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**14. ABSTRACT**

Adstiladrin (nadofaragene firadenovec) is an adenoviral gene therapy construct that was developed for the local treatment of patients with high-risk non-muscle invasive bladder cancer. The overall goal of the work that is being performed at Johns Hopkins is to perform tissue- and urine-based genomic studies on the samples collected within the context of the Phase III trial to identify biomarkers that predict response and/or resistance. Emerging evidence indicates that urine tumor DNA measurements can be used to distinguish the effects of surgery (TURBT) versus intravesical BCG. Therefore, using separate sources of funding and in collaboration with Convergent Genomics, we performed pilot retrospective studies on urine collected in the Phase II trial to determine whether urine tumor DNA characteristics correlated with recurrence-free survival. This work was successful, setting the stage for validation (pending DOD approval of a revised SOW) with the samples from the Phase III trial.

**15. SUBJECT TERMS**
Bladder cancer, interferon, gene therapy

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## TABLE OF CONTENTS

1. **INTRODUCTION**. In Year 2 we worked with the team at MD Anderson to design and analyze experiments to characterize the effects of adenoviral or lentiviral interferon gene delivery on gene expression and growth inhibition in preclinical human and mouse models in vitro and in vivo; the results of these experiments are summarized in part in the Annual Report submitted by their team. In addition, we used a separate source of funding to perform a pilot/feasibility study to correlate urine tumor DNA (utDNA)with response (determined by biopsy) and recurrence-free survival in pre- and post-treatment samples collected from the patients who were enrolled in the Phase II clinical trial of Adstiladrin. These samples were sent to Convergent Genomics for analysis using their UroAmp platform. We plan to revise our SOW and do the same with the urine collected in the Phase III trial based on the results we obtained from the Phase II trial and our other collaborative work summarized below. We also performed feasibility studies to determine whether flash-frozen bulk urine and/or exosomes isolated from it can be used to generate high-quality RNA for next generation whole transcriptome sequencing.

Based on work we performed in another DOD TTSA (CA170270), we have concluded that we will need the utDNA data to design and interpret the results of the RNA and DNA sequencing studies we will perform using the pretreatment biopsies. In the other project, which was designed to identify biomarkers of response and resistance to BCG, we performed whole transcriptome and panel DNA sequencing on pretreatment tissues from 110 patients treated with BCG at Johns Hopkins and correlated molecular subtypes and immune-related gene expression signatures with recurrence-free survival at 2 years. Using the UROMOL classifier, Woonyoung Choi in our group observed that patients with Class 2a tumors had significantly better outcomes than did patients whose tumors were assigned to the other molecular subtypes. However, she was unable to reproduce the finding in several other public cohorts, including another that we had generated at Northwestern University as part of the project. One major issue associated with identifying biomarkers of response to intravesical therapies is that it has been impossible to accurately measure local disease burden in patients before and after surgery and intravesical therapy.

Convergent Genomics is a spin-off from Oregon Health and Sciences University. They have been working with us as members of the Genitourinary Cancers Committee of the Southwest Oncology Group (SWOG) to profile pre- and post-treatment urine collected in SWOG's Phase II clinical trial of adjuvant atezolizumab in patients with BCG unresponsive disease. Strikingly, the work demonstrated that 15% of patients had no residual disease after surgery. Another 14% of patients had complete or partial responses to atezolizumab as measured by decreases in utDNA, and 71% of patients had either stable DNA levels or increases in levels on therapy. Overall, pre- and post-treatment utDNA levels were strongly prognostic of recurrence-free survival; in separate work Convergent has observed the same in patients treated with BCG. Based on this work, the DOD TTSA CA170270 team competed successfully for NCI BIQSFP funding to support performance of UroAmp in patients treated with BCG in the Phase III S1602/PRIME clinical trial.

2. **KEY WORDS**. Urine, liquid biopsy, DNA mutations, low-pass whole genome sequencing, RNAseq.

3. **ACCOMPLISHMENTS**.

**What were the major goals of the project?**

Specific Aim 1: *Perform genomic and metabolomic analyses on tumors and urine from the Phase 3 clinical trial.*

Major Task 3: Prepare RNA and DNA from FFPE unstained slides (n = 151 tumors)
Major Task 4: Perform whole transcriptome RNAseq on RNA from FFPE unstained slides (n = 151 tumors)
Major Task 5: Perform bladder cancer panel DNA sequencing on DNA from FFPE tumors
Major Task 6: Isolate RNA from urine sediment (n = 302 samples)
Major Task 7: Isolate cell-free DNA from urine supernatants (n = 302 samples)
Major Task 8: Perform whole transcriptome RNAseq on RNA from urine sediment samples
Major Task 9: Perform panel DNA sequencing on cell-free DNA from urine supernatant samples
Major Task 10: Isolate extracellular vesicles from urine supernatants (n = 302)
Major Task 11: Isolate total RNA from urine extracellular vesicles
Major Task 12: Perform micro RNA expression profiling on RNA from urine extracellular vesicles
Major Task 13: Perform mass spectrometry on urine supernatants (n = 302 samples)
Major Task 14: Data processing and analysis

Major Task 15: Manuscript preparation and submission

We decided to delay processing the pretreatment tumor tissues and longitudinal urine samples from the Adstiladrin Phase III trial **(Specific Aim 1)** pending analysis of UroAmp urine profiling. Specifically, in Major Tasks 7 and 9 we proposed to isolate cell-free DNA from all of the available pre- and post-treatment urine samples using an in-house ThermoFisher high definitiation (HD) panel sequencing approach so that we could associate our biomarkers with a quantitative measure of disease burden. However, since then we have established a collaboration with Convergent Genomics, who have developed a clinical CAP-CLIA assay to do this, and we have evaluated its performance in urine collected from two completed Phase II clinical trials. Here we will summarize the results as justification for our decision to postpone the processing of the Phase III Adstiladrin samples.

Urine tumor DNA profining in S1605. S1605 was SWOG's single-arm Phase II clinical trial of the anti-PDL1 antibody, atezolizumab, in patients with BCG unresponsive NMIBC. A total of 129 eligible patients were enrolled in the trial, and the 6 month complete response rate (27%) and 12- and 18- month recurrence free survival were similar to those observed in patients treated with pembrolizumab (PMID: 37596191). Urine was collected from patients treated with at least one dose of intravenous atezolizumab at baseline and before the 5$^{th}$ cycle of therapy (3 months). The uCGP was assessed using UroAmp (Convergent Genomics). Risk scores for recurrence at baseline and at 3 months were calculated by a prespecified machine learning algorithm incorporating alterations in 60 genes and low pass whole genome sequencing, and categorized as high versus low. Molecular response was classified based on change in uCGP between the two time points. Risk scores and molecular response were submitted to SWOG for clinical correlation with EFS using a Cox model, adjusting for CIS status. Time to event was calculated from date of study entry (baseline risk) or from the 2$^{nd}$ collection time (3-month risk) to first high grade (HG) recurrence or persistent CIS at 3 months. Death unrelated to bladder cancer and patients last known to be alive without HG recurrence were censored at date of last visit.

Samples were provided at baseline in 89 patients and before the 5$^{th}$ cycle in 77; 68 had both samples available for paired analysis. The risk score at baseline was classified as high in 69% of samples (73% for CIS with or without Ta/T1 and 62% for Ta/T1). At 12 and 18 months the EFS probabilities were 26% and 23% for high-risk

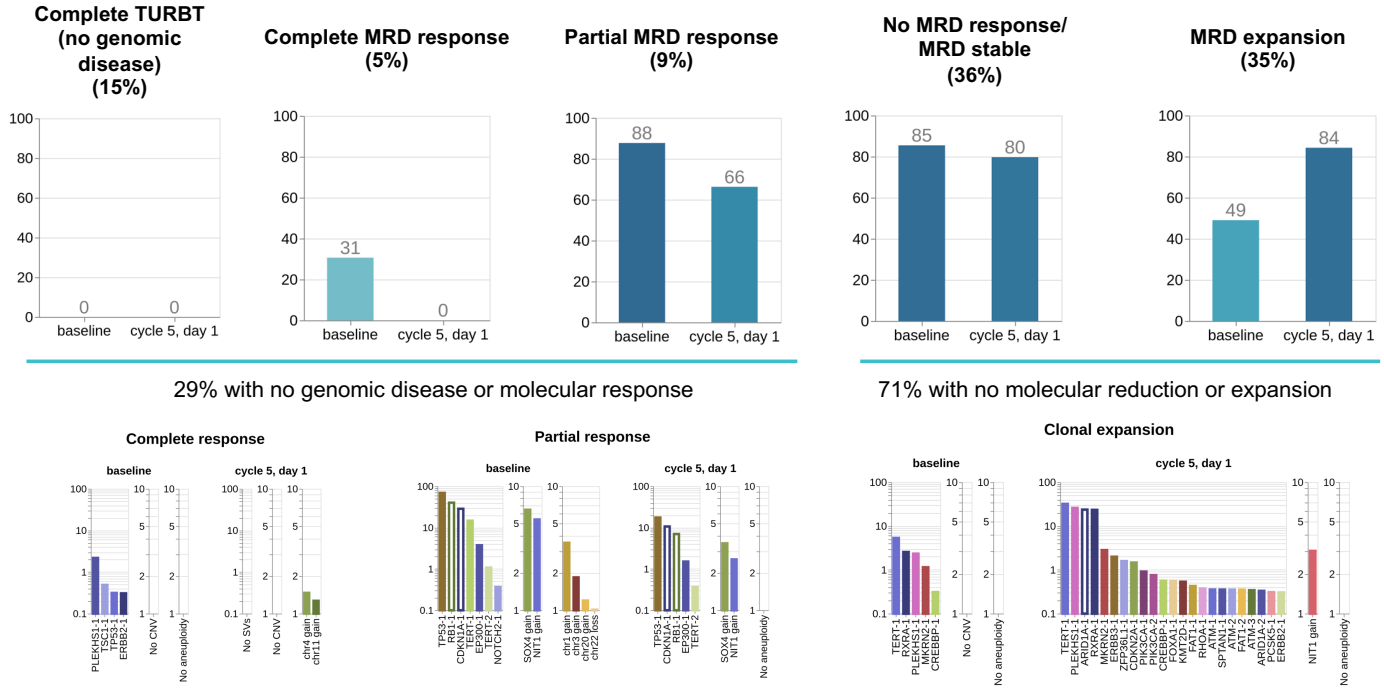


Figure 1. *Urine tumor DNA characteristics in the longitudinal samples collected in S1605.* Top panels: examples of genomic responses to surgery and atezolizumab. Bottom panels: specific mutations and copy number alterations detected in the urine.

and 67% and 51% for low-risk patients, respectively, with a HR of 2.82 (95% CI: 1.58, 5.03; p<0.001). The risk score at the 3-month timepoint was classified as high in 81% of samples, and the EFS probabilities at 12 and 18 months after urine collection were 26% and 22% for high-risk and 80% and 72% for low-risk patients,

respectively, with a HR of 3.39 (95% CI: 1.41, 8.13; p<0.006). Molecular response to treatment was classified as complete (CR) in 8%, partial (PR) in 14%, stable (SD) in 25% and progression (PD) in 46%, with 7% having no detectable genomic abnormalities at both time points. Clinical recurrence was observed by 18 months in 0/6 patients with CR, 7/9 (78%) with PR, 11/14 (79%) with SD and 25/33 (76%) with PD by genomic profile.

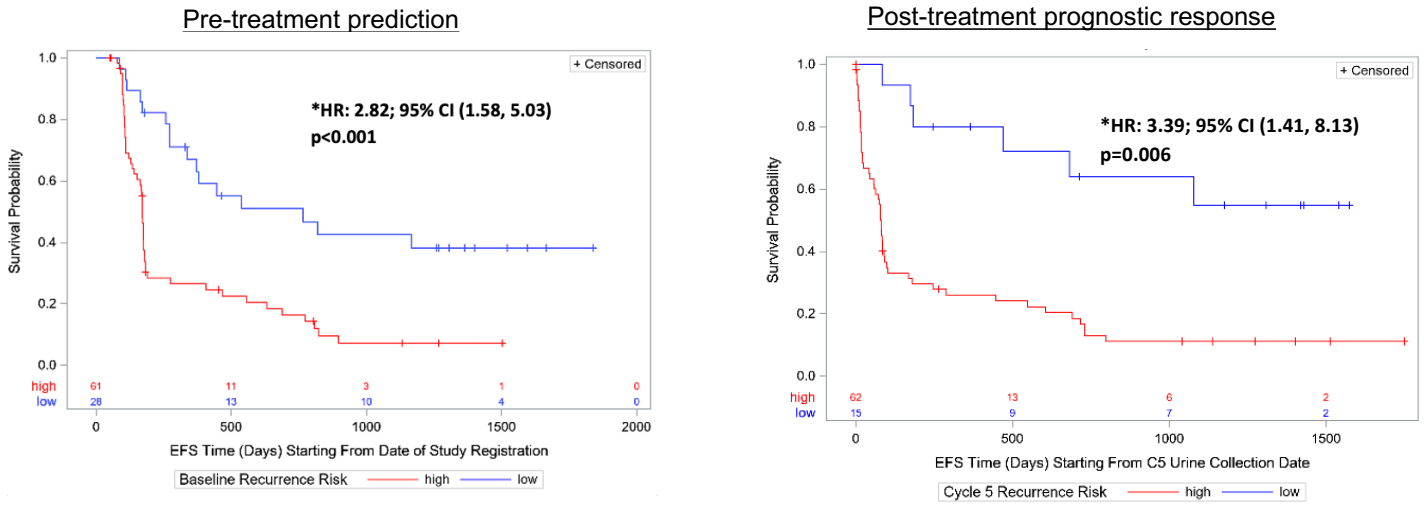


**Figure 2.** *Correlation of UroAmp risk categories with recurrence-free survival*. The risk calculation is based on genomic disease burden and the prognostic impact of specific genomic features (mutations and aneuploidy).

Urine tumor DNA profiling in the Phase II trial of Adstiladrin. This was an open-label, multicenter, parallel-arm, phase II study (NCT01687244) of 43 patients with BCG-unresponsive NMIBC who received intravesical nadofaragene. The primary endpoint was 12-month HG-recurrence-free survival (RFS). All patients who received at least one dose were included in the urinary minimal residual disease (uMRD) analysis. Urine samples were collected prior to induction and at 3 months. uMRD testing was done using the UroAmp MRD assay, which identifies single-nucleotide variants, copy-number variants, insertion-deletions, copy-neutral loss of heterozygosity, microsatellite instability, and aneuploidy. Among evaluable patients (n=35), initial pathological stages were Ta (n=3), T1 (n=9), and Tis (n=23), with concomitant CIS in six patients. In pre-treatment urine (n=32), TP53, TERT, PIK3CA, ARID1A, PLEKHS1, ELF3, and ERBB2 were among the most prevalently mutated genes. Most CNVs occurred in SOX4 and NIT1. uMRD identified patients with high (72%) and low (28%) recurrence risk in both pre- and post-induction collections. At 12 months, post-induction RFS rate was 100% for low-risk and 38% for high-risk patients ($P = 0.038$, log-rank test). Pre-induction RFS was 56% for low-risk and 22% for high-risk ($P = 0.097$, log-rank test). Using matched pre- and post-induction urine (n=15), quantitative drug response was measured and patients categorized as MRD Negative (7%), Complete Responder (13%), Partial Responder (27%), Stable (20%), or Refractory (33%). Recurrence correlated broadly with response groups: MRD Negative and Complete Responder groups did not recur on study, while 7 of 12 patients in the other groups recurred.

**What opportunities for training and professional development has the project provided?**
None.

**Research planned for the next reporting period.**

We plan to revise the SOW and HRPO documents to incorporate UroAmp analyses of the Phase III urine samples. We plan to complete ongoing feasibility studies aimed at determining which RNA and protein analyte(s) can be measured effectively in the urine, including a new plan to incorporate Olink inflammatory panel protein measurements. We plan to perform RNA, DNA, and protein measurements on "extreme responders" – i.e., those patients who had complete or partial genomic responses to Adstiladrin in the Phase III – using newer spatial technologies for the RNA and protein. Overall, we plan to revise and complete the major tasks outlined in Specific Aim 1.

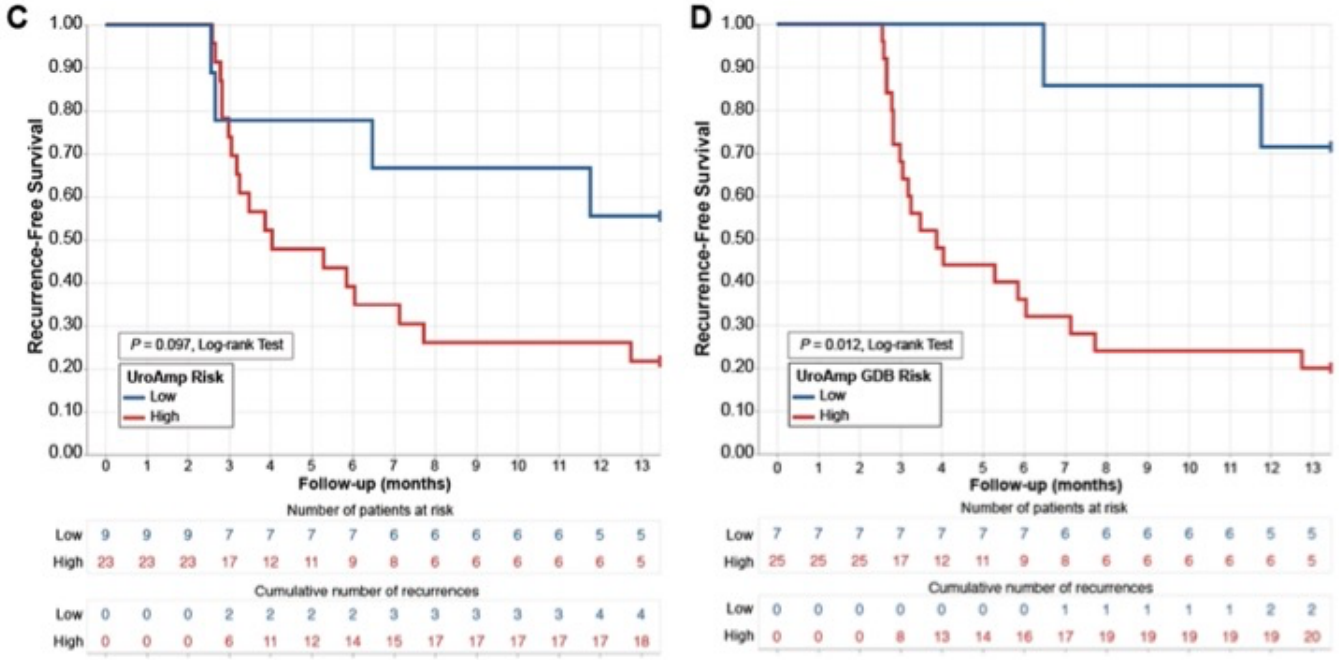


**Figure 3.** *Urinary MRD and recurrence-free survival in the Phase II Adstiladrin trial*. Bulk urine collected before treatment was analyzed using Convergent's UroAmp platform. Left panel (C): RFS by UroAMP low and high recurrence groups. Right panel (D): RFS by genomic disease burden (GDP)-enhanced recurrence groups.

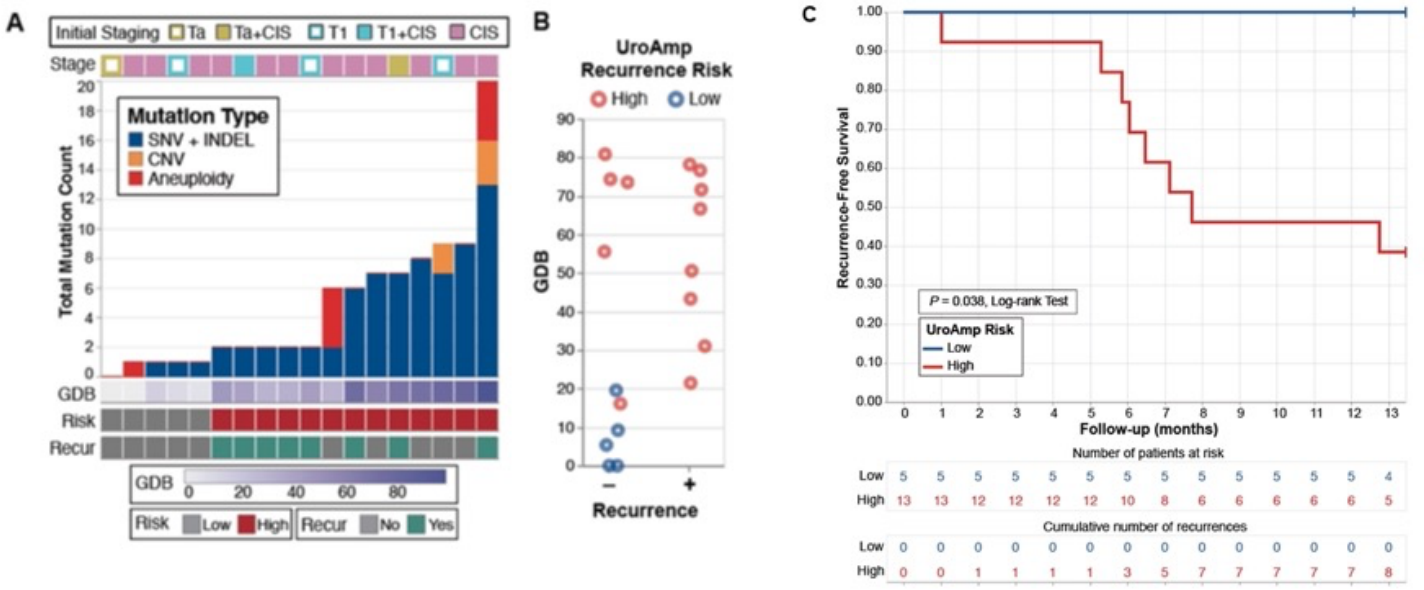


**Figure 4.** *Post-treatment urinary MRD is associated with RFS in the Adstiladrin Phase II trial*. (A) Genomic and clinical characteristics of the cohort. GDP, genomic disease burden. (B) Association between GDP and recurrence risk. (C) Twelve-month RFS stratified by UroAmp risk score.

## 4. IMPACT

**What was the impact on the development of the principal discipline(s) of the project?**

The urine tumor DNA profiling experiments have completely changed our thinking about how the therapies that are being developed for NMIBC impact cancer recurrence. In past studies we assumed that the drugs we employ in the adjuvant setting (i.e., BCG, intravesical chemotherapy, Adstiladrin, and immune checkpoint inhibitors) were the dominant determinants of cancer recurrence; we now know that surgery has at least as much impact (and possibly more). Therefore, if we are to identify novel biomarkers of response to these therapies, we need to know which patients are benefiting from them. We expect that focusing on those patients with residual genomic disease after surgery will enable us to better distinguish responders from non-responders.

**What was the impact on other disciplines?**
Nothing to report.

**What was the impact on technology transfer?**
Nothing to report.

## 5. CHANGES/PROBLEMS

As discussed above, we plan to change the SOW to incorporate the UroAmp and possibly Olink tests into the studies we plan to perform with urine. We are completing pilot studies to determine whether high-quality whole transcriptome RNAseq can be performed on RNA isolated from exosomes or sediment prepared from flash-frozen bulk urine; so far it appears that processing sediment immediately after urine collection may be required, and we now plan to make the UroAmp analyses the top priority, so we will need to reevaluate our plans for the other biomarkers based on available volumes of urine. (A major advantage of the metabolomic and Olink profiling is that they each require less than 1 ml of raw urine.)

**Actual or anticipated problems or delays and actions or plans to resolve them.**
We delayed starting the tissue and urine profiling studies so that the feasibility of UroAmp urine tDNA profiling could be tested. The results have changed our thinking about how to identify the patients who are or are not benefiting from Adstiladrin, and we are reprioritizing the biomarker studies because of feasibility and input from other investigators. Revising the SOW and completing the remaining experiments in the next project period should be feasible.

**Changes that had a significant impact on expenditures.**
Not applicable.

**Significant changes in use or care of human subjects.**
None. We will request to make changes to our approved HRPO in the coming months.

**Significant changes in use of biohazards and/or select agents.**
Not applicable.

## 6. PRODUCTS

**Publications, conference papers, and presentations.**
An abstract and oral presentation describing the S1605 UroAmp study were presented at the 2023 International Bladder Cancer Network (IBCN) Annual Meeting in September. Abstracts describing the S1605 and Adstiladrin Phase II UroAmp studies were submitted for the 2024 ASCO GU conference in February. Manuscripts describing both studies have been drafted with a plan to submit them to *Clinical Cancer Research* before the end of 2023.

**Books or other non-periodical, one-time publications.**
A review article describing the development of Adstiladrin was submitted to *Nature Reviews in Urology*.

**Other publications, conference papers and presentations.**
None.

**Technologies or techniques.**
None.

**Inventions, patent applications, and/or licenses.**
None.

**Other Products.**
None.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS.

| | |
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| *Nearest person month worked:* | *12* |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

None.

**What other organizations were involved as partners?**
None.

## 8. SPECIAL REPORTING REQUIREMENTS

An independent Annual Report detailing the studies performed by the Partnering PI's group at MD Anderson Cancer Center was submitted separately.

## 9. APPENDICES

Alexis R. Steinmetz, Sharada Mokkapati, David McConkey, Seppo Yla-Herttuala, and Colin Dinney, "The evolution of nadofaragene firadenovec: a brief review and the path forward." *Nature Reviews in Urology*, submitted.

**The evolution of nadofaragene firadenovec: a brief review and the path forward**

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**Abstract (max 250 Words)**

Since it was first described as a therapeutic approach for bladder cancer in 1976, Bacillus Calmette-Guérin (BCG) has remained the mainstay of treatment for non-muscle invasive bladder cancer (NMIBC) despite high rates of cancer recurrence and progression as well as ongoing drug shortages. The intravesical gene therapy nadofaragene firadenovec (rAd-IFNα/Syn3) is the newest agent to be FDA approved for NMIBC unresponsive to frontline treatment with BCG and the first gene therapy developed for bladder cancer. The non-replicating recombinant adenovirus vector delivers a copy of the human interferon alpha-2b gene into urothelial and tumor cells, causing them to express these pleotropic cytokines with potent antitumor effects. The development of this therapy represents an important landmark in urologic oncology and several decades of research dedicated to the study of interferon's direct and indirect antitumor properties. The data gathered from the Phase I, II, and III clinical trials continue to provide additional insights into the precise mechanisms underlying both the efficacy of and resistance to nadofaragene. Ongoing studies of prognostic biomarkers have been promising; these will ultimately improve patient selection and allow for the modulation of factors in the tumor or immune microenvironment to further increase therapeutic response.

**Keywords (4 -10)**
BCG unresponsive bladder cancer, antiangiogenic, interferon, immunogene therapy, non-muscle invasive bladder cancer

## Background

The search for effective, bladder-sparing therapies for patients with non-muscle invasive bladder cancer (NMIBC) has occupied this disease space for several decades. Transurethral resection of visible tumor followed by intravesical immunotherapy with Bacillus Calmette-Guérin (BCG)—a weakened strain of the bacterium *Mycobacterium bovis*—is the current standard of care for patients with high-risk disease (carcinoma in situ, high-grade Ta or T1 tumors) [1]. The clinical activity of BCG against bladder cancer was described in 1976, and it has remained the frontline treatment for these patients for almost sixty years [2]. However, even with adequate BCG therapy, most patients will eventually recur and many will progress to incurable disease [3, 4].

Researchers have extensively studied alternative and second-line treatment options, and until recently only two agents were FDA approved for BCG-unresponsive disease. The cytotoxic anthracycline Valrubicin was approved in 1998, despite a dismal complete response rate of 10% at one year (and disease-free survival of 4% at 2 years) [5]. Over a decade later, the single-armed trial KEYNOTE-057 led to the FDA approval of pembrolizumab, a PD-1 inhibitor with a somewhat more durable response that came at the cost of systemic immune-related toxicities [6]. Neither of these treatments have been widely adopted by practicing urologists, partially due to the suboptimal response rates and adverse effect profiles. For patients who are unfit or unwilling to undergo major surgery with radical cystectomy, treatment options have remained extremely limited.

In December 2022, the first intravesical gene therapy nadofaragene firadenovec (rAd-IFNα/Syn3; sold under the brand name Adstiladrin by Ferring Pharmaceuticals) was approved for adult patients with high-risk BCG unresponsive NMIBC with CIS with or without papillary tumors [7]. This novel interferon alpha (IFNα) therapy was developed at the University of Texas M.D. Anderson Cancer Center (MDACC) in collaboration with the gene therapy company Canji Inc. and demonstrated promising durable responses in the Phase III trial: 45.5% of patients with CIS and 60% of those with Ta/T1 who achieved a complete response (CR) at three months

remained recurrence free at 12 months. The breakthrough approval of rAd-IFNα/Syn3 represents nearly twenty-five years of dedicated effort examining tumor immunology and the role of angiogenesis in NMIBC. The Phase I,II, and III clinical trial data have also provided new insights into mechanisms of tumor immunobiology that may explain therapeutic susceptibility and resistance, and ultimately improve outcomes for patients with NMIBC.

**Why interferon?**

The evolution of IFN-based gene therapy can be traced back to the initial discovery of type 1 IFNs by British virologist Alick Isaacs and his Swiss postdoctoral student, Jean Lindenman in 1957. Their seminal work described a substance produced by virus-infected cells—"interferon"—that was capable of interfering with viral growth [8]. Over a decade later in 1969, American immunologist Ion Gresser began publishing his pioneering work demonstrating that these pleiotropic cytokines could also prolong survival in mice inoculated with tumor cells, providing the early evidence for antitumor properties of IFN [9]. A series of lab experiments that began in 1980 revealed that IFNα inhibited angiogenesis, and throughout the 1980s data emerged suggesting that intravesical IFN may have a role in reducing recurrence in bladder cancers [10].

Isaiah Fidler (1936-2020) dedicated his career at MDACC to studying cancer metastasis and tumor biology; work from his lab demonstrated that IFN had a potent anti-angiogenic effect in colorectal cancer [11]. When urologic oncologist Colin Dinney joined faculty at MDACC, they began focusing on IFN's impact on angiogenesis in bladder cancer [12]. This set the stage for almost 25 years of multi-institutional collaborations dedication to understanding the immunostimulatory, antiangiogenic, and apoptotic effects of IFN in the setting of bladder malignancy (Figure 1).

**Development of an orthoptic model to study IFN's ability to inhibit angiogenesis**

Researchers at MDACC successfully developed an orthotopic model of bladder cancer by implanting a human bladder cancer cell line into the bladders of athymic, nude mice. Through in vivo cycling (where metastases are isolated and cells are reinjected into the bladder wall), aggressive variant cell lines with high tumorigenic and metastatic potential were characterized to better understand the molecular basis of tumor progression [13]. This early work demonstrated

that metastatic tumors were highly vascularized and overexpressed pro-angiogenic molecules, such as basic FGF (bFGF).

This in vivo model was then used to determine whether the systemic administration of IFNα could decrease the growth of bladder tumors. Treatment with IFNα led to suppression of bFGF (and to a lesser extent, VEGF), which correlated with reduced microvessel density and decreased tumor growth [12]. This observation was independent of cell lines' susceptibility to the antiproliferative effects of IFNα, supporting the idea that the inhibition of angiogenesis was a key antitumor mechanism of IFNα.

Subsequent experiments confirmed these findings and identifed the optimal dosing and schedule of IFNα while further exploring its anti-angiogenic properties [14]. Mice with orthoptic implants were treated with subcutaneous injections of human IFNα, which acted to inhibit the expression of pro-angiogenic factors bFGF, VEGF, and MMP-9; expression inversely correlated with the inhibition of tumor-induced neovascularization and tumor growth. Moreover, researchers found that the optimal schedule of IFNα required daily dosing to downregulate these factors. The need to maintain continuous low levels of IFNα to maximize its effect made this therapy an ideal candidate for viral-mediated gene therapy, where bladder and tumor cells would constantly produce the cytokine without the need for daily re-dosing.

**Developing and optimizing gene therapy for NMIBC**

Intravesical IFN was also studied as a therapy for superficial bladder cancer to avoid the significant systemic toxicities associated with IFN. Moreover, intravesical instillation allowed for direct contact with the urothelium and tumor. However, early reports of trials of intravesical IFNα were discouraging, presumably due to the inability to sustain sufficient dwell time and exposure to urothelial cells [15]. Meanwhile, gene therapy had emerged in the 1960s-1970s, and by the 1990s adeno-associated virus-mediated delivery of antiangiogenic factors was emerging as an antitumor strategy [16].

The viral delivery of genes ensures active proteins exist inside transfected cells and are also released into the microenvironment. In the case of bladder cancer therapy, this provides continuous exposure to IFN even with time-limited exposure of the agent to the bladder [15]. Gene therapy also takes advantage of IFN's ability to directly impede tumor growth, elicit an effective bystander effect, and activate the innate and adaptive immune responses; every tumor

cell does not need to take up the vector for it to be efficacious [17]. A series of experiments using human and murine Ad-IFNβ were conducted to study the in vitro and in vivo effects of Ad-IFN and confirm the antiangiogenic effects that had been previously described [18]. Human bladder cancer cells (resistant to the antiproliferative effects of IFN) were infected with Ad-IFNβ, and orthotopically implanted into nude mice. The investigators also directly injected these vectors into human xenografts in nude mice. This study took advantage of the species specificity of Ad-IFNβ to demonstrate that the effect of IFN went well beyond direct toxicity. Murine Ad-IFNβ had no effect on angiogenesis factor expression by the human tumor cells, as it was targeting the microenvironment and not the human tumor cells. Ad-IFNβ effectively inhibited tumor growth and metastasis through antiangiogenic effects (e.g. inhibiting angiogenesis factor production, inducing endothelial cell apoptosis) and neovascularization, and also activated host effector cells [18].

The growing interest in Ad-IFN gene therapy for NMIBC led investigators to search for ways to enhance viral transgene expression, either by modifying the adenoviral vector, using alternative vectors, or removing the glycosaminoglycan (GAG) layer with detergents or chemicals. The GAG layer served as a barrier to cell entry, leading to insufficient gene delivery. The solution to this dilemma was discovered somewhat serendipitously by investigators from Canji. Connor et al. (2001) used a rat model to screen compounds that would increase viral transduction and subsequently gene transfer [19]. One detergent tested called BigCHAP showed initial promise, although results were inconsistent when BigCHAP was used from different pharmaceutical producers. Testing different commercially available preparations revealed that the active ingredient in BigCHAP was actually a contaminant of the manufacturing process—an excipient called Syn3 [19].

The impact of dosing schedule and the ability of Ad-IFNα/Syn3 to provide continuous production and secretion of adequate IFNα levels was assessed in pre-clinical translational studies done in preparation of the Phase I/II clinical trials [20-22]. Recombinant Ad-IFNα (rAd-IFNα) was installed intravesically into rats at varying concentrations, and then re-dosed at both short and long intervals [21]. As hypothesized, the bladder acted as a sort of bioreactor after rAd-IFNα/Syn3 administration, and human IFNα was detectible in the urine for seven days. Longer intervals between doses improved the duration and magnitude of adenoviral gene expression, and redosing at 90 days was optimal to create sustained levels of IFNα in the urine. Importantly,

intravesical exposure resulted in only minimal levels of IFNα protein in systemic circulation, and only one intravesical instillation was required [20, 21] .

**Nadofaragene for BCG unresponsive NMIBC**

The Phase I dose-escalating clinical trial of Ad-IFNα/Syn3 began in April 2011 [23]. Although the study was initiated prior to the formalized FDA definition of BCG failure, it was conducted in patients with NMIBC who had recurred despite standard therapy with BCG. The primary objective was to assess safety of this therapy, while the secondary endpoints were rAd-IFNα gene expression and evidence of clinical activity at three months. The investigators found that Ad-IFNα/Syn3 was well tolerated, and there were no dose-limiting toxicities identified. Consistent with the preclinical studies, effective gene transfer was demonstrated by elevated IFNα levels in the urine (in a dose-dependent manner). Most importantly, the study demonstrated the promise of Ad-IFNα/Syn3 in this difficult-to-treat patient population: 36% of patients maintained a CR at 12 months.

The Society of Urologic Oncology Clinical trials consortium oversaw the randomized, open-label, parallel arm Phase II trial of Ad-IFNα/Syn3 for forty-three patients with high grade BCG refractory or relapsed NMIBC [24]. Lack of high-grade disease recurrence at 12 months was the primary end point. Treatment response, incidence and time to cystectomy, and concentration of IFNα in the urine were secondary endpoints. Trial investigators reported a 30% CR for CIS and a 50% HG RFS for HG Ta/T1 at 12 months. Again, treatment with Ad-IFNα/Syn3 was well-tolerated, and no patients discontinued because of an adverse event.

This promising data from the Phase I/II trials supported the registration of a multi-institutional Phase III study in 2016, which would lay the groundwork for the FDA approval of Ad-IFNα/Syn3 as the first gene therapy for bladder cancer [25]. Ultimately 151 patients were included in the efficacy analysis. Participants received a single intravesical dose followed by repeat dosing at 3,6, and 9 months in the absences of high-grade recurrence. Late recurrences beyond 12 months were rare, and very few patients progressed (5%)—the majority of those who progressed (75%) had high grade T1 disease. Overall, treatment with Ad-IFNα/Syn3 was safe, well-tolerated, and efficacious. There was no observed pattern of immune-related adverse events, treatment-related deaths, or deaths from bladder cancer.

**Biomarkers to predict response and further elucidate underlying mechanisms**

The identification of clinical characteristics or biomarkers that accurately predict complete and durable responses can improve patient selection and provide additional information about the mechanistic properties of Ad-IFNα/Syn3, particularly in understanding its ability to decrease angiogenesis and induce cell death pathways. Analysis of urine samples from patients in the Phase II clinical trial demonstrated that Ad-IFNα/Syn3 induced a sustained IFN phenotypic response in the bladder; there was increased expression of T cell and checkpoint markers, and no single immune cell was responsible for the effect of the therapy. Four days after intravesical instillation, levels of IFNα2 as well as CXL10 (a desirable chemokine attractant of CD8+ T cells) had increased significantly and remained elevated [17]. Other studies have shown that BCG does not induce CXL10 expression, which has been hypothesized to contribute to suboptimal therapeutic responses to BCG [26]. The levels of other urinary cytokines—TRAIL, CCL2, IL-6 and G-CSF—were also trending upwards after exposure to Ad-IFNα/Syn3. Notably, patients who were considered complete responders had a significant correlation of increased urinary IFNα2 and IL-6 levels from day 4 compared to pre-treatment—this was not significant in non-responders [17].

Let7f is another potential urinary biomarker that is currently under investigation in patients with BCG-unresponsive NMIBC treated with Ad-IFNα/Syn3. Let7f is a type of non-coding micro RNA capable of post-transcriptional gene regulation that has been identified as a possible tumor suppressor in multiple human malignancies, including NMIBC [27]. Loss of let7 leads to overexpression of oncogenic targets, making it a promising therapeutic target and potential biomarker. Detectable Let7f levels in the urine from trial patients correlated with response to Ad-IFNα/Syn3 and were prognostic of treatment response at 12 months [28].

Further exploration of Phase III data demonstrated that anti-adenoviral antibodies are predictive of a durable response [29]. While baseline antibody titers were not predictive, post-treatment serum antibody levels did correlate with response. Antibody levels greater than 800 and peak antibody fold change greater than eight were associated with higher likelihood of a durable response. Now that Ad-IFNα/Syn3 is commercially available, it will be increasingly possible to validate these findings in larger cohorts.

**Conclusion**

Although BCG became one of the most successful cancer immunotherapies on the market, even with adequate treatment most patients will eventually recur—and many will progress to incurable disease. For this reason, the addition of this adenoviral vector-mediated gene therapy to the BCG-unresponsive armamentarium has been an important advancement in the management of this challenging disease. Currently, studies are underway investigating methods to modulate factors inherent to Ad-IFNα/Syn3 sensitivity via combination therapies (e.g. with EGFR or PDL-1), novel vectors (e.g. lentiviral-IFNα) and additional biomarkers (particularly those related to the molecular luminal/basal profile of NMIBCs). While the response rates are better than any previously FDA approved treatments for BCG-unresponsive NMIBC, the continued development of innovative therapeutic strategies will improve patient selection and oncologic outcomes as well as provide novel insights into the molecular mechanisms of NMIBC.

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# Nadofaragene firadenovec-vncg: Timeline of Development & the Path Forward

Basic Research and Preclinical Testing → Clinical Trials: Confirmation of Safety & Efficacy in Humans → New drug application review & FDA Approval → Post- marketing surveillance & optimizing IFN therapy

### 1950s- 1960s

- **Discovery** of interferon
- Evidence for **antitumor** properties

### 1990s - early 2000s

- Dose-dependent **anti-angiogenic effects** of IFN confirmed in human BLCA xenografts
- Sustained levels of IFN required; **adenoviral-mediated gene transfer** to bladder tested in vivo
- Recombinant Ad-IFNα produces biologically active protein in vitro and in vivo; **optimized with Syn3**
- Delivery/efficacy of **AdIFNα/Syn3 confirmed** in orthotopic BLCA xenografts

### 2009

**Investigational new drug application** submitted to FDA by Ferring Pharmaceuticals

### 2011

**Phase I**

Non-randomized, open-label, dose-escalating, multi-center trial:

No dose limiting toxicities

Dose-dependent IFNa levels in urine confirmed effective gene transfer

36% of patients had CR at 12 months

### 2012-2015

**Phase II**

Parallel-arm, open-label, multicenter trial

30% CR for CIS and a 50% HG RFS for HG Ta/T1 at 12 months

Overall, 35% of patients had CR at 12 mo

No patients discontinued due to adverse events

### 2016-2019

**Phase III**

Single-arm, open-label, repeat-dose, multicenter trial:

53% CR rate at 3 mos for patients with CIS. 46% of these patients maintained the complete response through to 12 mos

No study related deaths

### 2022

**Secondary analysis of trial data:**
serum and urinary biomarkers may predict durable response - e.g. Let7f micro RNA, anti-adenoviral antibodies

**December 2022: FDA Approval**
Nadofaragene approved for high-risk BCG-unresponsive NMIBC with CIS with or without papillary tumors.

### 2022 - present

Development of **novel vectors** for the delivery of IFN gene therapy, i.e. lentivirus-IFN improved survival of tumor-bearing mice and upregulates IFN target genes

Study of **combination therapies** - EGFR, anti-PDL1 - significant improvement in survival; other studies underway

Ongoing investigations of sensitivity of molecular subtypes to IFN families (IFNα/β/λ)

**Figure 1.** Key milestones in the evolution of nadofaragene firadenovec-vncg and the path forward. IFN, interferon. BLCA, bladder cancer. FDA, Food and Drug Administration. CR, complete response. CIS, carcinoma in situ. NMIBC, non-muscle invasive bladder cancer. BCG, Bacillus Calmette-Guérin.