating an obstacle for the drugs that rely on penetration or adherence to the bladder wall to ensure efficient treatment and prolonged drug retention time (Yoon *et al.*, 2020; Khizer *et al.*, 2021). Drug residence time can also be affected by frequent drug wash-out and dilution due to bladder filling and voiding (GuhaSarkar and Banerjee, 2010). In order to prolong drug retention time, bladder can be drained of urine prior to drug instillation and/or fluid intake can be limited during the treatment period. However this can be challenging for elderly patients and therefore frequent drug dosing is often used to maintain therapeutic levels of the drug in the bladder (Tyagi *et al.*, 2016; Yoon *et al.*, 2020). Research has shown that mucoadhesive materials can interact with GAG layer components via free hydroxyl or carboxyl groups, therefore allowing prolonged drug attachment to the urothelium (GuhaSarkar and Banerjee,

2010). Materials such as chitosan and its derivatives, poly (ethylene glycol) (PEG), poloxamers, dimethyl sulfoxide (DMSO) and polydopamine have demonstrated mucoadhesive and mucopenetrative properties when used for intravesical drug delivery to bladder using nanotechnology (GuhaSarkar and Banerjee, 2010; Ways, Lau and Khutoryanskiy, 2018; Poinard *et al.*, 2019).

Nanotechnology combined with pharmaceutical and biomedical sciences enhanced the development of next generation drug products, that provide the patient with higher drug efficacy and improve the safety and toxicology profiles (Onoue, Yamada and Chan, 2014; Bobo *et al.*, 2016). Nanosized agents demonstrate large loading capacity, high metabolic stability, specific tissue targeting and controlled release compared to conventional low molecular weight agents (Armendáriz-Barragán *et al.*, 2016; Nakamura *et al.*, 2016). Active molecule encapsulation ensures that biodistribution of the particles relies on physical properties of the carrier rather than the drug, therefore improving the efficacy of the treatment (Armendáriz-Barragán *et al.*, 2016).

4. Bladder diseases: Key molecular mechanisms and therapeutic strategies

4.1 Bladder cancer (BC)

BC is characterised by the uncontrolled growth of abnormal cells in the urothelial lining of the bladder with a significant prevalence worldwide estimated by 380,000 cases and 150,000 deaths per year (Knowles and Hurst, 2015). BC is commonly classified into two categories: non-muscle-invasive bladder cancer (NMIBC), which makes up to 70% of the cases and muscle-invasive bladder cancer (MIBC), displaying the remaining 30% of the cases (Martínez-Fernández *et al.*, 2015). The stage of BC is determined based on the invasive nature of the tumour. Stages of Ta (Non-invasive papillary carcinoma) and T1 (Tumour invades

subepithelial connective tissue) are commonly diagnosed in NMIBC. However, stages of T2 (Tumour invades muscularis propria), T3 (Tumour invades perivesical tissue) and T4 (Tumour has spread beyond the fatty tissues and nearby organs or structures) are discerned in MIBC (GuhaSarkar and Banerjee, 2010; Knowles and Hurst, 2015; Martínez-Fernández *et al.*, 2015).

Smoking is considered the most significant environmental risk factor for BC. Tobacco smoking is responsible for more than 50% of BC cases and an estimated death rate of 31% and 14% in male and female smokers, respectively (Kolawole *et al.*, 2017). Epigenetic studies support the premise that cigarette smoking is associated with the genesis of the epigenetic alterations in bladder cancer besides being a direct mutagenic carcinogen (Crawford, 2008). Hence, smoking cessation is considered the primary preventive therapy for bladder cancer.

BC has been characterised by a vast number of mutations and epigenetic and signalling pathways alterations (Knowles and Hurst, 2015; Martínez-Fernández *et al.*, 2015; Tran *et al.*, 2021). Furthermore, the emerging hypothesis is that NMIBC and MIBC have different molecular drives and alterations, emphasising the need for an advanced understanding of the BC origin to provide novel therapeutic strategies with better therapeutic outcome (Tran *et al.*, 2021). These alterations in the epigenetic machinery have been found to affect DNA methylation, chromatin remodelling and histone modifications, the expression of non-coding RNAs, and the expression of transcriptional regulators such as the Kruppel-like factors (KLFs) (Knowles and Hurst, 2015; Martínez-Fernández *et al.*, 2015; Tran *et al.*, 2021). The genes that govern the organisation of the chromatin and the histone modifications were found to be frequently altered in BC by mutations or a change in their expression or function (Martínez-Fernández *et al.*, 2015; Kolawole *et al.*, 2017; Martinez *et al.*, 2019). The origin, genesis,

contribution of these alterations in BC and the therapeutic strategies will be discussed below to assess their use as actionable targets for BC management **(Figure 3).**

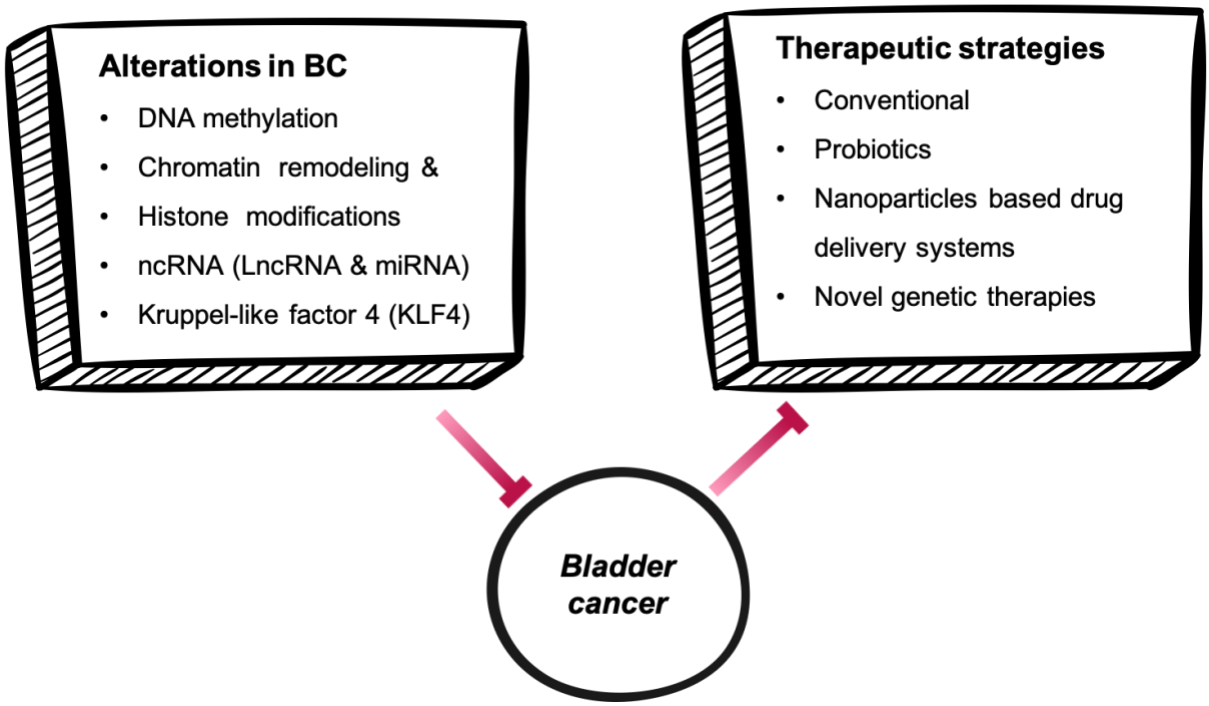

Figure 3. The key molecular mechanisms of bladder cancer development correlated and the available therapeutic strategies.

4.1.1 Key molecular mechanisms of bladder cancer correlated with potential gene therapeutic targets

4.1.1.1 DNA methylation in BC

The addition of a methyl group to the 5' position of a cytosine ring of a DNA molecule by a covalent bond is defined as DNA methylation. Normal DNA methylation is a vital process for development; however, alterations in this process can indicate the development of diseases, including cancer (Knowles and Hurst, 2015; Martinez *et al.*, 2019). Alterations in normal DNA methylation process were reported in 50-90% of BC cases (Martinez *et al.*, 2019). Hypermethylation of the promoter sites of specific genes that act as tumour suppressors is an example of these alterations in BC, which negatively affect these genes expression resulting in BC development and progression (Martinez *et al.*, 2019).

The modern whole-genome methylation assays have established the relation between BC methylome and BC diagnosis and response to therapy as an alternative way for the expensive, invasive diagnostic procedures. Hence, the methylated status of a gene set was used as a diagnostic tool in primary BC. For instance, methylation of *IPF1*, *TAL1*, *GALR1*, *TJP2* and *PENK* was higher in MIBC tumours than in NMIBC (Martinez *et al.*, 2019).

Furthermore, the degree and extent of hypermethylation were used to indicate the stage and grade of BC. For instance, most hypermethylation alterations were reported in early BC (carcinoma in situ), and more altered methylation was reported with high grade and invasive tumours compared to low-grade tumours (Martinez *et al.*, 2019). Additionally, the patients' response to Bacillus Calmette-Guerin (BCG) therapy was correlated with the methylation status of MSH6 and THBS1 to distinguish responders to therapy (Martinez *et al.*, 2019). Accordingly, understanding the underlying DNA methylation changes correlated with BC has provided an advanced insight into novel diagnostic and screening procedures that accurately predict the BC prognosis with lower costs.

4.1.1.2 Chromatin remodelling and histone modifications in BC

Polycomb repressor complex (PRC), including its two classifications (PRC1 and PRC 2) involved in histone modifications has gained attention due to its broad implications in malignancies (Martínez-Fernández *et al.*, 2015). PRC2 is responsible for a group of biological process, including cell differentiation and stemness. Four different proteins form the structure of the PCR2 in mammals, including EED (Embryonic Ectoderm Development), SUZ12 (Suppressor of Zeste 12 Homolog), EZH2 (Enhancer of Zeste Homolog 2) and RBBP7/4 (Retinoblastoma Binding Protein 7/4)(Martínez-Fernández *et al.*, 2015).

EZH2 is the catalytic subunit of PRC2 responsible for the catalysis of the (H3K27me3), an epigenetic mark responsible for the repression of gene expression of affected regions in the genome (Martínez-Fernández *et al.*, 2015). The overexpression of PRC1 and PRC2 were correlated with various types of tumours, including BC. Thus, EZH2 specifically as a subunit of PRC2 has significant implications in tumorigenesis, which has drawn extensive investigations into its role in tumorigenesis (Martínez-Fernández *et al.*, 2015; Martinez *et al.*, 2019). This resulted in several conclusions agreed that the EZH2 role in tumorigenesis is not confined only to the epigenetic silencing through histone methylation. However, it can expand to include modulation of other cellular proteins, gene expression activation for different pathways, and silencing of several miRNAs such as the family of mir-200 (Martínez-Fernández *et al.*, 2015; Martinez *et al.*, 2019). Thus, the potential of EZH2 to silence miRNAs and tumour suppressor genes explains its consideration as an oncogenic factor.

The exact details of the EZH2 roles as an epigenetic silencer or gene expression activator are out of the scope of this review article; however, several review articles have detailed the roles of EZH2 and its interaction with different pathways (Martínez-Fernández *et al.*, 2015; Martinez *et al.*, 2019; Tran *et al.*, 2021). The focal point of this discussion is to explain the role of EZH2 in BC prognosis and emphasise the potential of using EZH2 as a potential therapeutic target for BC using novel therapies.

4.1.1.3 Non-coding RNA (ncRNA) in BC

ncRNAs are molecules that are not translated into proteins but instead play regulatory roles in cellular functions by adjusting DNA expression (Martinez *et al.*, 2019). Long non-coding RNA (lncRNA), small interfering RNA (siRNA), micro-RNA (miRNA; miR) are all types of ncRNA that play a prominent role in BC development and progression (Knowles and Hurst, 2015;

Martinez *et al.*, 2019). lncRNAs are made up of more than 200 nucleotides and responsible for essential biochemical processes. The contribution of lncRNAs in carcinogenesis was studied by comparing their expressions in tumour tissues compared to healthy controls to find out that lncRNAs are differentially expressed in several types of tumour tissues. In BC specifically, it was found that deregulation of lncRNAs can lead to carcinogenesis in different ways, including induction of metastasis and sustained proliferative signalling (Martinez *et al.*, 2019).

Furthermore, several lncRNAs were identified for their contribution in BC, including lncRNA-UCA1 that was reported to induce epithelial-mesenchymal transition (EMT) and promote BC cell migration and invasion (Bhan, Soleimani and Mandal, 2017). This was attributed to its potential in targeting the miR-145–ZEB1/2–FSCN1 pathway and miR-582-5p, besides its role in modulating the miR-143/ HMGBG1 signalling pathway (Martinez *et al.*, 2019). In addition, LncRNA-H19 is another contributor reported to be overexpressed in BC, leading to increased miR-675 expression, thus inhibiting *TP53* activation. Furthermore, the lncRNA-H19 was found to inhibit E-cadherin and target miR-29b-3p, resulting in EMT and metastasis (Bhan, Soleimani and Mandal, 2017). More details about Several other lncRNAs involved in BC development and progression can be found in these articles (Bhan, Soleimani and Mandal, 2017; Xie *et al.*, 2017; Martinez *et al.*, 2019).

miRNAs are the second main class of ncRNAs with a prominent role in the epigenetic aetiology of BC. These are 21-24 nucleotides that mediate gene silencing by targeting messenger RNAs (mRNAs) of multiple genes (Knowles and Hurst, 2015; Bhan, Soleimani and Mandal, 2017; Xie *et al.*, 2017; Martinez *et al.*, 2019). Additionally, they play an integrated role with lncRNAs in oncogenic pathways. The contribution of miRNA in BC development is attributed to the

type of miRNA since they can be divided into oncogenic miRNA or tumour suppressor miRNAs.

In order to clarify the contribution of the alterations in miRNA expression in BC, it is necessary to explain the functions of these biomolecules. For instance, the family of miR-200, including miR-200a, miR-200b, miR-200c, miR-429 and miR-141, is a tumour suppressor family found to efficiently inhibit the EMT process by regulating ZEB1 and ZEB2 transcription factors (Martínez-Fernández *et al.*, 2015; Martinez *et al.*, 2019). This family was found to be downregulated in BC, which was responsible for poor prognosis in BC and was later used as prognostic biomarkers. miR-34 is a metastatic suppressor miRNA in BC that functions by directly targeting CD44, a cell surface transmembrane glycoprotein that is considered a vital gene in cancer development due to its critical role in several cellular functions, including cancer cell growth, metastasis, and resistance to apoptosis (Yu *et al.*, 2014; Liang *et al.*, 2021). miR-34 was found to be significantly downregulated in BC tissues and cell lines (Yu *et al.*, 2014). Others include miR-100, miR-101 and miR-214, which were found to be downregulated in BC, correlated with an unfavourable prognosis. On the contrary, as summarised in **Figure 4**, miR-452, miR-21, miR-222, miR-182, miR-133b, miR-155, miR-145, and miR-152 upregulation in BC were found to contribute to poor prognosis (Xie *et al.*, 2017; Martinez *et al.*, 2019).

Some miRNAs were found to target the fibroblast growth factor receptor (FRGR3) pathway, including miR-99a, miR-100, miR-101, and miR-145 (Martinez *et al.*, 2019). FGFR3 showed an increased expression in BC, which was later correlated with the stage of the BC (van Rhijn *et al.*, 2020). FGFR3 overexpression can be attributed to the downregulation of miR-99a and miR-100 in BC, which negatively regulate the expression of FGFR3 (Knowles and Hurst, 2015) **(Figure 4)**. This attests that FGFR3 is another potential target in BC. Although it is not

well understood whether these alterations in miRNAs expression are causative of or associated with the disease development, understanding these alterations will provide an insight into novel therapeutic approaches that can potentially target these alterations to restore the homoeostasis and block BC metastasis.

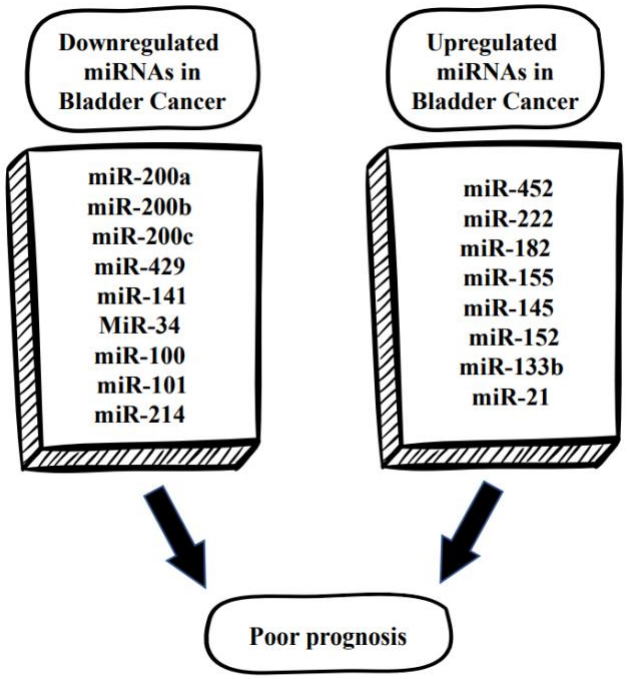

Figure 4. The alterations in miRNAs expression in bladder cancer, resulting in poor prognosis.

4.1.1.4 Kruppel-like factor 4 (KLF4) in BC

Kruppel-like factors (KLFs) are transcriptional regulators of cell differentiation and proliferation. KLF4 and KLF5 were found to have a distinct tissue specificity by being highly restricted to the epithelial cells of several organs and were correlated with colon carcinogenesis (Ohnishi *et al.*, 2003). Several studies have investigated their contribution in BC to determine whether they can be considered potentially actionable BC targets. For example, a study conducted by Ohnishi et al. demonstrated that KLF4 and KLF5 were highly expressed in normal bladder epithelium; however, only KLF4 was downregulated in bladder cancer tissues

and cell lines (Ohnishi *et al.*, 2003). This study has revealed that transducing bladder cancer cells with the KLF4 gene suppressed cell growth and induced apoptosis suggesting that the inactivation of KLF4 can contribute to bladder carcinogenesis. Several studies have confirmed these findings and emphasised that downregulation of KLF4 has been observed in urothelial carcinoma of the bladder and associated with local recurrence of urothelial carcinoma of the bladder (Li *et al.*, 2014; Tseng *et al.*, 2016). Hence, several studies suggested the use of KLF4 as a potential predictive biomarker for metastasis and death(Ohnishi *et al.*, 2003; Leng *et al.*, 2013; Tseng *et al.*, 2016). Accordingly, KLF4 can be considered as a potential target in BC.

4.1.2 Therapeutic strategies for bladder cancer

4.1.2.1 Conventional therapeutic procedures

The adopted therapeutic strategy is chosen according to the BC category (GuhaSarkar and Banerjee, 2010; Martínez-Fernández *et al.*, 2015). NMIBC is usually treated by transurethral resection of the tumour followed by chemotherapy instilled locally by intravesical instillation as adjuvant therapy (GuhaSarkar and Banerjee, 2010; Knowles and Hurst, 2015; Martínez-Fernández *et al.*, 2015). Although these therapeutic procedures are employed in NMIBC, there is a high recurrence rate and probability of developing MIBC (Martínez-Fernández *et al.*, 2015). Additionally, the efficiency of drug delivery using intravesical instillation is limited by the bladder permeability barrier, which requires frequent catheterization and may result in bladder fibrosis (GuhaSarkar and Banerjee, 2010; Kolawole *et al.*, 2017). Hence, this developed the need for integrating the intravesical instillation with sustained-release drug delivery systems to extend the drug retention times at the site of action, improve treatment efficiency, and reduce dosing frequency.

For MIBC, the procedure in most cases implies cystectomy accompanied by a combination of chemotherapeutic drugs (Martínez-Fernández *et al.*, 2015). Despite using these therapeutic procedures, apparently, the metastasis and mortality rates among the patients are still high, which explains the need for novel therapeutic strategies that can tackle the origin of the disease and cease its progression.

4.1.2.2 Probiotics

The early evidence that probiotics could be used to reduce the risk of bladder cancer, came in late 1990s and early 2000s when several studies reported that the use of *Lactobacillus casei* strains inhibited the experimentally induced tumours in the bladders of animal models (Tomita *et al.*, 1994; Ohashi *et al.*, 2002; Feyisetan, Tracey and Hellawell, 2012) and acted as immunomodulators by enhancing host immune system (Kato, Endo and Yokokura, 1994; Ohashi *et al.*, 2002; Naito *et al.*, 2008). Additionally, the relationship between consumption of fermented milk products, containing bacterium *Lactobacillus* strains, and reduced bladder cancer incidents was observed (Ohashi *et al.*, 2002; Zhang *et al.*, 2019).

Randomised controlled trial (RCTs) conducted by Naito et al., demonstrated that superficial cancer recurrence was significantly decreased when intravesical chemotherapy was combined with orally administrated *L. casei* strain Shirota (LcS) (Naito *et al.*, 2008). These results supported the findings of previously reported double-blind trials by Aso et al., where bladder cancer patients were separated into two groups and orally administrated either LcS or placebo, to observe if probiotic strain enhances prevention from cancer recurrence (Ohashi *et al.*, 2002; Hoesl and Altwein, 2005; Aragón *et al.*, 2018).

Several studies have focused on using probiotics as a bladder cancer treatment, instead of using them as the cancer preventative option. A study done in early 2000s provided evidence that the use of certain probiotic strains, such as LcS, enhances innate immune response, as well as stimulates cytokine production, which potentially plays an important role in anti-tumour activity in murine cancer (Matsuzaki and Chin, 2000). Based on these findings, study by Seow et al. set to investigate how conventional bladder cancer treatment by Bacillus Calmette-Guerin (BCG) immunotherapy compares with live and lyophilised *Lactobacillus rhamnosus GG* (LGG) treatment of bladder tumours in mice animal models (Seow *et al.*, 2010). Results revealed that LGG therapy has increased chemokine XCL1 levels in the bladder, therefore enhancing recruitment of T and natural killer (NK) cells, which facilitates with tumour regression. When compared with BCG treatment, probiotic treatment has recruited more immune cells into the bladder and produced higher levels of TNF-α, which can induce tumour regression. This data suggests that probiotics could be a safe and effective way to treat bladder cancer, and therefore, could potentially match the efficacy of BCG immunotherapy.

Following these results, a study by Cai et al. used different concentrations of LGG to stimulate dendritic cell (DC) maturation and cytokine production for short and prolonged time periods, which were then compared with BCG therapy (Cai *et al.*, 2016). Results demonstrated that strongest anti-tumour effect was obtained by using a short exposure of a small dose of LGG to activate neutrophils, which in turn stimulated DCs to modulate T cell activation. These conditions were reported to be more efficient in mice bladder tumour treatment compared to BCG treatment.

Some evidence shows that probiotic strains could slow down the development of the tumours in the bladder. A study by Sivan et al. demonstrated that use of *Bifidobacterium 7* has delayed

the bladder tumour outgrowth compared to no treatment in murine animal model (Sivan *et al.*, 2015). However, research done by Nada et al. showed that probiotic strains *Lactobacillus acidophilus* and *Bifidobacterium longum* did not have any effect on bladder cancer cell viability and tumour angiogenesis, compared to the significant effects that probiotics had on gastric cancer cells (Nada *et al.*, 2020). These conflicting results could suggest that anti-tumour activity could be related to specific probiotic strains.

Up to date research demonstrates that probiotic treatment can be used as a preventative measure against BC recurrence, as well as potentially halt tumour progression. Additionally, several studies show successful immune system activation by probiotics, which therapeutic effects could potentially reach the efficacy of BCG immunotherapy. However, further research in animal models, along with additional human RCTs are crucial to confirm the probiotics efficacy against BC.

4.1.2.3 Nanoparticles based drug delivery systems

Drug delivery using nanotechnology in order to treat cancer has emerged in early 2000s and since then researched different types of nanocarriers to bring therapeutic agents to the target site (Barani *et al.*, 2021). Intravesical instillation of drugs has been proven to be more effective than oral drug administration, however combined with nanotechnology it further enhanced drug permeability into the tumour tissue and reduced adverse effects (Chen *et al.*, 2015). However, due to bladder filling and voiding, the short drug retention time in the bladder still remains one of the biggest limitations of this treatment method (Barani *et al.*, 2021; Sahatsapan *et al.*, 2021). Recent studies have investigated use of different mucoadhesive materials to prolong nanoparticle retention time in the bladder, as well as provide sustained drug release to minimise frequent dosing (Ali *et al.*, 2020; Xu *et al.*, 2020; Sahatsapan *et al.*, 2021). In addition,

these studies demonstrated enhanced safety profiles and treatment efficacy against tumour cells, and study by Xu et al. even demonstrated a direct and more precise tumour tissue targeting by encapsulated drugs.

Nanoparticles used for bladder cancer treatment can be made from different materials, which means that the therapy they are delivering differs too **(Figure 5)**. Metallic nanoparticles can be modified with antibodies and ligands, which would only bind to the specific tumour tissue, that way preventing healthy tissue from harm (Chen *et al.*, 2015; Jain, Kathuria and Momin, 2021). Use of photothermal therapy excites the gold nanoparticles, therefore destroying cancer cells without harming surrounding tissues (Chen *et al.*, 2015). However, further *in vivo* studies are required to confirm safety of this treatment, along with nanoparticle clearance after the treatment. In contrast, polymeric nanoparticles have been demonstrated to be safe and biodegradable option used for drug delivery purposes, with some polymers, such as poly (lactic-co-glycolic acid), already approved for use by FDA (Bobo *et al.*, 2016). Chemotherapeutic drugs for bladder cancer treatment can be efficiently delivered in polymeric nanoparticles, due to their small size, reduced side effects and systemic exposure, sustained drug release and longer retention times when mucoadhesive materials are added to the formulation (Chen *et al.*, 2015; Barani *et al.*, 2021; Jain, Kathuria and Momin, 2021). Several studies have looked into encapsulating different therapeutic agents into polymeric nanoparticles and treatment efficacy against tumour cells in the bladder, reporting promising results of reduction in tumour growth, extended drug release and retention in the bladder and efficient drug penetration into the urothelium layer (Martin *et al.*, 2013; Jin *et al.*, 2014). Similarly to polymeric nanoparticles, drug delivery by liposomes offer enhanced drug bioavailability, high encapsulation into particle core, and therefore enhanced therapeutic effect (Kashyap *et al.*, 2021). Some studies have reported that encapsulation of BCG cell wall

skeleton, which is the main immune active centre of BCG and is an alternative treatment option to live BCG, in liposomes has shown enhanced antitumor effects without usual toxicity of BCG treatment (Nakamura *et al.*, 2014; Whang *et al.*, 2020).

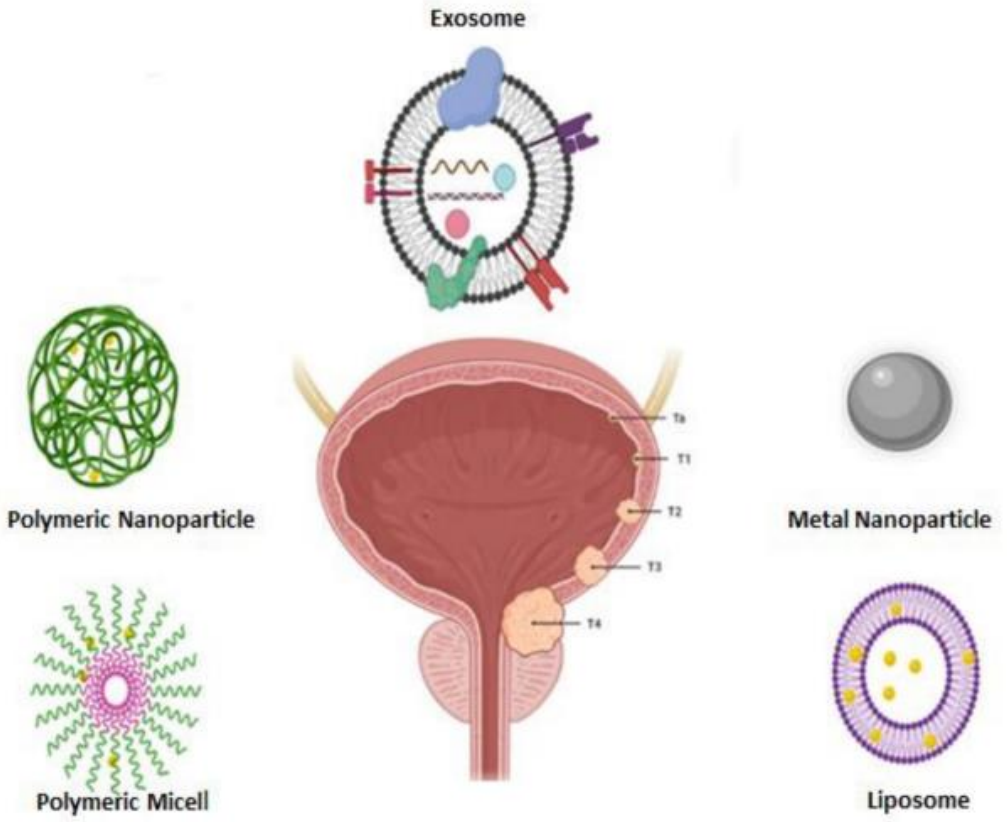

Figure 5. Different types of nanosized drug carriers that can be used to deliver drugs or other active agents into the tumour site to treat bladder cancer. Reprinted with permission from Barani et al. (Barani *et al.*, 2021).

Although up to date research looks incredibly promising in developing a highly robust and safe way of anti-tumour and chemotherapy drug delivery to the bladder to treat cancer, some limitations still remain. Main problems occur due to safety profiles of these therapies, where sufficient drug concentrations need to be delivered using non-toxic concentrations of polymeric/metallic/lipid materials. In addition, time and efficiency of drug delivery vesicle clearance from the body needs to be investigated to ensure long-time treatment safety.

4.1.2.4 Novel genetic therapies

The gene expression studies have identified several actionable targets in BC, as discussed in **section 4.1.1**. This introduced a novel insight into therapeutic strategies that can target the molecular mechanisms responsible for the disease progression. As mentioned in **section 3.1**, various genetic alterations were reported in BC cases. Accordingly, several studies have focused on targeting these alterations to restore homeostasis and cease the progression of the disease. This section of the review is focused on discussing the novel therapeutic strategies that can efficiently modify the alterations addressed in **section 4.1.1**, besides the recent studies that have tested the efficiency of the suggested therapies.

Following the discussion in **section 4.1.1.2**, EZH2 is among the potential oncogenic actionable targets in BC due to its overexpression in BC, resulting in silencing miRNAs and tumour suppressor genes. Several studies have looked into the interactions between EZH2 and miRNAs to examine the potential of miRNAs in downregulating EZH2 in BC. Therefore, several studies have looked into the interactions between EZH2 and miRNAs to examine the potential of miRNAs in downregulating EZH2 in BC (Martínez-Fernández *et al.*, 2015). Thus, these investigations resulted in a miRNA-EZH2 network explaining the interactions between EZH2 and several miRNAs. As shown in **Figure 6**, several miRNAs were found to directly regulate the EZH2 expression post-transcriptionally. These include miR-101, miR-26a, miR-214, miR-217, miR-124, miR-138, miR-98, miR-25, miR-30d, miR-199a, miR-29, miR-144 and Let 7 family (Martínez-Fernández *et al.*, 2015). Accordingly, these findings have paved the way for several studies interested in screening the alterations in the regulatory mechanisms of gene expressions in BC. Among the alterations that were reported by these studies was the downregulation of miR-101-3p in BC (Martínez-Fernández *et al.*, 2015; Li, Xie and Zhang, 2019). miR-101-was found to negatively regulate EZH2 expression besides acting as a tumour

suppressor gene in several malignant cancers, including BC (Martínez-Fernández *et al.*, 2015; Li, Xie and Zhang, 2019).

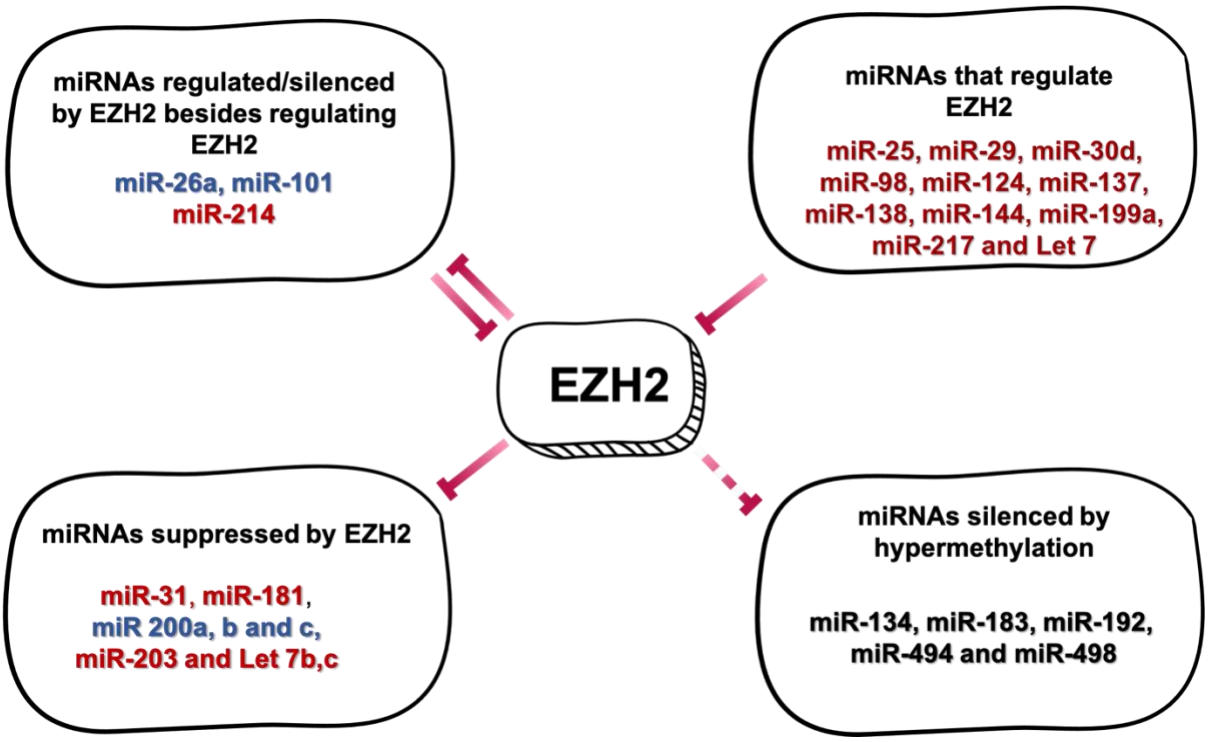

Figure 6. EZH2-miRNA network. In red, the miRNAs with experimental evidence of EZH2 interaction; those that are also observed in BC are denoted in blue; and in black those miRNAs without direct evidence of repressed expression by EZH2. Reprinted with permission from Martínez-Fernández et al.(Martínez-Fernández *et al.*, 2015).

Additionally, miR-101-3p was found to inhibit the invasion and migration of bladder cancer 253J-BV cells demonstrated by a study conducted by Ma et al. (Ma *et al.*, 2017). According to the miRNA-EZH2 network established by several studies, EZH2 was suggested as a potential target of miR-101-3p (Li, Xie and Zhang, 2019). Hence, miR-101-3p downregulation was accompanied by a commensurate increase in EZH2 expression, which is reported to be abnormal in several malignant cancers. Despite these findings, a recent study has investigated the regulatory roles and molecular mechanism of miR-101-3p in bladder urothelial carcinoma (BUC) chemoresistance, which often results in chemotherapy failure. Therefore, the study has

revealed the therapeutic potential of miR-101-3p in improving the therapeutic outcome of chemotherapy by increasing the sensitivity of BUC to cisplatin (CDDP) through targeted EZH2 silencing (Li, Xie and Zhang, 2019). Hence, these findings suggest a new therapeutic strategy that modifies the molecular mechanisms correlated with BC by miRNAs delivery. As mentioned above, several miRNAs besides miR-101-3P were found to regulate EZH2 directly; however, there is still a need for systemic studies to assess the efficacy of delivering these miRNAs in modifying the genetic alterations correlated with BC and restoring homeostasis.

As shown in **Figure 6**, EZH2 regulates many miRNAs by epigenetic repression. These miRNAs generally act as tumour suppressors (Martínez-Fernández *et al.*, 2015). Among these miRNAs is the miR-200 family that was found to be downregulated in BC and correlated with poor prognosis in BC, as mentioned in **section 4.1.1.2.** A study conducted by Wang et al. confirmed the correlation between EZH2 and miR-200 family, by demonstrating that increasing the expression of EZH2, a hallmark of NMIBC at high risk of recurrence, results in a significant decrease in miR-200 family expression and the knockdown of EZH2 increases the expression of miR-200 family in BC cell lines (Wang *et al.*, 2010). Despite these findings, several studies have investigated the role of miR-200c delivery in modifying the progression of the disease. The results demonstrated that miR-200c can efficiently inhibit the BC progression by inhibiting the invasion and proliferation of BC cells through several pathways other than the EZH2 regulation, such as the downregulation of BMI-1 and E2F3 and by targeting lactate dehydrogenase A (Liu *et al.*, 2014; Yuan *et al.*, 2017). These findings revealed the clinical implications of miRNAs in BC; however, there is a need for further studies to develop a delivery system that provides efficient miRNAs delivery and localization to the site of action.

As discussed in **section 4.1.1.3,** miRNA-34 is one of the miRNAs that was found to be downregulated in BC and has been linked to chemotherapy resistance in several types of cancer. In addition, a study conducted by Yu et al. has revealed the role of miR-34a as a potential therapeutic target in BC, which was linked to its role as an antimetastatic microRNA and suppressor of angiogenesis in bladder cancer by directly targeting CD44 (Yu *et al.*, 2014). Previous studies demonstrated a piece of accumulated evidence that CD44 was linked with lower survival rates and lower response rates in BC (Liang *et al.*, 2021). The study conducted by Yu et al. using in vivo experiments confirmed that miR-34a was frequently downregulated in bladder cancer tissues and demonstrated that miR-34a could suppress cell migration and invasion (Yu *et al.*, 2014). In addition, the study provided clear evidence that miR-34a functions as an antimetastatic miRNA by suppressing CD44, which regulates a group of genes involved in BC. Therefore, this study provided a novel therapeutic strategy by revealing the role of miR-34a in modifying one of the dysregulated mechanisms underlying tumour metastasis in BC.

Despite these findings, CD44 targeting has attracted increasing attention as an effective target in BC. Therefore, a recent study conducted by Liang et al. has developed a novel self-cross-linkable chitosan-hyaluronic acid dialdehyde nanoparticles for CD44-targeted siRNA delivery to treat BC (Liang *et al.*, 2021). This study has shown the value of coupling a therapeutic siRNA that aims to downregulate the expression of Bcl-2 with a hyaluronic (HA) vehicle possessing natural CD44 targeting properties (Liang *et al.*, 2021). Accordingly, the study has confirmed the upregulation of CD44 expression in bladder tumours then demonstrated the efficiency of the developed NPs in inhibiting the targeted oncogene (Bcl-2) and tumour growth after being taken up by T24 cells through CD44 receptor-ligand-mediated endocytosis (Liang *et al.*, 2021). Thus, this study provided a novel gene delivery system that effectively targets BC

with high CD44 expression and casts light on using polymeric biocompatible nanoparticles for gene delivery as a novel strategy to inactivate oncogenic miRNAs or restore tumour suppressor miRNAs in BC.

Fibroblast growth factor receptor 3 (FGFR3) is another gene that was reported to be dysregulated in BC and highlighted as a potential target of therapeutic strategies for BC, as mentioned in **section 4.1.1.3**. FGFR3 belongs to a family of structurally related tyrosine kinase receptors (FGFR1-4), which regulate several biological processes, including proliferation, differentiation, migration and apoptosis (Wu *et al.*, 2014). FGFR3 is considered an oncogene in most low-grade NMIBC and up to 40% of invasive bladder tumours (Wu *et al.*, 2014). In addition, FGFR3 was reported to be one of the most frequently mutated genes and potential targets in BC since its activation was found to mediate growth and neoplasia in several types of cancer, including BC (Wu *et al.*, 2014; van Rhijn *et al.*, 2020). These findings have raised the need for further studies to investigate the regulatory mechanisms of FGFR3 and identify gene candidates that can regulate its expression in BC. Therefore, a study conducted by Wu et al. was among the first to address the correlation between miRNA-99a and FGFR3 expression in BC (Wu *et al.*, 2014). miRNA-99a was found to be downregulated in several types of cancer, including BC, as was further confirmed by Wu et al. study (Wu *et al.*, 2014). Furthermore, this study indicated that miRNA-99a could efficiently suppress BC cell proliferation, migration and invasion by directly downregulating FGFR3. These findings suggest that miRNA-99a may be an effective treatment for BC; however, further studies are needed to explore the possibility of delivering it using a biocompatible delivery vehicle to the site of action.

Finally, it is noteworthy to address the dysregulation of KLF4 observed in BC and the suggested therapeutic strategies targeting KLF4 as a potential target in BC. KLF4 is a

transcriptional regulator associated with cell arrest that is significantly downregulated in bladder tumours; hence, several studies were done to investigate the role of KLF4 in BC (Shields, Christy and Yang, 1996; Segre, Bauer and Fuchs, 1999; Ohnishi *et al.*, 2003). These studies concluded that increasing the expression of KLF4 in bladder cancer cell lines results in the suppression of urothelial cancer cell growth, migration and invasion (Ohnishi *et al.*, 2003; Li *et al.*, 2014; Xiao *et al.*, 2014). Accordingly, these studies provided solid evidence that KLF4 functions as a tumour suppressor in bladder carcinogenesis and paved the way for other studies to identify the genes regulating the KLF4 expression. Therefore, a study conducted by Xiao et al. was among the first studies to address a direct correlation between miRNA-10b and KLF4 in BC (Xiao *et al.*, 2014). This study has demonstrated that miRNA-10b is significantly upregulated in BC cell lines and metastatic tissues and that the increased expression of miRNA-10b results in enhanced BC cell migration and invasion, addressing by this that miRNA-10b may function as oncogenes in BC cells. Interestingly, the study has revealed that miRNA-10b functions by downregulating E-cadherin through targeting KLF4, promoting by this migration and invasion in BC cells (Xiao *et al.*, 2014). This suggests that KLF4 is a direct functional target of miRNA-10b and proposes a novel therapeutic strategy to block BC cell metastasis.

In summary, this section of the review is focused on addressing the potential actionable targets in BC and the underlying regulation mechanisms besides proposing novel strategies that rely on delivering miRNAs to restore homeostasis and modify the epigenetic alterations. Thus, identifying these miRNAs could pave the way for future studies to develop miRNAs delivery vehicles that provides efficient miRNAs delivery and localization to the site of action as a novel therapeutic strategy for BC.

4.2 Bladder interstitial cystitis

Interstitial cystitis (IC) or painful bladder syndrome (PBS) can be used interchangeably to describe chronic symptoms of discomfort or pain coupled with increased urinary frequency in the absence of etiological factors for these symptoms, such as infection (Rosamilia, 2005; van de Merwe *et al.*, 2008; Dasgupta and Tincello, 2009; GuhaSarkar and Banerjee, 2010; Nirmal *et al.*, 2012). IC causes massive damage to the bladder lining, resulting in a group of chronic complex symptoms. These include urinary frequency accompanied with bladder pressure or discomfort, urgency or nocturia and pelvic pain in 70% of the cases (Rosamilia, 2005; Dasgupta and Tincello, 2009; Nirmal *et al.*, 2012). This section of the review is focused on explaining the aetiologies of IC, addressing the biomarkers correlated with the disease and discussing the proposed therapeutic strategies **(Figure 7)**.

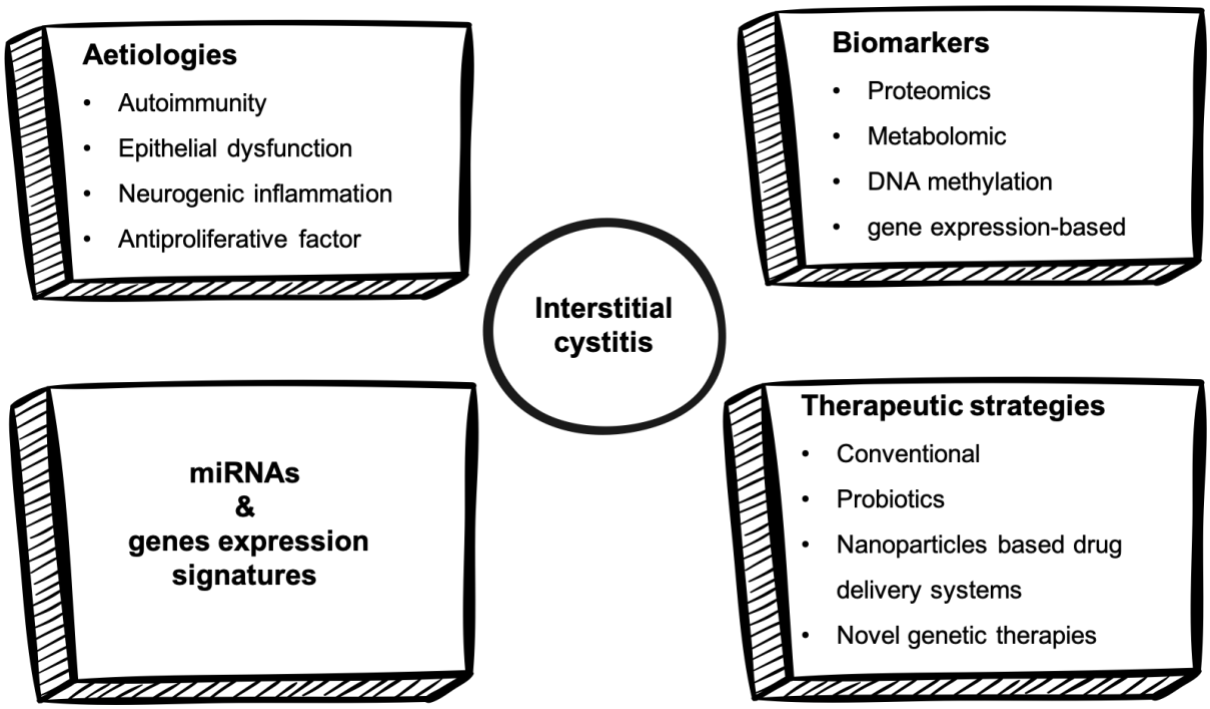

Figure 7. A summary of the aetiologies, biomarkers and the therapeutic options for interstitial cystitis.

4.2.1 Aetiologies of IC

The causes of the syndrome remains poorly understood, which hinders the epidemiology studies due to the inconsistencies in the diagnostic criteria (Rosamilia, 2005; van de Merwe *et al.*, 2008; Kim, Kim and Kim, 2020). As a result, several theories of pathogenesis were proposed to explain the complex aetiologies of the syndrome, including autoimmunity, neurogenic inflammation, epithelial dysfunction, allergic reaction and genetic factors (Rosamilia, 2005; Dasgupta and Tincello, 2009; Nirmal *et al.*, 2012). In this section, these theories will be briefly explained as follows.

4.2.1.1 Autoimmunity

Immunoglobulins such as IgG, IgA and IgM and T lymphocytes were found to be significantly higher in interstitial cystitis besides an increase in the number of CD8+ and CD4+, T lymphocytes, B-lymphocytes and plasma cells within the urothelium and submucosal layer of the bladder in IC compared to the normal bladder wall (Dasgupta and Tincello, 2009). Additionally, there is an association between several autoimmune diseases, such as systemic lupus erythematosus, Sjogren's syndrome and IC (Rosamilia, 2005). However, it is still unknown whether these findings are causative or reactive since no consistent profile of immune activity was reported in IC (Rosamilia, 2005; Dasgupta and Tincello, 2009). Thus, the pathogenesis of the disease cannot be explained or correlated to any of these findings.

4.2.1.2 Epithelial dysfunction/permeability

A dysfunctional epithelium alters the bladder permeability, allowing transepithelial migration of solutes, such as potassium and other toxic substances, to enter the subepithelial layer of the bladder wall (Dasgupta and Tincello, 2009; GuhaSarkar and Banerjee, 2010; Nirmal *et al.*, 2012). Consequently, this might result in depolarizing the subepithelial afferent nerves, provoking by this the sensory symptoms (Nirmal *et al.*, 2012). The disruption of the GAG layer

is the fundamental theory of dysfunctional epithelium. Typically, this GAG layer forms the superficial layer of the extracellular matrix and functions as a protective layer to prevent the reabsorption of other urinary constituents into the bladder wall (Dasgupta and Tincello, 2009). However, the underlying molecular mechanisms behind the disruption of this layer in IC are still unknown. Moreover, there was no significant difference in total glycosaminoglycans and hyaluronic acid between controls and interstitial cystitis patients. Hence, using the level of proteoglycan as a biomarker of IC was not found useful (Dasgupta and Tincello, 2009). Accordingly, no direct correlations can be drawn from this theory to be implemented in the diagnosis or the therapeutic strategy of IC.

4.2.1.3 Neurogenic inflammation

An increased number of activated mast cells in bladder urothelium were found to be related to IC (Dasgupta and Tincello, 2009; Nirmal *et al.*, 2012; Song *et al.*, 2019). The secretions of mast cells contain several nociceptive and inflammatory molecules such as substance P and nerve growth factor (NGF), resulting in increased proliferation of the nerve fibres (Dasgupta and Tincello, 2009; Nirmal *et al.*, 2012; Jiang *et al.*, 2013). Additionally, it was found that histamine released via mast cells could activate the pain-sensing C fibres located within the uroepithelium and submucosa of the bladder (Nirmal *et al.*, 2012). The theory of mast cell's role in neurogenic inflammation was supported by several studies that demonstrated positive correlations. These findings include detecting an elevated level of urinary methyl histamine, a major metabolite of the mast cell mediator histamine and the unique mast cell enzyme in IC, besides an enhanced sensitivity of the perivascular sensory nerve terminals to substance P, the neuropeptide substance in the untreated IC patients (Dasgupta and Tincello, 2009; Nirmal *et al.*, 2012; Kuo, 2014).

4.2.1.4 Antiproliferative factor (APF)

The change in the bladder epithelial permeability was among the fundamental theories that aim to explain the cause of IC; however, there was a need to understand the mechanisms that initiate this change in IC. Therefore, several studies were conducted to investigate the underlying mechanisms, which conclusively addressed a correlation between the presence of APF peptide that inhibits the proliferation of bladder epithelial cells in vitro and the consequent changes in specific epithelial cell growth factor levels, and symptoms of IC (Rosamilia, 2005; Dasgupta and Tincello, 2009; Nirmal *et al.*, 2012; Kuo, 2014). These findings suggest that the change in bladder urothelial permeability can be related to the inhibition of normal bladder epithelial regeneration by APF, which might initiate the pathogenesis of IC.

4.2.2 IC biomarkers

The pathogenesis of IC is complex and is believed to be multifactorial; however, the underlying mechanisms of the genesis are unknown; which results in variable therapeutic outcomes. Thus, identifying IC biomarkers would provide the clinicians with a non-invasive diagnostic tool for clinical management and provide an insight into the molecular aetiology of IC (Kuo, 2014; Kim, Kim and Kim, 2020). Several proteomic biomarkers, metabolomic biomarkers, serum biomarkers and DNA methylation biomarkers were identified in IC for assisting diagnostics with improved precision.

As proteomics biomarkers, several biomarkers were investigated in urine specimens, including nerve growth factor (NGF), Interleukin-6 (IL-6), histamine, antiproliferative factor (APF), macrophage inhibitory factor (MIF) and heparin-binding (HB)-EGF (Kuo, 2014; Kim, Kim and Kim, 2020). A study conducted by Tonyali et al. reported a significant increase in NGF/Cr in IC patients compared to the control group (Tonyali *et al.*, 2018). Another study conducted

by Lamale et al. reported an increase in the urinary concentrations of histamine and IL-6 in IC patients (Lamale *et al.*, 2006). Moreover, urinary macrophage inhibitory factor (MIF) concentration was found to be significantly higher in IC patients with Hunner lesions compared with patients without Hunner and with controls (Kim, Kim and Kim, 2020). Furthermore, as explained in **section 4.2.1.4** , several studies demonstrated that the increased level of APF is correlated with IC (Rosamilia, 2005; Kuo, 2014; Kim, Kim and Kim, 2020). Additionally, as for serum biomarkers, several serum pro-inflammatory cytokines and chemokines were found to be significantly elevated in IC cases such as IL-1β, IL-6, TNF-α and IL-8 (Lamale *et al.*, 2006; Kuo, 2014; Song *et al.*, 2019).

Etiocholan-3alpha-ol-17-one (Etio-S) was among the metabolomic biomarkers that were found to be elevated in IC patients, as demonstrated by Parker et al. study (Parker *et al.*, 2016; Kim, Kim and Kim, 2020). These studies collectively demonstrated that urine and serum biomarkers could be used to monitor IC disease as a potential diagnostic and prognostic tool.

Therefore a study conducted by Bradley et al. aimed to develop a methylation profile that could be used as a non-invasive biomarker as well as providing an insight into the underlying genesis mechanisms by investigating the feasibility of using urine samples to perform a DNA methylation study in females with IC (Bradley, Megan S., Emily E. Burke, Carole Grenier, Cindy L. Amundsen, Susan K. Murphy, 2018). The study has demonstrated the feasibility of DNA methylation profiling from voided urine specimens in women with IC and addressed a dysregulation in the MAPK pathway supported by finding several gene members of this pathway with differentially methylated CpG sites in cases relative to controls (Bradley, Megan S., Emily E. Burke, Carole Grenier, Cindy L. Amundsen, Susan K. Murphy, 2018). In addition to DNA methylation biomarkers, some studies were focused on investigating gene expression-

based biomarkers. For example, a study conducted by Ogawa et al. demonstrated an overexpression in genes related to inflammatory responses in IC such as T-helper type 1-related chemokines and cytokines such as CXCR3 binding chemokines and TNFSF14 (Ogawa *et al.*, 2010). Although identifying the IC biomarkers might not explain the underlying genesis mechanisms or the aetiology of the disease, it offers a solution for the inconsistencies encountered with the diagnostic criteria.

4.2.3 miRNAs and genes expression signatures in IC

To provide an extensive insight into the underlying molecular mechanisms of IC, studies have focused on performing a comparative gene expression profile study of bladder biopsies from patients with ulcerative IC and control patients. Gamper et al. study was one study that focused on investigating any significant differences in mRNA levels between bladder cells from IC patients compared to healthy controls and hypothesised that B-cell invasion into the bladder wall could be the dominant feature of ulcerative IC. (Gamper *et al.*, 2009). This suggestion was based on the study findings, which showed that 25 proteins with the highest IC-to healthy expression ratios are expressed in B-leukocytes besides finding nine of the top 25 gene expressions (*IGHM*, *MS4A1*, *FCRL3*, *IGHG1*, *PAX5*, *BLK*, *IGHA1*, *IGL@* and *FCRLA*) to be in the top 100 probe sets for B-lymphocytes (Gamper *et al.*, 2009). Additionally, the study revealed that cell adhesion leucocyte chemotaxis is among the immune reaction processes dominant in ulcerative IC as six cytokine/chemokine genes encoding CXCL13, CCL18, IL-8, CCL19, CXCL6 and GROa ranked within the top seven genes of this process (Gamper *et al.*, 2009). Therefore, these findings highlight that the comparative gene expression profile studies might provide an insight into the molecular mechanisms behind the IC development, which could be translated into novel therapeutic strategies.

Another study conducted by Arai et al. has focused on investigating the molecular pathogenesis of IC based on miRNA expression signature (Arai *et al.*, 2018). This study revealed that 366 miRNAs (203 and 163 downregulated and upregulated, respectively) were dysregulated in IC tissues (Arai *et al.*, 2018). miR-320 family miRNAs (miR-320a, miR-320b, miR-320c, miR-320d and miR-320e) specifically were found to be significantly downregulated in IC (Arai *et al.*, 2018) **(Figure 8)**. Moreover, the study identified several genes that are targeted by the dysregulated miRNAs in IC tissues. These genes are related to the E2F family that function as transcriptional factors responsible for cell proliferation, differentiation and apoptosis. The study revealed that *E2F-1, E2F-2, and TUB* were putative targets of the miR-320 family (Arai *et al.*, 2018). Hence the study showed that protein expression of E2F transcription factor 1 (E2F-1), E2F transcription factor 2 (E2F-2) and tubby bipartite transcription factor (TUB) were strongly expressed in IC urothelial cells compared with normal bladder and BC tissues (Arai *et al.*, 2018).

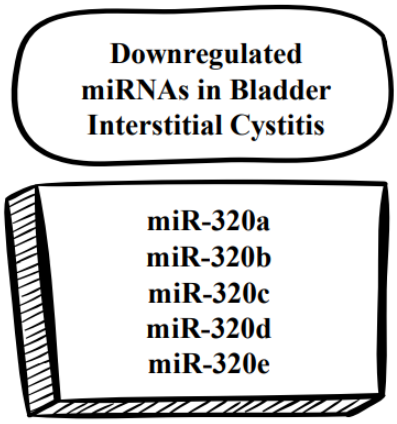

Figure 8. miRNAs expression signature in bladder interstitial cystitis.

E2Fs overexpression has been correlated with cancer development; additionally, *E2F-1* and *ETF-2* overexpression have been correlated with inflammation associated with traumatic spinal cord injury (SCI) in a mouse model (Arai *et al.*, 2018). Collectively, the findings suggest that overexpression of *E2F-1* and *E2F-2* could promote inflammation associated with IC. Although

these findings cannot explain the development process of IC, they provide an insight into the miRNA regulatory networks in IC that could provide new prognostic markers and therapeutic targets for IC.

4.2.4 Therapeutic strategies for interstitial cystitis

4.2.4.1 Conventional therapeutic procedures

Treatment of interstitial cystitis (IC) can vary depending on the case, as some treatments are not as effective for every patient. Most commonly, conventional painkillers are suggested to relieve the symptoms of pelvic pain, however if the treatment is not effective, often oral or intravesical instillations of pentosan polysulfate sodium (PPS) are suggested (Parsons, 2006). PPS is thought to replenish and restore damaged GAG layer by coating bladder lining and reducing potassium leakage into the bladder muscle (Hanno, 1997; Tseng, 2014). The treatment was reported to take effect after prolonged time period of periodical PPS administration, with quickest results observed after 4 weeks, but best response is usually achieved with prolonged therapy time from 3 to 8 months (Parsons *et al.*, 1993; Hanno, 1997; Nickel *et al.*, 2005).

Intravesical DMSO instillations is a FDA and European Association of urology approved alternative method to treat IC cases (Lim *et al.*, 2017; Colemeadow, Sahai and Malde, 2020). It is thought to work by reducing inflammation, relaxing bladder muscle layer and eliminating pain (Tseng, 2014; Colemeadow, Sahai and Malde, 2020). Several RTCs have confirmed DMSO treatment significant against placebo group (Perez-Marrero, Emerson and Feltis, 1988; Sairanen *et al.*, 2009). DMSO treatment can be combined with other IC treatment options, such as triamcinolone, heparin, hydrocortisone and/or bupivacaine, to enhance the effect of the

therapy (Lim *et al.*, 2017), however not all combinations provided long lasting results and therefore patients needed to be retreated (Stav *et al.*, 2012; Gafni-Kane *et al.*, 2013).

However, due to unknown aetiology of the IC condition, combined with varying symptoms and their fluctuating mild to severe persistence, it becomes challenging to select a correct and highly treatment option for every patient. Therefore, there is an unmet need to establish a robust, precise and safe treatment option for all IC cases.

4.2.4.2 Probiotics

Currently, most of the research on IC is focused in understanding the pathophysiology of this condition and finding effective treatment options. Application of complementary medicine to treat IC or provide complementary therapy can be challenging due to undefined disease aetiology, different symptoms and disease phenotypes that are observed in IC patients, and lack of overall treatment efficacy. However, couple of studies have investigated the use of probiotics, reporting beneficial outcomes. Two case studies by Mansour et al. have reported significant decrease in IC symptoms after prolonged treatment of combined therapy by probiotics, cranberry tablets, garlic and parsley tablets, magnesium tablets, primrose oil and L-arginine (Mansour *et al.*, 2014). In addition, another study has demonstrated that 58.8% of IC patients reported positive improvement after probiotic treatment (O'Hare *et al.*, 2013). However, these findings need to be tested in well-designed RCTs in order to prove probiotic effectiveness when treating IC patients.

4.2.4.3 Nanoparticles based drug delivery systems

At the moment, intravesical injections of botulinum toxin A (BoNT/A) has received a lot of attention on being a promising treatment of IC cases. It works by suppressing release of

neurotransmitters, which in turn relaxes the striated and smooth muscles and controls local inflammation (Chen and Kuo, 2020). By decreasing bladder detrusor overactivity, BoNT/A treatment can reduce urgency to urinate, urinary incontinence and daily frequency (Kuo, 2020). However, this treatment can result in complications like as haematuria and UTI, along with other side effects, such as the extremely painful procedures of injection, which are required to prolong efficacy of the treatment (Jhang and Kuo, 2021). Although intravesical drug administration has shown positive outcomes, the treatment efficacy could be enhanced if urothelium permeability could be improved (Chuang, Tyagi, *et al.*, 2009). In order to achieve that, along with safe and efficient treatment by BoNT/A, scientists have been looking into combining this therapy with nanotechnology.

Introducing Botox protein into a liposome, a small lipid bilayer structure that can adhere to the apical membrane and luminal cells of the bladder, could facilitate treatment penetration into the bladder wall and its activity on nerve plexus (Chen and Kuo, 2020). Although some studies suggested that intravesical liposome treatment alone has achieved beneficial results in IC treatment (Fraser *et al.*, 2003; Chuang, Lee, *et al.*, 2009), couple of studies have demonstrated that treatment of BoNT/A encapsulated in liposomes have improved active agent uptake and decreased IC symptoms, demonstrating great safety profile of the treatment (Chuang, Tyagi, *et al.*, 2009; Chuang and Kuo, 2017). However, this treatment lacked significance in RTC when compared with placebo treatment group. Scientists suggest that the difficulty of proving treatment efficacy in IC in placebo-controlled study can be challenging due to the different bladder dysfunction symptoms, patient phenotypes and natural history of IC (Chuang and Kuo, 2017). Although liposome delivery of active therapeutic agents shows promising results, single treatment for heterogenous conditions of IC could be the limiting factor of treating this condition.

4.2.4.4 Novel genetic therapies

There are growing appeals for developing novel gene therapies that can regulate the IC on the molecular level in order to attempt to arrest or repair the defect that produces the disease. Several recent experimental studies in the literature could contribute to advance this growing field. For example, a study conducted by Lv et al. has focused on investigating the role of miRNA-214 in the epithelial-mesenchymal transition (EMT) process and the development of interstitial cystitis (IC) in postmenopausal women (Lv *et al.*, 2017). Several studies have suggested that miRNA-214 is involved in the pathogenesis of BC; however, this study revealed the role of miRNA-214 in IC by targeting Mitofusin 2 (Mfn2)(Lv *et al.*, 2017). Mfn2 is known to participate in regulating cell proliferation and apoptosis and was found to have tumour promoting effects in human cancer, which makes it one of the therapeutic targets for the treatment of BC (Lou *et al.*, 2015). However, the study conducted by Lv et al. has investigated the interaction between miR-214 and Mfn2 in the IC of postmenopausal women (Lv *et al.*, 2017). The study demonstrated that suppressing miR-214 expression by targeting Mfn2 could promote the EMT process and contribute to bladder wall fibrosis and IC in postmenopausal women (Lv *et al.*, 2017). Therefore, this study provides a novel insight into IC treatment; however, further studies are needed to illustrate the therapeutic implications of miRNA-214 in IC treatment.

Another study conducted by Song et al. has investigated the effect of miRNA-132 in the inflammatory response and detrusor fibrosis in IC through the Janus kinase signal transducer and activator of transcription (JAK-STAT) signalling pathway in rat models (Song *et al.*, 2019). JAK/STAT signalling pathway plays a prominent role in immune and inflammatory responses, and the blockage of this signalling pathway was correlated with reduced bladder

hyperreflexia and urinary bladder inflammation (Song *et al.*, 2019). miRNA-132 has been found to play a critical role in angiogenesis and inflammation and was found to provoke an inflammatory response and detrusor fibrosis in IC by activating the JAK-STAT signalling pathway (Song *et al.*, 2019). Additionally, the results demonstrated that overexpression of miR-132 increased inflammatory cell infiltration, collagens I and III expressions, and mast cell growth besides increasing the level of several inflammatory mediators, including IL-6, IL-10, IFN-γ, TNF-α, and ICAM-1(Song *et al.*, 2019). Thus, this study offers another therapeutic approach in IC; however, further studies are needed to examine the efficacy of this treatment in clinical practice.

The (JAK-STAT) signalling pathway has been considered by another recent study conducted by Hou and co-workers; however, the study has examined the effect of another miRNA, which is miRNA-495 (Hou, Li and Huo, 2021). Therefore, the study has focused on investigating the effects of miRNA-495 on the inflammatory response and bladder fibrosis in rats with ulcerative IC via the JAK-STAT pathway by targeting Janus kinase 3 (JAK3), which is a nonreceptor kind of tyrosine kinase responsible for T cell regulation (Henkels *et al.*, 2011; Hou, Li and Huo, 2021). The results demonstrated a group of findings that emphasise the role of miRNA-495 in ameliorating the inflammatory response and bladder fibrosis in ulcerative IC rat models (Hou, Li and Huo, 2021). First, the study's findings suggest that miRNA-495 overexpression could ameliorate the inflammatory response and bladder fibrosis associated with IC via in-activation of the JAK-STAT signalling pathway by downregulating JAK3 in a rat model with IC (Hou, Li and Huo, 2021). Second, rats transfected with overexpressed miRNA-495 and silenced JAK3 showed reduced mRNA expression levels of several inflammatory mediators, including IL-6, IL-8, IL-10, IL-17, and TNF-α. Therefore, the study suggests that miRNA-495 could alleviate the symptoms of IC by inhibiting JAK3, suggesting by this another novel

therapeutic approach for IC. However, this needs to be further studied to check the clinical efficacy of miRNA-495 in treating IC (Hou, Li and Huo, 2021).

This section of the review is focused on addressing the novel genetic therapeutic strategies in IC and emphasise the need for further studies to assess the translation of these strategies into clinical practice. Additionally, it is noteworthy to mention that there is a need for further studies to develop novel delivery systems for miRNAs mimics/inhibitors that can efficiently target the dysregulated pathways in IC.

4.3 Overactive bladder syndrome

Overactive bladder (OAB) is a disorder characterised by urgency, frequency and nocturia with or without urge urinary incontinence (UUI) (Tiwari and Naruganahalli, 2006; Andersson *et al.*, 2021). UUI is defined as an involuntary leakage preceded or accompanied by an abrupt and strong desire to void (Tiwari and Naruganahalli, 2006). Fifteen to twenty five percent of the general population older than 40 years are diagnosed with OAB. The prevalence increases with age and is generally higher in women than men (Andersson *et al.*, 2021). The disease impacts the patient's social, occupational, psychological and sexual aspects, reducing the quality of life (Tiwari and Naruganahalli, 2006). Additionally, several medical complications were correlated with OAB, such as falls, fractures and skin and urinary tract infections (Tiwari and Naruganahalli, 2006). Therefore, despite the disease's debilitating symptoms, several studies have been focused on identifying the underlying cause of OAB and developing novel therapeutic strategies (Fraser *et al.*, 2002; Christ, 2004; Tiwari and Naruganahalli, 2006; Andersson *et al.*, 2021). Although several mechanistic hypotheses were suggested, the exact cause or causes of OAB development have not yet been identified.

The filling cycle of a normal bladder has a change in internal volume from nearly zero to more than 400 mL (Lukacz *et al.*, 2011; Andersson *et al.*, 2021). To facilitate bladder emptying, detrusor smooth muscle cells undergo change from low tension to rapid contraction resulting in voluntary or involuntary voiding. The contractile function within the detrusor muscle is enabled by the organizational structure of the detrusor muscle, made up of contractile units in a syncytium connected by gap junctions (Wang, Brink and Christ, 2006). Recent research has highlighted that there could be several potential subtypes of OAB, that have different underlying pathophysiologies resulting in this bladder dysfunction (Peyronnet *et al.*, 2019). Emerging evidence supports that increased afferent nerve signalling, resulting from urothelial/suburothelial dysfunction, contributes to unrestricted detrusor contractions and is the cause of OAB. Hence OAB is characterised as a sensory-symptom complex instead of being caused by detrusor activity.

Normally, the emptying contraction is induced by the excitatory input from the parasympathetic system to the muscle units, and the bladder emptying is initiated by the release of acetylcholine from the pelvic nerve stimulating the muscarinic (M3) receptors on the detrusor smooth muscle cell (Andersson and Arner, 2004; Andersson *et al.*, 2021). This triggers an intracellular signalling cascade leading to simultaneous depolarization of the detrusor muscle cells and opening of voltage-dependent Ca2+ channels at the cell surface. This high influx of extracellular calcium sensed by calmodulin protein leads to myosin light chain kinase activation and muscle contraction (Andersson and Arner, 2004). This process should be followed by the relaxation of the detrusor muscle mediated in part by the K+ channels. Hence, the opening of the K+ channel allows for K+ ions efflux, resulting in hyperpolarization of the cell membrane and diminished calcium levels, and thus the spontaneous activity and the detrusor muscle tone are reduced (Petkov, 2014). When OAB symptoms are experienced, this

mechanism functions during the filling phase and does not affect the emptying contraction (Andersson *et al.*, 2021). Therefore, the involuntary detrusor contractions characteristic of bladder overactivity is considered the root cause of OAB. In addition, other neurological disorders, such as Parkinson's disease, Alzheimer's disease, stroke and spinal cord injury, are considered triggering factors for OAB by dysregulating the reflexes to the bladder and urethra, resulting in neurogenic detrusor overactivity (Tiwari and Naruganahalli, 2006). This section of the review is focused on explaining the genetic pathways involved in OAB development, and the proposed therapeutic strategies for OAB **(Figure 9)**.

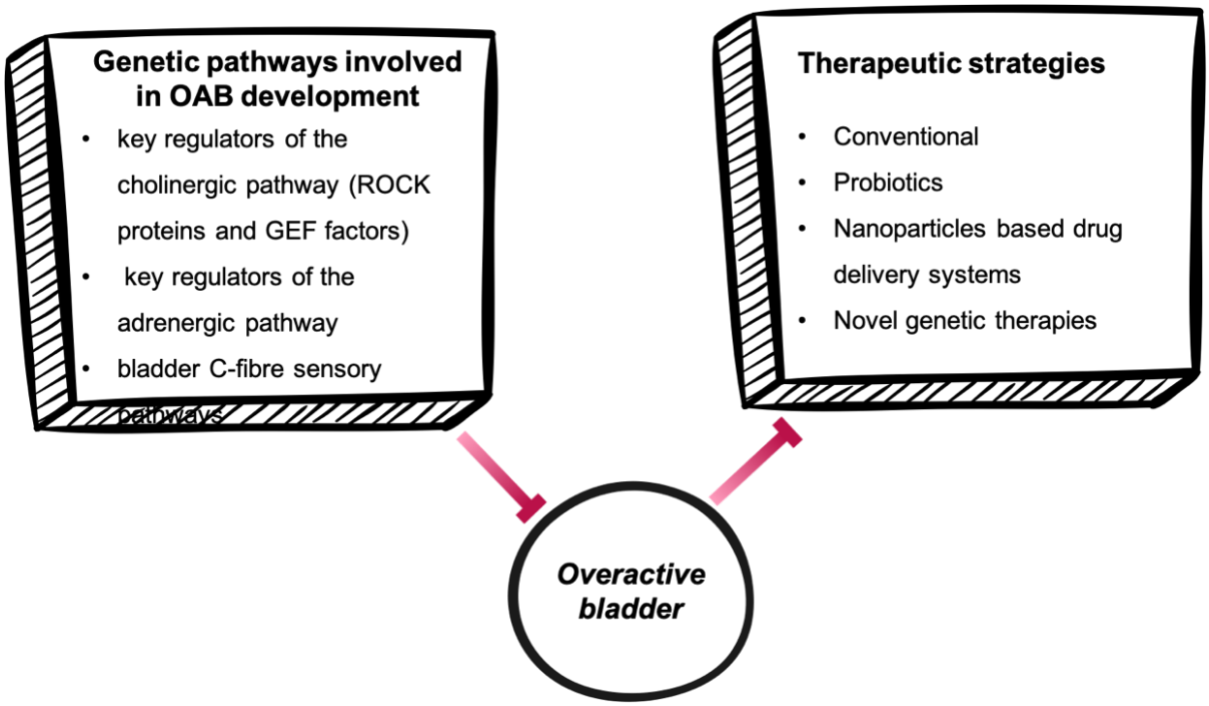

Figure 9. A summary of the genetic pathways involved in OAB and the proposed therapeutic strategies.

4.3.1 Genetic pathways involved in OAB development

In addition to the suggested mechanisms of OAB development, recent studies have been focused on understanding the molecular basis of OAB and identifying the genetic factors and alterations associated with OAB as novel diagnostic and therapeutic strategies. Accordingly, several genes were suggested to have a critical role in the basis of the OAB disease. For

example, Rho-related kinase (ROCK) proteins and Guanine nucleotide exchange factors (GEF) are key regulators of the cholinergic pathway, controlling the bladder contractions through the muscarinic (M3) receptors, as already discussed (Yoshimura and Chancellor, 2003). In addition, ROCKs are regulators of several cellular processes, including growth, apoptosis, metabolism and migration by controlling the actin cell skeleton and cell contraction (Yoshimura and Chancellor, 2003). Specifically, ROCK2 has a pivotal role in regulating smooth muscle contraction by myosin 6 phosphatase inactivation and direct MLC phosphorylation (Puetz, Lubomirov and Pfitzer, 2009). Therefore, due to the role ROCK2 plays in smooth muscle contraction, it has been screened for being an effective gene in the basis of OAB (Matsushita *et al.*, 2010; Fırat *et al.*, 2019).

In addition to the ROCKs, GEFs are also regulatory factors that facilitate the GDP separation from Rho and GTP binding; therefore, they activate small GTPases and regulate multiple cellular responses in response to various extracellular stimuli (Fırat *et al.*, 2019). ARHGEF10, a member of the GEF family, is responsible for the myelination of the peripheral neurons and the regulation of the Rho activity. Therefore, overexpression of ARHGEF10 increases the GTP-dependent Rho and the functional disorder of these proteins causes an excessive contraction in the smooth muscle, suggesting it is a pathway worth considering when studying OAB (Yoshimura and Chancellor, 2003; Fırat *et al.*, 2019).

Other pathways controlling the relaxation of the bladder are believed to be involved in the development of OAB. Fundamentally, the adrenergic receptors control smooth muscle relaxation in which the β3 subtype plays a significant role (Yoshimura and Chancellor, 2003). The adrenergic receptor β3 (ADRβ3) gene encodes the β3 receptor protein (Emorine *et al.*, 1989). The ADRβ3 gene regulates several tissues, including smooth muscle and adipose tissue,

through relaxation or thermogenesis. More importantly, they have a major role in maintaining the smooth muscle tone, especially in the bladder (Fırat *et al.*, 2019). Therefore, the hypofunction of this receptor is correlated with disruption of the bladder detrusor muscle and urinary tract dysfunction (Fırat *et al.*, 2019). Furthermore, several studies reported a correlation between the overexpression of the Nerve growth factor (NGF) and the emergence of hyperexcitability in bladder C-fibre sensory pathways, resulting in symptoms common to OAB (Christ, 2004; Kashyap *et al.*, 2013). However, this sensory abnormality contribution is plausible, but the evidence is far from conclusive.

4.3.2 Alterations in miRNAs levels in OAB

Several recent studies have been focused on the miRNA levels in OAB and their potential to be used as auxiliary parameters for evaluating the molecular mechanisms of OAB. For example, a study conducted by Firat et al. investigated the relationship between the alterations in miRNA levels in OAB and the pathogenesis of the disease to provide a better understanding of the underlying mechanism of the disease progression and the potential gene targets (Fırat *et al.*, 2019). The study demonstrated upregulation in the expression of Let-7b-5P and miRNA-98-5P targeting the ADRβ3 gene and miRNA-92a-3P targeting the ARHGEF10 gene (Fırat *et al.*, 2019). Thus, elevated miRNA levels targeting the ADRβ3 receptor gene may result in OAB symptoms due to the inhibition of the target gene. Additionally, the expression of the miRNA-142-3P and miRNA-200c-3P were significantly upregulated in OAB patients (Fırat *et al.*, 2019). However, the expression level of miRNA-139-5P targeting the ROCK2 gene, which has an influential role in the cholinergic pathway, was significantly downregulated in OAB patients (Fırat *et al.*, 2019). Additionally, miRNA-98-5P showed a high diagnostic accuracy; however, combining miRNA-98-5P and miRNA-139-5P showed the highest diagnostic accuracy (Fırat *et al.*, 2019). Although, these findings suggest that miRNAs could be used as diagnostic

biomarkers of OAB, the clear benefit to the improvement of the treatment using the biomarkers is still debateable.

Another study conducted by Zhang et al. reported a significant upregulation of miRNA-34a in the bladder tissues of OAB patients, suggesting that miRNA-34a increased the susceptibility to OAB due to its role in regulating the cholinergic signalling pathway (Zhang *et al.*, 2012). Furthermore, Kashyap et al. demonstrated a dysregulation in miRNA-132 and miRNA-221 in rat OAB models, and these miRNAs were effective in bladder hypertrophy and dysfunction (Kashyap *et al.*, 2016). The findings of this study were further confirmed by Chermansky et al. study which reported a dysregulation of miRNA-221 and miRNA-125b in OAB patients (Chermansky *et al.*, 2018). Collectively, these studies address the role of several miRNAs in OAB development, which could be used as diagnostic biomarkers and therapeutic targets in OAB. **Figure 10** shows a summary of the upregulated and downregulated miRNAs in OAB.

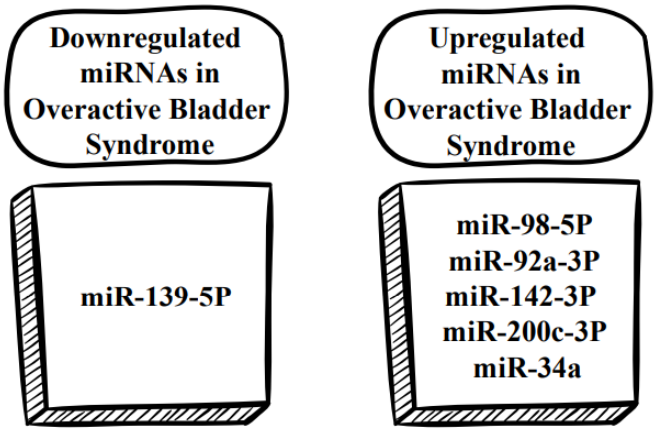

Figure 10. Alteration in miRNAs expression in overactive bladder syndrome.

4.3.3 Therapeutic strategies for OAB

4.3.3.1 Conventional therapeutic procedures

OAB treatment is mainly directed at relieving symptoms rather than reversing pathophysiological abnormalities (Lightner *et al.*, 2019). According to NICE guidelines, lifestyle changes, physical and behavioural therapies are suggested as first-choice treatment, since improvement of symptoms could be achieved by pelvic floor muscle training (Kegel exercises) and/or bladder training. However, if these methods do not show any symptom improvement, pharmacological intervention is recommended. NICE recommends drugs with antimuscarinic action as first-choice treatment, because they block muscarinic receptors in the bladder, therefore reducing contraction of bladder muscle and urinary frequency, along with urgency incontinence. Antimuscarinic drugs such as oxybutynin, propiverine, tolterodine and darifenacin have been extensively used in treating OAB cases and their effectiveness have been proven in multiple RCTs (Yamada *et al.*, 2018). However, treatment with some of these drugs usually present with side effects. For instance, orally administrated oxybutynin encounters first-pass metabolism, resulting in release of metabolite N-desethyloxybutynin which contributes to occurrence of dry mouth (Zacchè, Srikrishna and Cardozo, 2015). To reduce side effects, some studies have investigated the efficacy of intravesically delivered antimuscarinic drugs, with some promising reports showing reduced side effects and drug levels in the serum, as well as increased efficiency of the treatment (Fowler, 2000; Reitz and Schurch, 2004; Evans, 2005; Hayashi *et al.*, 2007). However, intravesical use of antimuscarinic drugs to treat OAB has not been licenced yet.

Alternatively, mirabegron and BoNT/A injections are a recommended treatment when antimuscarinic drugs do not provide expected effect. Mirabegron is a β3-adrenoceptor agonist responsible for mediating detrusor muscle relaxation and increase in bladder capacity (Khizer *et al.*, 2021). Multiple trials have demonstrated significantly reduced number of incontinence episodes and micturitions in 24 hours compared to placebo group (Chapple *et al.*, 2014; Khizer

et al., 2021). Although some side effects were reported after this treatment, in general the use of Mirabegron was established to be well tolerated and safe (Chapple *et al.*, 2014; Khizer *et al.*, 2021).

Scientific evidence has shown that BoNT/A injection into the detrusor muscle can reduce urinary urgency and incontinence episodes by blocking neuromuscular transmission and paralysing the muscle (Kuo, 2009; Wyndaele and Hashim, 2020). This is achieved by synaptic vesicle protein SV2 that belongs to BoNT/A cleaving cytosolic translocation protein SNAP-25, which in turn inhibits the process of exocytosis and inhibits the release of neurotransmitters (Chuang *et al.*, 2014). Several RTCs have demonstrated the effectiveness of BoNT/A in reducing urinary incontinence and frequency compared to placebo, usually for 6 months, but some studies showed effects up to 9 months (Hsieh *et al.*, 2016). Local adverse effects such as increase in post-void residual volume (PVR), higher risk of UTI and catheterisation were reported, however no systemic effects were observed (Hsieh *et al.*, 2016). Study by Mohee et al. reports that although patients seemed to tolerate side effects well during early stages of the treatment, by 3 years of treatment more than half of the patients discontinued BoNT/A injections due to tolerability issues (Mohee *et al.*, 2013).

4.3.3.2 Probiotics

Although no RTCs or studies have been done to investigate treatment of OAB using probiotics, some studies have reported differences between patients with healthy urinary microbiota and those suffering from a urinary incontinence (UI). Study by Pearce et al. found that *Lactobacilli crispatus* were more frequently found in healthy urinary microbiota, while *L. gasseri* was more frequently cultured in microbiota of UI patients (Pearce *et al.*, 2014, 2015; Komesu *et al.*, 2019). In addition, other bacteria, such as *Actinobaculum*, *Aerococcus* and *Oligella*, whose

species contain emerging uropathogens, have also been more frequently sequenced in UI cases. Based on these results, authors hypothesised that higher frequency in some bacterial species could be responsible for bladder dysfunctions (Pearce *et al.*, 2014). In contrast, a study by Karstens et al. reports that lower diversity of bacterial species in microbiome were linked to more severe OAB symptoms and higher number of incontinence episodes (Karstens *et al.*, 2016). Previous studies agree that significant differences in certain bacterial species in the urinary microbiota could be used as markers for the disease, as well as potential treatment targets. Based on this, a study by Curtiss et al. have confirmed previously reported findings and have statistically proven that *Lactobacilli* is less frequently found in urinary microbiota of OAB patients, compared to control group patients (Curtiss *et al.*, 2017). These findings suggest that *Lactobacilli* bacteria could have protective role in urinary microbiota (Curtiss *et al.*, 2017; Fok *et al.*, 2018). Therefore, further research should investigate beneficial effects of probiotics in keeping healthy urinary microbiota and potential treatment of OAB symptoms (Curtiss *et al.*, 2017).

4.3.3.3 Nanoparticle based drug delivery system

Similar to IC (**Section 4.2.4.3**), BoNT/A delivery by liposomes have been also investigated for OAB treatment. Although these two bladder dysfunctions are considered to be different diseases, the overlap in most common symptoms, such as urinary urgency and frequency, means that some of similar treatment methods could be used (Macdiarmid and Sand, 2007). A pilot study by Kuo et al. and RTC by Chuang et al. have reported safe and efficient treatment of OAB with BoNT/A delivered by liposomes (Chuang *et al.*, 2014; Kuo *et al.*, 2014). However, both studies reported that one of the bladder dysfunction conditions, urgency urinary incontinence, did not show significant improvement. The authors of the pilot study demonstrated that the treatment had no associated adverse effects, as well as suggested that

BoNT/A successfully integrated into urothelium cells through endocytosis. The RTC conducted by Chuang et al. demonstrated BoNT/A encapsulated liposome treatment only on OAB patients that had refractory to antimuscarinic drugs (Chuang *et al.*, 2014). Limited duration of follow up period of this trial has resulted in no clear results of long-lasting efficacy of BoNT/A encapsulated liposome treatment. SNAP-25 protein, that is cleaved upon BoNT/A administration, have not demonstrated decreased levels 3 months after the treatment, however it is possible that SNAP-25 proteins have recovered in the meantime (Chuang *et al.*, 2014; Kuo *et al.*, 2014).

Apart from BoNT/A, other therapeutic agents were attempted to be delivered to bladder using liposomes in order to treat OAB cases. A study performed on normal rat model demonstrated that capsaicin-loaded liposomes were successful in blocking voiding reflex for prolonged period of time (Tyagi *et al.*, 2004). It is important to note that capsaicin is a chemical isolated from red pepper and is a natural alternative to pharmaceutical in order to treat voiding dysfunctions (Fraser *et al.*, 2003). However, intravesical treatment of capsaicin has been reported to often result in severe discomfort and pain, due to the delivery of therapeutic agent including use of ethanol, which would be irritating on the urothelium. Study by Tyagi et al. demonstrates that capsaicin delivered by liposomes was just as effective in reducing voiding frequency as intravesical capsaicin instillations, however the study does not mention any data on toxicity and potential side effects of this treatments (Tyagi *et al.*, 2004). In addition, no further studies have investigated capsaicin use for OAB treatment.

Although some promising attempts were reported on drug delivery by liposomes to treat OAB cases, no further trials and studies have further investigated these findings. The main limitation of mentioned systems is the long-term effectiveness of the treatment, as drug delivery by

liposomes does not ensure sustained therapeutic agent release or prolonged BoNT/A effect on detrusor muscle.

4.3.3.4 Novel genetic therapies

Few gene therapy approaches were tested for their efficacy in controlling OAB and ameliorating its symptoms. Using ion channel gene therapy for controlling the detrusor overactivity/bladder hyperactivity is one approach (Christ, 2004). This approach uses K-channel gene therapy with the large conductance, calcium-sensitive K channel (i.e., hSlo, Maxi-K or BKCa, or KCa) since K channels are essential modulators of detrusor smooth muscle cell tone because of their functional antagonism of transmembrane calcium flux (presumably diminished during Maxi-K-induced hyperpolarization) (Christ, 2004). A study conducted by Christ et al. showed that a single bladder instillation of hSlo ameliorates the bladder hyperactivity produced by six weeks of partial urethral outlet obstruction (PUO) (Christ *et al.*, 2001). Additionally, rats injected with pVAX/hSlo exhibited almost complete ablation of hyperactivity without any detectable effects on other parameters of bladder function, suggesting that this approach may be an effective therapeutic modality for treating urinary incontinence driven by bladder hyperactivity (Christ *et al.*, 2001).

Other studies have focused on targeting the NGF gene since its overexpression in the bladder is implicated as the mediator of symptoms associated with OAB (Kashyap *et al.*, 2013). Fundamentally, NGF can be downregulated directly by antibodies or indirectly by blocking the translation of NGF mRNA using a sequence-specific gene silencing (antisense) (Kashyap *et al.*, 2013). However, it could be challenging to ascertain a delivery system that can load the genetic molecule and deliver it efficiently across the urothelium. Therefore, a study conducted by Kashyap et al. has developed a novel intravesical therapy for OAB by targeting the

intracellular synthesis of NGF in the urothelium using cationic liposomes complexed with antisense OND targeting NGF (Kashyap *et al.*, 2013). The study demonstrated that the increased bladder NGF content after acetic acid irritation could be blocked by local instillation of antisense OND complexed with liposomes (Kashyap *et al.*, 2013).

As mentioned in **section 4.3.2**, several miRNAs were addressed as potential therapeutic targets; however, there is a need for studies to investigate the complexation of miRNAs mimics/antisense into efficient delivery systems to develop novel non-invasive therapeutic approaches for OAB.

5. Conclusion and future outlook

The ability of the current treatment options for bladder diseases to provide a highly effective, precisely targeted and long-lasting effect is limited by the challenges linked to the bladder anatomy and physiology. Targeted drug delivery is challenging due to the location of the bladder in a human body, therefore meaning that drug efficacy is often significantly reduced due to the first-pass metabolism, drug dilution during urine storage and frequent voiding. In addition, unknown aetiology of these diseases limits the therapy options to mostly symptomatic treatment rather than targeting the site of pathology origin. In this article, we explored the genetic background of each bladder disease and the underlying alterations in miRNAs levels associated with each disease. Additionally, this review addresses the novel nanoparticle formulations employed as novel and efficient gene delivery systems for targeting bladder diseases. Many studies have shown the advantages of using nanoparticles as drug delivery systems, and many nanomedicine products have already entered the market. Furthermore, with deeper insights into disease aetiology, nanoparticles can become a valuable carrier system to deliver active agents/genes to the target tissue.

Although probiotic therapy has been reported to have promising preventative and therapeutic outcomes on bladder diseases, scientific evidence is struggling to confirm the benefits of this naturopathy treatment. The lack of data in recent years and conflicting results reported in a small number of trials suggests that probiotic treatment efficacy depends on each patient and disease phenotype. In contrast, probiotics have demonstrated a naturally enhanced immune system with no treatment-related toxicity, which indicates a safe, natural adjuvant therapy for minor beneficial effects.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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