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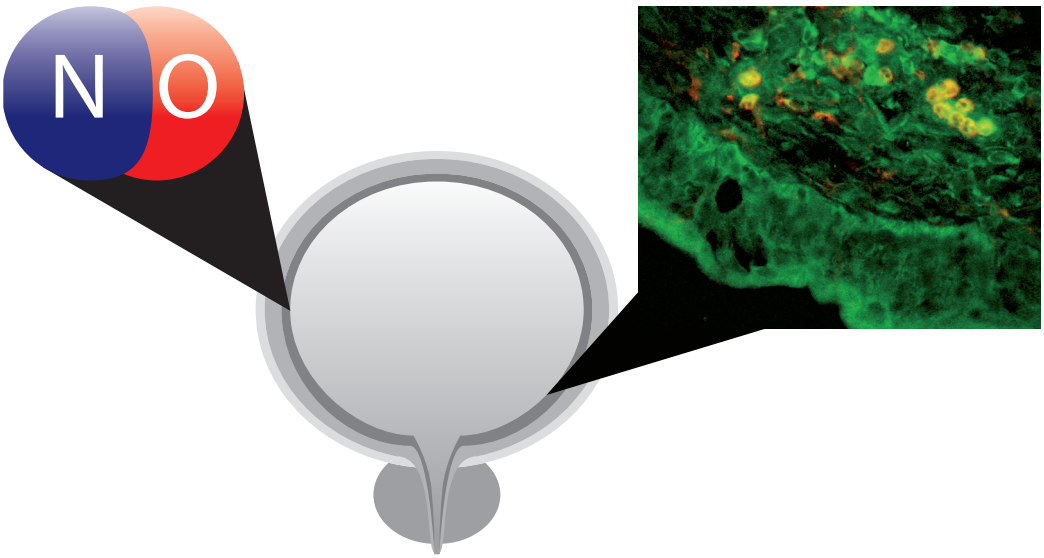
On the role of nitric oxide in lower urinary tract disease



Lotta Renström Koskela

Karolinska Institutet

From the Department of Molecular Medicine and Surgery,
Section of Urology
Karolinska Institutet, Stockholm, Sweden

ON THE ROLE OF NITRIC OXIDE IN LOWER URINARY TRACT DISEASE

Lotta Renström Koskela

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ABSTRACT

Nitric oxide (NO) is an important biological molecule with a variety of functions. Among other, it is a signalling molecule capable of inducing smooth muscle relaxation and vasodilatation, it regulates proliferation, can induce apoptosis and act as an effector molecule in host defence reactions and in immune regulatory processes. High levels of NO are also seen in inflammatory diseases and NO is thought to play a role in tumour biology. The present thesis mainly focuses on the role of NO in the pathogenesis of bladder pain syndrome/interstitial cystitis (BPS/IC), the role for NO in bladder tumour biology and its potentially cytotoxic effects following Bacillus Calmette Guérin (BCG) treatment.

In bladder biopsies from patients with classic BPS/IC we found an increased inducible nitric oxide synthase (iNOS) expression at both transcriptional and protein levels compared to controls. These findings were correlated with high levels of endogenously formed NO in the same patients. iNOS expression was localized to the urothelium and macrophages both in the urothelial layer and in the submucosa.

Local NO formation in patients with bladder tumours of different stage and grade was increased in patients with a carcinoma in situ (CIS) lesion alone or concomitant with a papillary tumour as compared to healthy controls and patients with papillary bladder tumours without concomitant CIS. The same relationship was observed for iNOS with higher levels of mRNA and protein expression in patients with CIS. After BCG treatment for bladder cancer, iNOS was up regulated in the urothelium but was also seen in immune competent cells in the submucosa. Luminal NO was significantly elevated, as was iNOS mRNA expression, in BCG treated patients compared to controls. Furthermore, iNOS protein expression was found in the BCG treated patients when biopsies were examined using Western blot technique. In patients with high-risk non-muscle invasive bladder cancer (NMIBC) polymorphisms in the iNOS and endothelial nitric oxide synthase (eNOS) genes influenced treatment response following BCG instillations.

In conclusion, our results demonstrate an elevation of NO levels in the bladder in patients with classic BPS/IC that in all probability originate from an increased expression of iNOS in urothelial and immune competent cells in the bladder wall. In addition, NO levels are higher in patients with CIS lesions than in patients with papillary bladder tumours and this increase is also likely due to an elevated expression of iNOS. Furthermore, NO levels are higher in the bladder after BCG treatment and are likely to reflect an increased expression of iNOS in bladder urothelial cells and immune competent cells in the submucosa. These findings are in line with previous results implicating that BCG may act through NO/NOS pathways, which is further supported by our observations that polymorphisms in the iNOS and eNOS genes may influence treatment outcome for BCG.

To my family

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV):

I. **Koskela, L. R.**, Thiel, T., Ehrén, I., de Verdier, P. J. and Wiklund, N. P. Localization and expression of inducible nitric oxide synthase in biopsies from patients with interstitial cystitis.
Journal of Urology, 180(2): 737-741, 2008.

II. Hosseini, A., **Koskela, L. R.**, Ehren, I., Aguilar-Santelises, M., Sirsjö, A. and Wiklund, N. P. Enhanced formation of nitric oxide in bladder carcinoma in situ and in BCG treated bladder cancer.
Nitric Oxide, 15(4): 337-343, 2006.

III. **Koskela, L. R.**, Poljakovic, M., Ehrén, I., Wiklund, N. P. and de Verdier, P. J. Localization and expression of inducible nitric oxide synthase in patients after BCG treatment for bladder cancer.
(Submitted).

IV. **Koskela, L. R.**, Ryk, C., Schumacher, M. C., Nyberg, T., Steineck, G., Wiklund, N. P. and de Verdier, P. J. Outcome after BCG treatment for bladder cancer may be influenced by polymorphisms in the NOS2 and NOS3 genes.
(Manuscript).

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LIST OF ABBREVIATIONS

BCG	Bacillus Calmette Guérin
BPS	Bladder pain syndrome
BPS/IC	Bladder pain syndrome / interstitial cystitis
cGMP	Cyclic guanosine-3', 5'-monophosphate
CIS	Carcinoma in situ
CSD	Cancer specific death
DMSO	Dimethyl sulfoxide
EAU	European Association of Urology
EDRF	Endothelium derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ESSIC	European Society for the Study of Interstitial Cystitis
HR	Hazard Ratio
IC	Interstitial cystitis
iNOS	Inducible nitric oxide synthase
NADPH	Nicotinamide adenine dinucleotide phosphate
NANC	Non adrenergic non cholinergic
NIDDK	National Institute of Arthritis, Diabetes, Digestive and Kidney Disease
NK	Natural killer cell
NMIBC	Non-muscle invasive bladder cancer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PDE	Phosphodiesterase
PPS	Pentosan polysulfate sodium
sGC	Soluble guanylate cyclase
SNP	Single nucleotide polymorphism
TB	Tuberculosis
TNM	Tumour Node Metastasis
WHO	World Health Organization
UTI	Urinary tract infection

INTRODUCTION

1.1 The history of nitric oxide

25 years ago nitric oxide (NO) was considered merely a pollutant gas, but through several independent discoveries the importance of NO as a biological messenger was discovered. In 1985, it was demonstrated that the ability to produce nitrite and nitrate was essential in macrophage induced bactericidal and tumouricidal activity (1, 2). Concomitantly, researchers attempted to characterize the chemical structure of endothelium derived relaxing factor (EDRF), discovered by Furchgott in 1980 (3). In 1987 NO was shown to be equivalent to EDRF (4, 5) and at the same time researchers demonstrated that NO was formed in macrophages (6). The discovery that NO could act as a signalling molecule was awarded the Nobel Prize in 1998 and NO had gone from being regarded a simple inorganic gas to become widely accepted as an important biological mediator with a multitude of functions. NO is involved in several biological processes, among others smooth muscle relaxation, regulation of vascular tone, host defence reactions and neurotransmission (7). NO is also a marker for objectively detecting inflammation in several organ systems, including the airways in asthmatic disease (8), the intestine in colitis (9) and the urinary bladder in cystitis of various origins (10, 11).

1.2 Nitric oxide synthases

NO is generated by a family of nitric oxide synthases (NOS). Three main isoforms, derived from separate genes, have been described and named after the cells in which they were first found (12). Two of the isoforms are constitutively expressed in normal cells; endothelial NOS (eNOS or NOS3) and neuronal NOS (nNOS or NOS1). Their activation is calcium and calmodulin dependent and occurs rapidly and transiently by stimuli that increase intracellular calcium levels. These intracellular Ca^{2+} fluxes can be caused e.g by activation of muscarine receptors situated on endothelial cells or by the arrival of action potentials at nerve endings and results in small amounts of produced NO (7, 12, 13). The third isoform is inducible (iNOS or NOS2) and, since calmodulin is tightly bound to this enzyme at all times, it is not dependent of free calcium levels and has therefore been referred to as calcium independent. iNOS was originally identified in activated macrophages and produces high levels of NO in a number of cell types as a response to inflammatory signals such as lipopolysaccharides and cytokines (7, 12, 13). Since iNOS, in contrast to eNOS and nNOS, is regulated at transcriptional

and posttranscriptional levels several hours can pass between iNOS activation and NO production. Once induced, iNOS produces large amounts of NO over a prolonged period of time (7, 13) and may be lethal to, or limit the growth of, invading organisms and tumour cells but may also have detrimental effects on normal cells (14).

The catalyzation of NO from the conversion of L-arginine to L-citrullin requires the presence of molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as well as calmodulin and several co-enzymes and co-factors (15-17). After its production NO can diffuse across the cell membrane of adjacent target cells and bind to intracellular soluble guanylate cyclase (sGC), thus leading to the formation of cyclic guanosine-3', 5'-monophosphate (cGMP) that acts as a second messenger through a variety of enzymatic reactions. These reactions may involve protein kinases, phosphodiesterases (PDE), or modulation of ion channels, leading to the effects generated by NO, e.g. relaxation of smooth muscle or inhibition of platelet aggregation. In host defence reactions the mechanism of action is not thought to be mediated through cGMP pathways. Instead intracellular iron loss and inhibition of mitochondrial respiration and DNA synthesis has been suggested (18) (Fig 1).

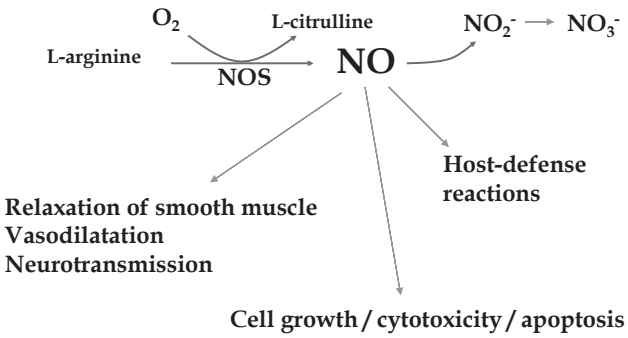

Fig 1.
Enzymatic formation of NO by the conversion of NOS.
With special thanks to Katarina Hallén

Human NOS genes are located on different chromosomes; nNOS is located on chromosome 12, iNOS on chromosome 17 and eNOS on chromosome 7 (19-23). They show a 50-60 % homology, are structurally related to each other and they all require dimerisation to become active (24) (Fig 2).

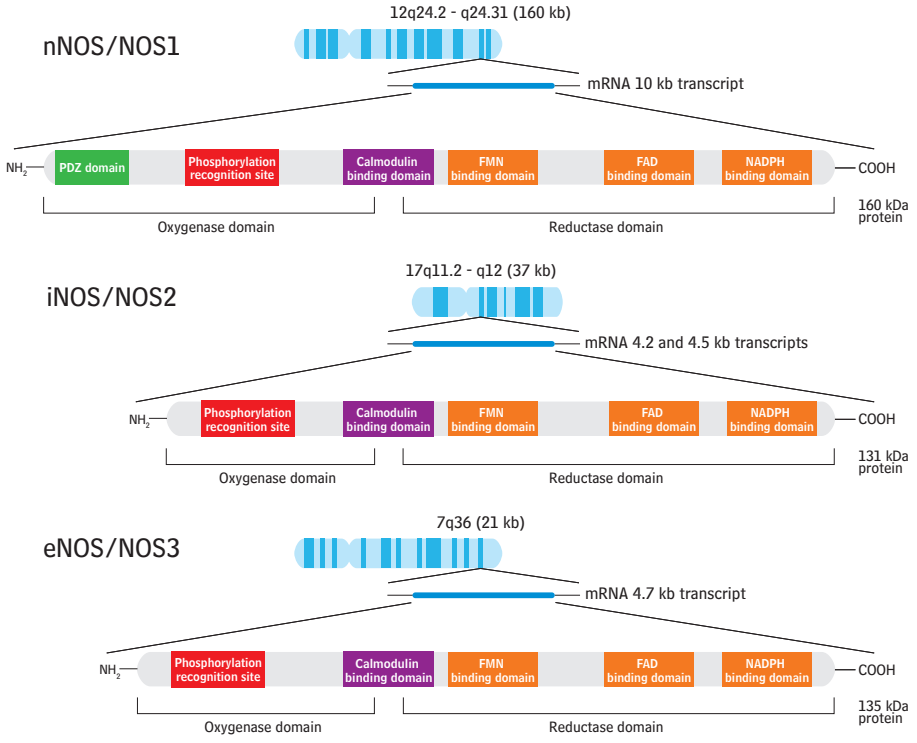

Fig 2. *A schematic picture of the three isoforms of NOS; their chromosome location, gene size, mRNA transcript and protein structure.*

1.3 NO in normal physiology of the lower urinary tract

NO has been identified as an important non-adrenergic, non-cholinergic (NANC) neurotransmitter in the lower urinary tract where it, among other, participates in the micturition reflex (25-27). Normal micturition is characterized by an initial drop in urethral pressure, followed by an increased intravesical pressure, resulting in emptying of the urinary bladder. It is thought that the drop in urethral pressure is caused by NO-mediated smooth muscle relaxation (27-30). In addition, it has been suggested that NO might also take part in the relaxation of the striated urethral muscle (31, 32). The role of NO on detrusor contractility is still a matter of controversy . NOS activity in the detrusor is much lower than in the urethra and bladder neck but experimental studies on both human and animals has suggested that NO may be involved in bladder relaxation (33, 34). Administration of NOS inhibitors decreases bladder capacity and results in hyperactivity of the bladder (34). NOSs are also present in the prostate and have been proposed to take part in

the regulation of prostatic smooth muscle tone, but are also belived to be involved in glandular function and local vascular perfusion in the prostate (35-37). With the discovery of its essential role in penile erection and the advent of systemic drug therapies for erectile dysfunction targeting the NO–cGMP pathway (sildenafil, tadalafil, and vardenafil) (38-40) NO has become widely known to the urologists. Prior to the discovery of NO it was known that penile erection was mediated through NANC neurotransmission. 1990, Ignarro et al., demonstrated that NO was endogenously formed and released from isolated strips of rabbit corpus cavernosum upon electrical field stimulation (38). This observation suggested that penile erection was mediated by cGMP dependent smooth muscle relaxation in the corpora cavernosa in response to neuronal release of NO. The NO-dependent signal system required for penile erection involves a complex biochemical pathway in which several targets are available for pharmacological manipulation. One of these is the PDE-5-enzyme, which converts cGMP to its inactive form. Inhibiting PDE-5 increases cGMP concentrations resulting in corporal smooth muscle relaxation and augmented penile erection (41, 42).

1.4 Interstitial cystitis

Interstitial cystitis (IC) is a chronic inflammatory disease of the urinary bladder. It is characterized by bladder pain, frequency, urgency and dysuria but has no pathognomonic findings upon clinical or microscopic evaluation. Several other diseases including bacterial cystitis, bladder outflow obstruction, pain syndromes, neurological disorders, radiation cystitis and malignancy affecting the bladder, can cause similar symptoms. One significant problem with IC has been the lack of a globally accepted definition of the disease and therefore epidemiological and clinical studies have been difficult to compare. For example, the reported incidence of IC varies greatly between Europe and North America (43, 44). To facilitate IC research studies the National Institute of Arthritis, Diabetes, Digestive and Kidney Disease (NIDDK) defined specific criteria for the diagnosis of IC in 1988 (45). These criteria were based on exclusion criteria rather than inclusion criteria. The National Institutes of Health Interstitial Cystitis Data Base study later demonstrated that a strict application of the NIDDK criteria might miss more than 60% of patients likely to have IC (46). This led to the consensus that these criteria are too strict and should be used only in research settings. The perception that the original term, interstitial cystitis, did not encompass the majority of cases of the clinical syndrome led to the reevaluation of the nomenclature. The name

of this disease has therefore undergone repeated revision, first to painful bladder syndrome (PBS) /IC introduced by the International Continence Society (47) and in 2008 the European Society for the Study of Interstitial Cystitis (ESSIC) proposed a change to bladder pain syndrome (BPS) and suggested that the diagnosis should be made on the basis of chronic bladder pain plus at least one other urinary symptom such as frequency or the urge to void, and that other diseases that could cause the same symptoms were excluded (48). The disease will be referred to as BPS/IC in the following text in this thesis. Traditionally, BPS/IC patients are divided into two subgroups, those with a classic or ulcerous form of BPS/IC and those with a non-ulcerous form, with about 90% having the latter form. The two subgroups differ in both clinical presentation, age distribution, histopathological and neuropathological features as well as in response to different treatments supporting the assumption of two different entities of BPS/IC (49-52). Patients with ulcerous BPS/IC present with a chronic destructive inflammation, mucosal ulcerations named Hunner's lesions and a decreased bladder capacity. At the end stage of the disease they develop a fibrotic bladder with minimal capacity resulting in severely comprised quality of life. Patients with non-ulcerative disease tend to be younger at diagnosis and signs of inflammation are scant, and the end stage with fibrotic bladder does not occur.

1.4.1 Aetiology

Although described for more than a 100 years ago (53) the aetiology and pathogenesis of BPS/IC has yet to be elucidated. Through the years extensive efforts have been made to establish the pathogenesis behind this disease and several theories have been put forward including inflammatory processes (54-56), infection (57), urothelial defects and damage to the protective glycosaminoglycan layer in the bladder (58, 59), immunological processes such as allergies and autoimmune mechanisms (60-62), hypoxia (63) and genetic susceptibility (64). In BPS/IC an increased mast cell count in the bladder wall has been found, particularly in the ulcerous form of BPS/IC. Mast cells are thought to play a pivotal role in the pathology of BPS/IC since they harbour a variety of inflammatory mediators that can cause several of the symptoms and histological findings in ulcerous BPS /IC such as pain, frequency, oedema, fibrosis and the production of new blood vessels in the lamina propria (65-71).

1.4.2 Diagnosis

The diagnosis of BPS/IC is often challenging since the symptoms can be caused by several other diseases e.g. carcinoma in situ (CIS). According to the ESSIC the diagnosis of BPS/IC in patients with chronic bladder pain (>6 months) accompanied by at least one other urinary symptom is based on clinical evaluation, physical examination, urine culture, residual urine, information on voiding patterns and cystoscopy with bladder distension and biopsies (48). Cystoscopic features that are accepted as positive signs for BPS/IC are Hunner's lesions or glomerulations after hydrodistension of the bladder. Biopsies are mainly performed to rule out malignancies such as CIS of the urothelium but can also provide information on histopathological features common in BPS/IC. The ESSIC has proposed that positive biopsy findings are inflammatory infiltrates, granulation tissue, mastocytosis and intra-fascicular fibrosis (48). On the basis of cystoscopy and biopsy findings sub classification of BPS/IC is possible (Fig 3).

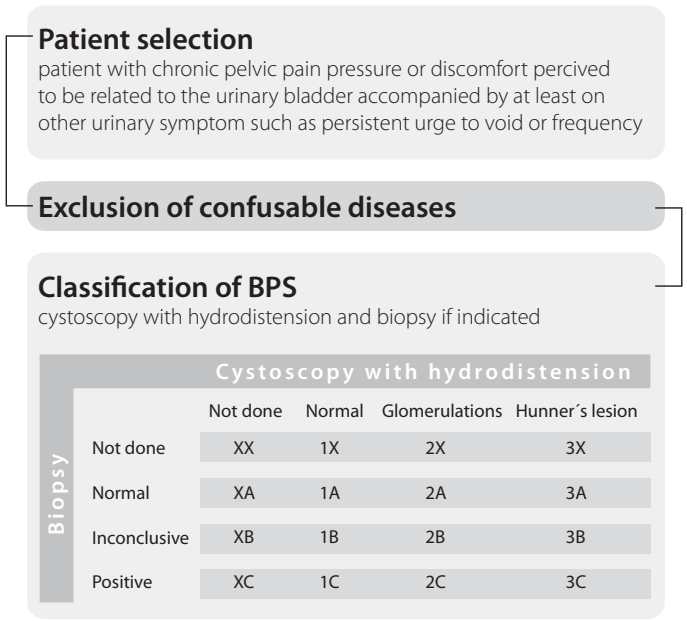

Fig 3. *Diagnosis and sub-classification of BPS/IC. Based on the findings on cystoscopy and biopsies subclassification is possible. Positive findings on biopsy are inflammatory infiltrates, granulation tissue, mastocytosis and intra-fascicular fibrosis. Adopted from the ESSIC proposal (van de Merwe et al. Eur Urol. 2008 Jan;53 (1):60-7).*

As for the role of cystoscopy and hydrodistension, views have been divided. Interestingly, a study by Zermann et al., in 1999, found petecial hemorrhages in patients undergoing sterilization in whom hydrodistention was performed. None of these patients showed any symptoms of BPS/IC (72).

1.4.3 Treatment

Treating BPS/IC is a great challenge when managing patients with this disease. The one symptom most difficult to control in these patients is the pelvic pain, which is thought to have nociceptive, visceral and neuropathic components. Several different treatment modalities are being used in clinical practice including both oral and intravesical treatment with pharmacological agents as well as surgical approaches.

The original two types of BPS/IC respond differently to treatments and a correct sub classification is essential when choosing therapy. For example patients with non-ulcerous BPS/IC do not respond well to reconstructive surgery by any method (73).

1.4.3.1 Oral drug treatment

Traditional pain treatment using non-steroidal anti-inflammatory drugs and opioids have been found to be ineffective or with limited success (74) and should be limited to patients awaiting further treatment. Since the number of mast cells have been shown to be increased in bladder biopsies from several patients with BPS/IC (56) antihistamines have been tried in BPS/IC treatment, although a randomized trial involving hydroxyzine failed to show a significant advantage compared to placebo (75). Amitriptyline is commonly used for the neuropathic component of pain seen in these patients and is recomended by the European Association of Urology (EAU). Pentosan polysulfate sodium (PPS) is another frequently used oral agent in treating the symptoms of BPS/IC. It is an oral heparinoid that is thought to augment the protective glucosaminoglycan layer of the bladder and was originally described in 1990 for the use of IC treatment (76). However, this treatment seems to improve urinary frequency more than pain (77). Glucocorticoids are potent anti-inflammatory drugs affecting the production of a wide range of inflammatory mediators and already in 1953 it was reported to have a temporary improvement of symptoms in patients with BPS/IC (78) but is not recommended by the EAU in their treatment arsenal (79). Immunosuppressive treatment with cyclosporine has been shown to possess superior effect to PPS in

randomized controlled trials but also has more side effects (80-82).

1.4.3.2 Intravesical drug treatment

Intravesical dimethyl sulfoxide (DMSO) was already in 1967 reported to improve BPS/IC symptoms by 75% (83) but more recent trials have not entirely been able to confirm this effect. Although 93% of the patients in a study from 1998 showed an improvement of symptoms the same study revealed that 59% relapsed in the following four weeks (84). Also intravesical PPS is commonly used.

1.4.3.3 Surgical treatment

Hydrodistension of the bladder is not only used as a diagnostic tool for BPS/IC but is also used in the treatment of BPS/IC. The mechanism of action is believed to be caused by damaging the submucosal neuronal plexa due to the mechanical stretch of the bladder wall. This, in turn, would thereby decrease pain transmission through the afferent fibers. The effect of the therapy range from 12-70% but the effect has been reported to be brief, with a duration of 3-6 months (85, 86). It is also possible to perform transurethral resection of the Hunner's lesions following bladder distension (87). As the disease progresses more radical surgery may be required, such as bladder augmentation and urinary diversion, although the success rate following surgery varies substantially between different series (25-96%) (88). In a recent study by Rössberger et al., it was shown that only patients with the ulcerous form of BPS/IC benefit from this kind of reconstructive surgery making it crucial to obtain a correct sub classification (73).

1.5 NO in bladder pain syndrome/interstitial cystitis

As already mentioned, NO is an objective marker for detecting inflammation (8, 9) and can be used in the diagnosis of classic BPS/IC. In patients with bladder inflammation luminal levels of NO are significantly increased compared to patients without inflammation of the bladder (10, 11). It is also possible to identify BPS/IC patients with classic ulcerous IC since they show increased endogenous formation of NO in the urinary bladder, which is not the case in the non-ulcerous form (89). This allows sub classification without performing hydrodistension and biopsies.

Measuring NO in the bladder is a relatively simple technique with few complications. It can also be used in the objective evaluation of different treatments. Hosseini et al., reported a decrease in luminal NO in the urinary

bladder after treatment with steroids for classic BPS/IC and that the decrease in NO correlated to a decrease in symptom score in the same patients (90).
NOS activity has been shown to be up regulated in the urothelium during bladder inflammation and it is therefore likely that the NO measured in the bladder from patients with BPS/IC originates from the bladder mucosa. However, it is possible that inflammatory cells in the bladder wall contribute to the luminal NO measured in patients with BPS/IC. Since NO has a very short half-life in biological tissues, it is not likely that NO produced deeper in the bladder wall would contribute to the rise in bladder luminal NO.

Whether elevated levels of NO are part of the pathogenesis in BPS/IC or simply a part of a secondary inflammatory response is yet to be elucidated.

1.6 Urinary Bladder cancer

Urinary bladder cancer is the ninth most common cancer worldwide, both sexes combined (91) with a male to female 3:1 ratio (92). In Sweden it is the sixth most common cancer form responsible for 4.5% of all new cancers. The incidence increases with age and is rare in individuals under the age of 45. Ninety percent of the bladder cancer cases are transitional cell carcinomas but squamous cell carcinomas and adenocarcinomas may also occur. Smoking is a well-established environmental risk factor for developing bladder cancer (93, 94), which is also the case with aromatic amines that commonly occur in iron and aluminium processing, industrial painting and printing (95). For squamous cell carcinoma of the bladder, infection with Schistosoma haematobium is the most common cause (96). Bladder cancer is not a hereditary disease but patients with a family history of bladder cancer have a slightly increased risk for disease development (97). Since not every patient with exposure to risk factors develop urinary bladder tumours and since some patients without risk factors develop the disease, it is evident that other factors than environmental influence the risk of cancer development. This could be caused by gen-environment and gene-gene interactions. It is also possible that this susceptibility can be caused by variations in DNA sequences e.g. polymorphisms.

Approximately 75-80% of the tumours present as non-muscle invasive bladder cancer (NMIBC) confined to the mucosa (Ta or CIS) or submucosa (T1). The remaining 20-25% consist of tumours invading the detrusor muscle or beyond (98) (Fig 4). For staging and grading, the tumour-node-metastasis (TNM)

classification is used together with the World Health Organisation (WHO) histological grading systems from 1973 and 2004 (99, 100). The recurrence rate for NMIBC is approximately 65% but only 10% progress to muscle invasive disease (101), with the exception of CIS. CIS is a flat, high grade, non-invasive lesion that may occur as a primary lesion in 1-4% of all bladder cancers, or concomitant with a papillary tumour in 13-20 percent of all bladder cancer patients (102, 103). Left untreated, CIS has a high risk of progression to invasive disease; approximately 50% of patients with untreated CIS develop invasive growth within 5 years (103, 104), and when occurring concomitantly with a high-grade pT1 papillary tumour, the risk for progression is even higher (105). Patients with muscle invasive tumours at diagnosis are more likely to progress despite radical surgery, radiation and/or chemotherapy (101).

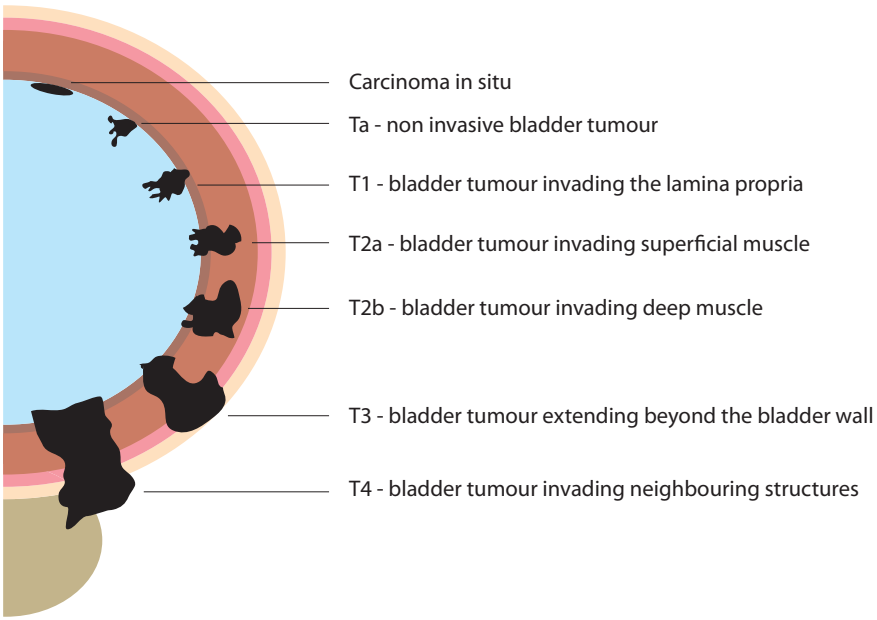

Fig 4. *Staging of bladder tumours.*

The diagnosis of bladder cancer is based on cystoscopic findings in combination with urinary cytology and histopathological examination of transurethral resection specimens or biopsies. The choice of treatment depends on several factors including stage, grade, the presence of a metastasis (distant or nodal) in combination with the general physical condition of the patient. For muscle invasive tumours, radical cystectomy with or without neo-adjuvant chemotherapy is the treatment of choice

provided that no metastases are present at diagnosis. When treating NMIBC it is important to establish the risk for recurrence and progression to optimize treatment results and avoid unnecessary over treatment. For this, the EAU has developed scoring systems and risk tables (Table 1). It is recommended in the EAU guidelines for NMIBC (98) to give an immediate intravesical instillation with a chemotherapeutic agent following the first transurethral resection to all patients. This is not the case in Sweden where post-operative instillations following the firs resection are not advocated in national clinical guidelines, but there are variations in clinical practise. In patients with low risk of recurrence and progression no additional treatment is recommended except for the initial post-operative instillation of chemotherapy. In patients with intermediate or high risk of recurrence and in intermediate risk of progression the immediate instillation of chemotherapy should be followed by further instillations of chemotherapy or maintenance with Bacillus Calmette-Guérin (BCG). In patients with high risk for progression BCG with maintenance for at least a year is indicated following a first initial chemotherapy instillation. It is also reasonable to propose immediate cystectomy to patients with NMIBC at high risk of progression.

Factor	Recurrence	Progression
Number of tumours		
Single	0	0
2 to 7	3	3
≥ 8	6	3
Tumour diameter		
< 3 cm	0	0
≥ 3 c m	3	3
Prior recurrence rate		
Primary	0	0
≤ 1 recurrence/yr	2	2
> 1 recurrence/yr	4	2
Tumour stage		
Ta	0	0
T1	1	4
Concomitant CIS		
No	0	0
Yes	1	6
Grade (1973 WHO)		
G1	0	0
G2	1	0
G3	2	0
Total score	**0-17**	**0-23**

Recurrence score	Probability of recurrence at 1 yr %	Probability of recurrence at 5 yr %	
0	15	31	**Low risk**
1-4	24	46	**Intermediate risk**
5-9	38	62	**Intermediate risk**
10-17	61	78	**High risk**

Progression score	Probability of progression at 1 yr %	Probability of progression at 5 yr %	
0	0.2	0.8	**Low risk**
2-6	1	6	**Intermediate risk**
7-13	5	17	**High risk**
14-23	17	45	**High risk**

Table 1. *Scoring system and risk table for NMIBC provided by the EAU (Babjuk et al. Eur Urol. 2008 Aug;54(2):303-14)*

1.7 NO in Tumour biology and especially in bladder cancer

Tumour growt h depends on various factors such as the properties of the tumour cells and their interaction with endothelial cells and tumour infiltrating immune cells (106). All of these cell types have been shown to produce NO in vitro (4, 6, 18, 107, 108). Several human cancers express iNOS (109, 110) suggesting that NO may be produced in tumour tissues. This opens for the possibility that NO could take part in tumour development and progression. Also bladder tumours have been shown to express iNOS in the urothelium (111) and in vitro studies have shown both calcium dependent and calcium independent NOS activity in both murine and human bladder cancer cell lines (MBT-2 and T24) (112). Diverging results on the role for NO in tumour biology have been reported. In some studies, NO seem to enhance tumour cell proliferation and angiogenesis (113) and, in others, increased NOS activity appears to correlate to a diminished metastatic ability (114). Other studies have reported that NO had no apparent effect on tumour growth (110). eNOS expression has been demonstrated in the endothelium of bladder tumour vessels (115) and endothelial derived NO, produced by eNOS, has been proposed to promote angiogenesis and cancer invasiveness (115, 116).

Endogenous NO production may influence cell growth and in 1995 Thomae et al., described a dual effect of NO on endothelial cell growth (117). It was noted that low concentrations of NO stimulated cell growth and high concentrations inhibited cell growth. In vitro studies on bladder cancer cells have suggested a similar role for NO in bladder cancer, promoting cell growth when produced at low concentrations whereas high concentrations result in cytostatic and cytotoxic effects (112, 118). Cytokine treatment of bladder cancer cell lines resulted in induction of calcium independent NOS activity with growth arrest and apoptosis as a result. When adding a NOS inhibitor apoptosis did not occur, suggesting that NO pathways are involved in this process (112, 118).

1.8 BCG treatment for bladder cancer

1.8.1 The history of BCG

In 1908 two researchers at the Pasteur Institute in France, Albert Calmette and Camill Guerin, began their pioneering work in searching for a vaccine against tuberculosis (TB) and in 1921 the vaccine was first tested in a human (119). The vaccine was named Bacillus Calmette-Guérin (BCG) and is a live, attenuated substrain of Mycobacteria Bovis. In the early 20th century, TB was noted to have antitumor effects. In an autopsy study from 1929, Pearl reported a lower frequency

of cancer in patients with TB. He also noted that patients surviving malignancies had higher incidence of active or healed TB (120). These observations encouraged researchers in their quest to use BCG as an anti tumour agent, and in the late 1950s BCG was found to activate macrophages and having the capacity to destroy cancer cells in mouse tumours (121). In the beginning of the 70ties, pioneering results on BCG as an effective cancer treatment (122, 123) generated enormous interest followed by several clinical studies, but BCGs promise as an effective anti tumour agent was not fulfilled with one notable exception, in bladder cancer. In 1976, Morales et al., developed a schedule for effectively treating non-muscle invasive bladder cancer (124). Although BCG is regarded the most successful immunotherapy agent for bladder cancer to date, its mechanism of action remains largely unknown. As reviewed by Brandau in 2007 (125), mycobacteria following BCG instillation are internalized into the urothelial cells after adherence to fibronectin. Proinflammatory cytokines are then secreted by the urothelial cells and act as chemotactants and attract innate immune cells e.g. neutrophiles and macrophages which further enhance the local production of cytokines and chemokines. The result is a strong non-specific inflammatory reaction with a Th1 response with activated cytotoxic T-cells and natural killer (NK) cells which together with macrophages are thought to eradicate bladder tumour cells (Fig 5).

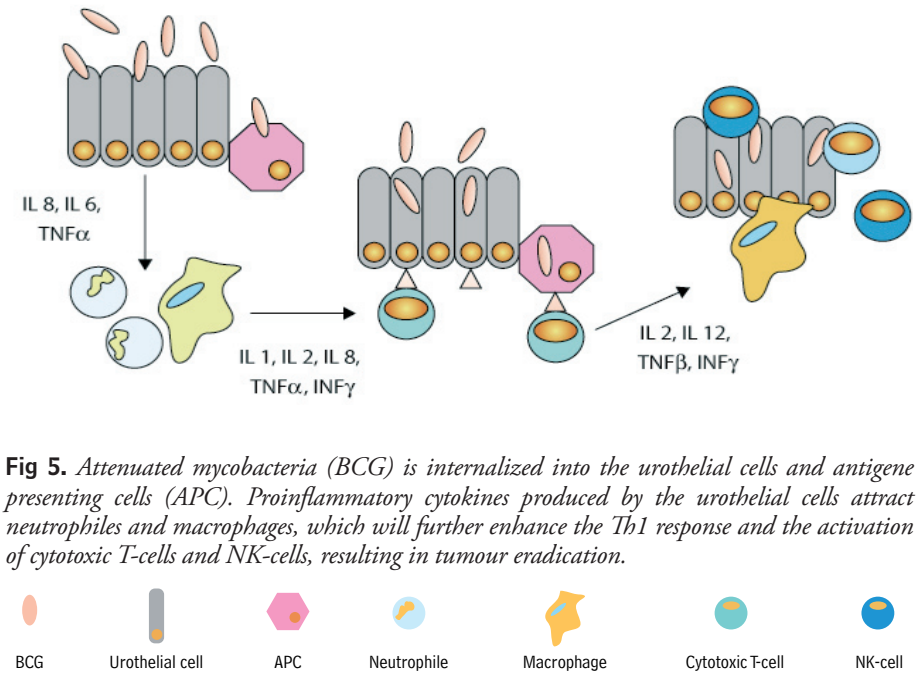

Fig 5. *Attenuated mycobacteria (BCG) is internalized into the urothelial cells and antigene presenting cells (APC). Proinflammatory cytokines produced by the urothelial cells attract neutrophiles and macrophages, which will further enhance the Th1 response and the activation of cytotoxic T-cells and NK-cells, resulting in tumour eradication.*

1.8.2 BCG treatment for bladder cancer

Treatment with BCG infused directly into the bladder is the most effective adjuvant intravesical treatment for preventing recurrence of NMIBC and is the golden standard for treating CIS (126). Although being a very effective treatment, not all patients with NMIBC should be treated with BCG due to their favourable prognosis and the risk for BCG toxicity. In patients with low risk of recurrence and progression, BCG may be considered overtreatment. In the EAU guidelines for NMIBC (98) BCG is recommended in patients at high risk of tumour progression, and is to be given after one immediate instillation of chemotherapy for at least one year. BCG can also be considered in patients with an intermediate or high risk of recurrence and an intermediate risk of progression. Also in this setting BCG is recommended after one immediate instillation of chemotherapy and should be given as maintenance for at least one year but other intravesical agents given as maintenance can also be used. To establish the risk for recurrence and progression in NMIBC and thus making a risk assessment the EAU has developed scoring systems and risk tables (Table 1).

BCG is given as an induction course with one instillation weekly for six weeks and should be followed by maintenance for at least one year (98). The optimal frequency, number of instillations and duration of maintenance BCG therapy has not been established. There are several local differences in treatment protocols between countries and even within countries.

Not all patients respond to BCG treatment, 30-35 % either relapse within the first five years after treatment or fail to respond entirely (127). Patients with high-risk NMIBC undergoing conservative treatment with BCG, should therefore be followed closely for early detection of BCG failure and subsequent disease deterioration. If signs of BCG failure are found radical surgery is the treatment of choice. The observation that radical surgery in BCG non-responders may have a less favourable prognosis than those undergoing immediate cystectomy (128), call for the identification of prognostic markers for those at risk for BCG failure.

1.9 NO in BCG treatment for bladder cancer

Elevated levels of NO have been reported in the bladder after BCG treatment (118, 129) and this increase in NO levels is seen after the first treatment and is sustained for up to six months. In vitro studies have revealed that several of the cytokines excreted in urine following BCG instillations (130, 131) are capable of evoking NO synthesis and NOS activity in both normal and urothelial tumour

cells with growth arrest and apoptosis as a result (112). When adding a NOS inhibitor apoptosis did not occur, suggesting that NO pathways were involved in this process (118). Furthermore, the application of NO donors may have an anti-proliferative effect on bladder cancer cells in vitro (112). This is in line with other studies which have detected iNOS protein and NOS activity in the bladder mucosa after BCG treatment (132). The mechanisms through which NO exerts its cellular cytotoxic effects have been attributed to intracellular iron loss with inhibition of mitochondrial respiration (1), inhibition of DNA synthesis by inhibiting ribonucleotide reductase activity (133), DNA strand breaks through nitrosylation of nucleic acids (134, 135) and a direct interaction with nuclear DNA causing DNA damage and mutations (135). Whether NO plays a role in the anti tumour activity that BCG exerts on tumour cells or is merely a response to the inflammatory reaction warrants further investigation.

1.10 Polymorphisms

A polymorphism is an inherited genetic variation in the base sequence of DNA co-existing in a population with a frequency >1%, and the polymorphism is present in every cell of an individual. Normally polymorphisms are not associated with severe diseases (136) and polymorphisms are common throughout the genome and can occur in both introns and exons. Single nucleotide polymorphism (SNP), the most common type of polymorphism, can occur as frequently as 1 per 300 base pairs (137). In every cell two alleles of a gene exist (one from the mother and one from the father). The most frequently occurring genetic variant in a population is considered common/normal and named the wild-type allele and the other alleles that are represented by fewer individuals in the population are called rare or variant alleles. Since the frequency of an allele varies in different populations the normal allele is population specific (Fig 6).

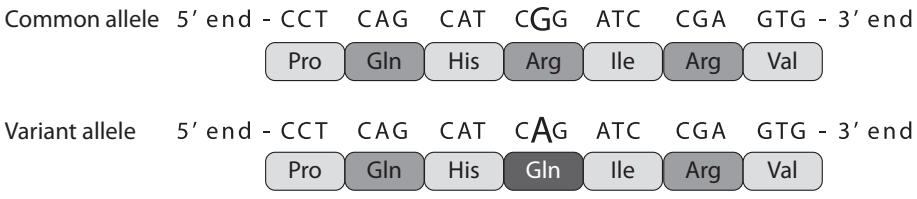

Fig 6. *A SNP in the DNA can cause a change of an amino acid.*
With special thanks to Charlotta Ryk.

Polymorphisms that occur within an enzyme may influence the enzyme activity. If the enzymes take part in processes involving cell cycle control, DNA repair mechanisms or the metabolism of toxic substances and medicines, the carriers of a variant allele might have a different susceptibility to disease development and to drug response. For example, a number of studies have shown an association between the medical outcome of a treatment and polymorphisms (138-140) and cancer risk has also been associated to different polymorphisms (141-145). However, it is important to keep in mind that the effect of a single polymorphism is normally modest in an individual, but may be rather significant on a population level.

1.10.1 Polymorphisms and cancer

The development of a cancer involves several steps including a promotion step, which may be initiated by a mutation that could increase genetic instability with the risk of further genetic alterations. Furthermore, the cancer cell has to gain properties that can allow it to proliferate independent of normal growth stimulation, acquire resistance to apoptosis, enable it to stimulate angiogenesis and give rise to the possibility of invading other tissues (146). Both environmental and genetic factors as well as gene-environment interactions can be involved in the development and progress of a cancer and polymorphisms in these genes can affect the susceptibility for developing a cancer. In bladder cancer 31% of the cases have been estimated to be caused by heritable factors (147). This suggests that low-penetrance genes and their polymorphisms could play an important role in bladder cancer, which is closely related to smoking habits and exposure to aromatic amines. For example a slow acetylation phenotype for the NAT2 gene has been correlated to bladder cancer risk in smokers (148). Furthermore, polymorphisms in DNA repair and in metabolic genes are associated with p53 mutations in urinary bladder cancer (149-152).

1.10.2 Polymorphisms in the iNOS and eNOS genes in bladder cancer

iNOS expression has been reported in several cancers including bladder cancer (109-111, 115). The iNOS (CCTTT)n promoter microsatellite polymorphism at -2,6kb has been suggested to be associated with gastric cancer and bladder cancer (153-155). In a recent study by Ryk *et al*., a long set (13 or more) of (CCTTT)n repeats were associated with a lower risk for developing bladder cancer but also with a higher risk for disease progression and cancer specific death once cancer had

emerged (155). In addition, eNOS polymorphisms have been associated to cancer risk and progression (156-160). Ryk *et al*., has recently found a correlation between bladder cancer and both the eNOS promoter polymorphism -786T>C and the intragenic eNOS polymorphism Glu298Asp (manuscript under revision in Nitric Oxide: Biology and Chemistry).

AIMS OF THE STUDY

The present work was carried through in order to study the role of nitric oxide in lower urinary tract disease. In particular, the following issues were addressed:

- To study whether high levels of endogenously formed NO also correspond to increased levels of iNOS at a transcriptional and protein level in patients with interstitial cystitis.

- To identify the localization of iNOS in the bladder mucosa in patients with interstitial cystitis.

- To analyze endogenous NO formation and iNOS gene expression at a transcriptional and protein level in patients with urinary bladder cancer of different stage and grade.

- To study the local NO formation and iNOS gene expression at a transcriptional and protein level in patients treated with BCG for urinary bladder cancer.

- To identify the localization of iNOS in the urinary bladder after BCG treatment for bladder cancer.

- To investigate if NOS2 and NOS3 polymorphisms influence the outcome after BCG treatment for bladder cancer.

MATERIALS AND METHODS

3.1 Study populations *(Paper I-IV)*

In **paper I** the study population consisted of 6 patients with classic BPS/IC and 8 control subjects without disease of the urinary bladder that were scheduled for endoluminal extraction for upper urinary tract stones.

In paper **II** NO was measured in 66 patients with transitional carcinoma of the bladder, 6 patients who had received BCG treatment and 6 tumour free control subjects with stress incontinence. The study population for real time PCR and Western blot consisted of biopsies from 28 patients with transitional carcinoma of the bladder, 3 patients who had received BCG treatment and 8 tumour free control subjects with upper urinary tract stone disease.

In paper **III** the study population consisted of 11 patients with bladder cancer who had received a six-week induction treatment with BCG and 11 tumour free control subjects without disease of the urinary bladder who were scheduled for endoluminal stone extraction in the upper urinary tract.

The study population in paper **IV** was selected from a population based material of 538 patients with a newly diagnosed bladder cancer. This cohort of patients had been prospectively collected from hospitals in Stockholm County between January 1995 and December 1996. Out of these 538 patients venous blood was available in 359 patients, and they were genotyped for NOS2 and NOS3 polymorphisms. Eightyeight of these patients presented with high-risk NMIBC, e.g. TaG3, T1 or primary CIS transitional cell carcinoma and were included in the present study. Fortyeight of the patients had received BCG treatment at some point. For these patients we have a clinical evaluation with up to 15 years of follow up.

For more detailed information on the study populations, see the individual papers.

3.2 Tissue collection *(Paper I, II and III)*

Biopsies from the urinary bladder were obtained during transurethral surgery from patients with urinary bladder cancer of different stage and grade, patients treated with BCG, patients with BPS/IC and from control subjects without disease of the urinary bladder undergoing transurethral surgery for upper urinary stone disease. The biopsies were snap frozen in liquid nitrogen and stored at –70°C until analyzed. Reagent strip urine analysis for urinary tract infection (UTI) was

negative in all patients and control subjects. Biopsies were obtained after informed consent and the local ethics committee approved the study protocol.

3.3 RNA extraction and cDNA synthesis *(Paper I, II and III)*

Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen®) and quantified by spectrophotometry. Two µg of total RNA were used for cDNA synthesis, using either the SuperScript® II RT kit (paper **I** and **II**) or the Superscript® III First-strand Synthesis SuperMix kit (paper **III**) according to the manufacturer's instructions (Invitrogen®, Life Technologies).

3.4 Real time Polymerase Chain Reaction *(Paper I, II and III)*

Fifty ng of cDNA were amplified by real time PCR with TaqMan universal PCR Master Mix (Applied Biosystems, Life Technologies) using 1mM primers and 0,5mM probes (Invitrogen® Life Technologies and Applied Biosystems, Life Technologies). iNOS primers and probe were custom made and the primers and probe for β-actin were purchased as assay on demand. Each patient sample was analyzed in duplicate using the ABI Prism 7700 Sequence Detector (paper **I** and **II**) or the 7900HT Fast Real-Time PCR System (paper **III**) (Applied Biosystems, Life Technologies). The PCR amplification was correlated against a housekeeping gene, β-actin, and all samples were analyzed in either a singleplex reaction with iNOS and β-actin in different wells or in a multiplex reaction with iNOS and β-actin amplified in the same well.

In paper **I** and **II** iNOS was quantified using a standard curve. In paper **III** the number of iNOS PCR cycles, e.g the CT-values, needed to detect iNOS expression was divided with the CT value for β-actin in each patient, and the difference between groups were calculated using the ΔΔCT method for relative comparison.

3.5 Western Blot *(Paper I, II and III)*

Frozen biopsies were pulverised in liquid nitrogen using a Braun Mikro-Dismembranator and then lysed in a modified RIPA buffer. The lysate was then centrifuged at 10.000 x g for 10 min at 4°C. The protein content of the supernatant fluid was determined with the Bradford protein assay according to the manufacturer's instructions (Bio-Rad Laboratories).

Equal amounts of protein from each sample was loaded onto a protein gel and

separated under reducing conditions by electrophoresis. Proteins were then transferred onto PVDF membranes (Bio-Rad Laboratories) using wet transfer and then blocked for one hour. The membranes were probed over night with either a mouse anti-human iNOS antibody (BD-Biosciences) or a mouse anti-human IgG1 β-actin antibody (Sigma-Aldrich). The membranes probed for iNOS was consecutively incubated with an anti-mouse biotinylated antibody followed by an anti-biotin-HRP antibody and the membranes probed for β-actin was directly incubated with an anti-mouse-HRP antibody. Blots were then developed with Western Blot detection reagents and photographed.

3.6 Immunohistochemistry *(Paper I and III)*

Biopsies were sliced in a cryostat in sections of 10µm and fixed in acetone. The sections were incubated with a rabbit polyclonal antibody raised to human iNOS (Santa Cruz Biotechnology, Inc.) and incubated over night at 4°C. To identify the inflammatory cells a mouse polyclonal antibody raised to human CD16 (Santa Cruz Biotechnology, Inc.) was also added. The sections were then rinsed and incubated for one hour with a goat anti rabbit antibody labelled with ALEXA Fluor 488 (Invitrogen®, Life Technologies) and a goat anti mouse antibody labelled with ALEXA Fluor 594 (Invitrogen®, Life Technologies) to identify iNOS and CD 16. The sections were mounted in Keisers glycerol gelatine (Merck). All micrographs of the immunolabeled sections were obtained using a digital camera system (Nikon microscope and camera), using appropriate filter settings for ALEXA Fluor 488 and ALEXA Fluor 594.

3.7 NO determinations in human urinary bladder *(Paper I-III)*

The NO concentration was measured by introducing a 100% silicon catheter into the bladder and then infusing 25mL room air into the catheter balloon. After 5 minutes incubation, the air was aspirated into a syringe and peak levels of NO were measured using a chemiluminescense NO analyzer (CLD 700, Eco Physics, Dürnten, Switzerland). Air from the examination room was also collected and analyzed in order to determine the NO concentration in the bladder by subtracting the NO level in the room air from the peak value in the air incubated in the catheter balloon. The detection limit for NO was 1 ppb and the analyzer was calibrated at known concentrations of NO in N_2, using an electromagnetic controller.

3.8 Genotyping methods *(Paper IV)*

3.8.1 Fragment analysis

PCR primers were designed with Primer3 software (http://(frodo.wi.mit.edu) and the forward primer was labelled with 6FAM™. PCR products were generated using 0.3 µM primer, AmpliTaq Gold® PCR buffer, MgCl2, DTP, and AmpliTaq Gold DNA polymerase (AppliedBiosystems). 1 µl of the PCR product was mixed with Hi-Di™ Formamide (AppliedBiosystems) and GeneScan™ 500 LIZ® Size Standard (AppliedBiosystems), heated for 3 minutes at 95° C, cooled on ice and analyzed using ABI Prism® 3730 Genetic Analyzer (AppliedBiosystems). Primary data were analyzed with GeneMapper®, version 4.0.

3.8.2 Allelic discrimination assay

TaqMan primers and probes were purchased from AppliedBiosystems and PCR was performed according to the manufacturers instructions, using 10ng DNA as template. The genotyping of amplified PCR products was scored by differences in VIC and FAM fluorescent levels in plate read operation on ABI PRISM 7900HT sequence detection system (AppliedBiosystems) using SDS-2.2.1 software.

3.8.3 DNA sequencing

Sequencing was used as quality control to verify authenticity of amplified sequences. ExoSAP-IT (GE Healthcare) treated PCR products, together with sequencing primer were added to the 5µl sequencing reactions, performed with BigDye^{R} Terminator Cycle sequencing kit (AppliedBiosystems), according to manufacturer's instructions. Sequencing reaction products were treated with BigDye XTerminator and loaded onto an ABI prism 3730 Genetic Analyzer (AppliedBiosystem). The data were analyzed using Sequencing Analysis 5.2 software (AppliedBiosystems) and 4Peaks.

3.9 Statistics *(Paper I-IV)*

In paper I and III the Mann-Whitney U-test for unpaired comparisons was used for statistical significance. Data was analyzed with a statistical software package (Sigma Stat).

In paper II two-tailed statistical significance were determined by comparison of mean values with analysis of variance (ANOVA) and for analyses with only two variables Students' t test for unpaired data was used. Data was analyzed using the same statistical software package as in paper I and III (Sigma Stat).

In paper **IV** all calculations were done with IBM SPSS Statistics, version 19.0 (IBM SPSS®). To assess the risk of cancer specific death and tumour progression over follow-up time we estimated hazard ratios (HR) using the Cox proportional hazards model. Plots of the Kaplan-Meier estimator with the two-sided log rank test were used to visualize the cumulative effect of polymorphisms over time.

For more detailed information on the experimental procedures, see the individual papers.

RESULTS

4.1 NO in PBS/IC *(paper I)*

In bladder biopsies from patients with classic BPS/IC we found an increased iNOS expression at both transcriptional and protein levels compared to controls. This corresponded to high levels of endogenously formed NO in the same patients.

Using real-time PCR iNOS expression at a transcriptional level was detected in all biopsies, including those from control subjects. iNOS mRNA expression was significantly higher in biopsies from patients with BPS/IC as compared to control subjects ($14.2x10^{-3}$ ± $9.2x10^{-3}$ vs $2.0x10^{-3}$ ± $1.1x10^{-3}$, $p>0.01$, Fig 7). Compared to controls endogenously formed NO was significantly increased in the urinary bladder in patients with PBS/IC (284 ± 218 vs 2 ± 1 ppb, $p<0.001$, Fig 7).

Biopsies from both controls and patients with BPS/IC were also examined with Western Blot technique for iNOS protein expression. iNOS protein expression was found only in biopsies from patients with BPS/IC, (Fig 7).

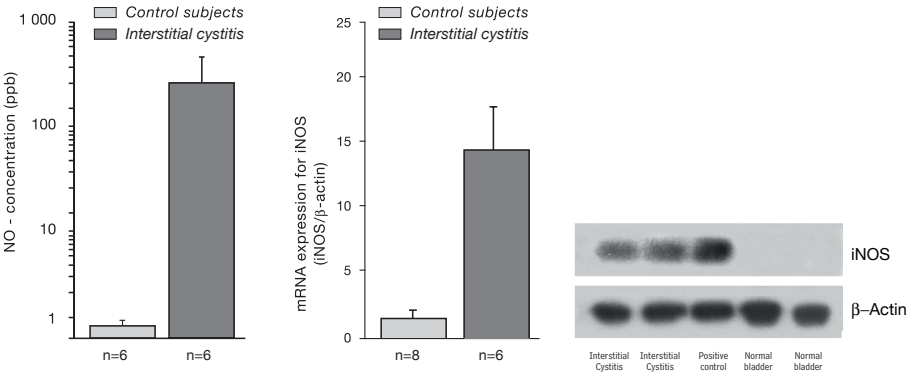

Fig 7. *(A) There was significantly higher endogenously formed NO in patients with PBS/IC vs controls, p<0.001 (B) Real-time PCR shows that mRNA expression for iNOS was significantley higher in patients with PBS/IC, p<0.01. (C) iNOS protein expression in biopsies from patients with BPS/IC and controls with normal bladder mucosa. RAW264.7 mouse macrophages stimulated with LPS and interferon γ served as positive control for iNOS protein expression.*

For iNOS localization in the bladder wall, immunohistochemistry was performed on bladder biopsies from the two groups. This showed a strong

immunolabeling of the urothelium in patients with BPS/IC compared to healthy controls (Fig 8). iNOS immunoreactivity in patients with BPS/IC was also found in inflammatory cells both in the submucosa and in the urothelium (Fig 8).

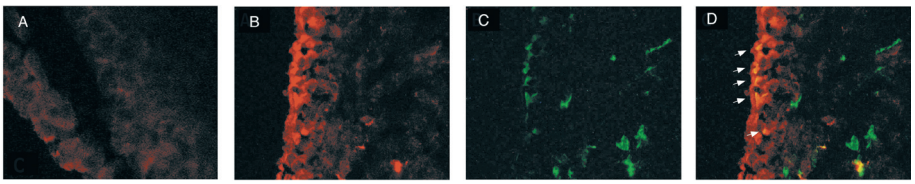

Fig 8. *Immunohistochemistry for iNOS in bladder biopsies from patients with BPS/IC (B-D) and a control subject (A). In picture B-D double immunolabeling for iNOS (red fluorescence) and the macrophagemarker CD16 (green fluorescence) show the co-localization of iNOS and iNOS expressing macrophages in the bladder wall, as indicated by arrows.*

4.2 NO in urinary bladder cancer *(paper II)*

In this study we found an increase in mRNA expression and protein levels for iNOS as well as elevated levels of endogenously formed NO in patients with CIS compared to patients with papillary tumours and healthy controls. No differences in NO production or iNOS gene and protein expression were seen in patients with papillary transitional cell carcinoma, regardless of stage and grade. Primarily our data showed increased NO production in GIII tumours compared to GI-GII tumours but further stratification showed that these augmented levels of NO production were found in patients with CIS lesions (alone or with a concomitant papillary GIII tumour).

Using real-time PCR mRNA expression was quantified in patients with bladder cancer of different grade and stage. No statistically significant differences were found between GI tumours ($2.4 \times 10^{-5} \pm 0.8 \times 10^{-5}$), GII tumours ($1.8 \times 10^{-5} \pm 0.6 \times 10^{-5}$) or GIII tumours ($9.2 \times 10^{-5} \pm 5.6 \times 10^{-5}$) $p=0.07$, although data indicated an increase in mRNA expression in GIII tumours (Fig 9). However, we observed significantly higher iNOS mRNA levels in biopsies from CIS lesions ($15.3 \times 10^{-5} \pm 9.9 \times 10^{-5}$) and they accounted for the difference seen in iNOS mRNA expression in GIII tumours (Fig 9). Tumour stage had no impact on iNOS mRNA expression in our study. The same observation was made when analyzing local NO production in the urinary bladder in patients with bladder cancer. NO concentrations were higher in patients with GIII tumours (12 ± 4 ppb) as compared to patients with GII (2 ± 1 ppb), GI tumours (2 ± 1ppb) and controls (3 ± 1ppb) $p<0.01$, (Fig 9).

As with iNOS mRNA expression, patients with CIS alone or with a concomitant papillary GIII tumour, accounted for the increase in luminal NO seen in patients with a GIII tumour. When data was divided into two groups, one with CIS and one with papillary GIII tumours without concomitant CIS, the NO levels found in patients with papillary tumours without concomitant CIS were the same as for GI and GII tumours (3 ± 1ppb, Fig 9).

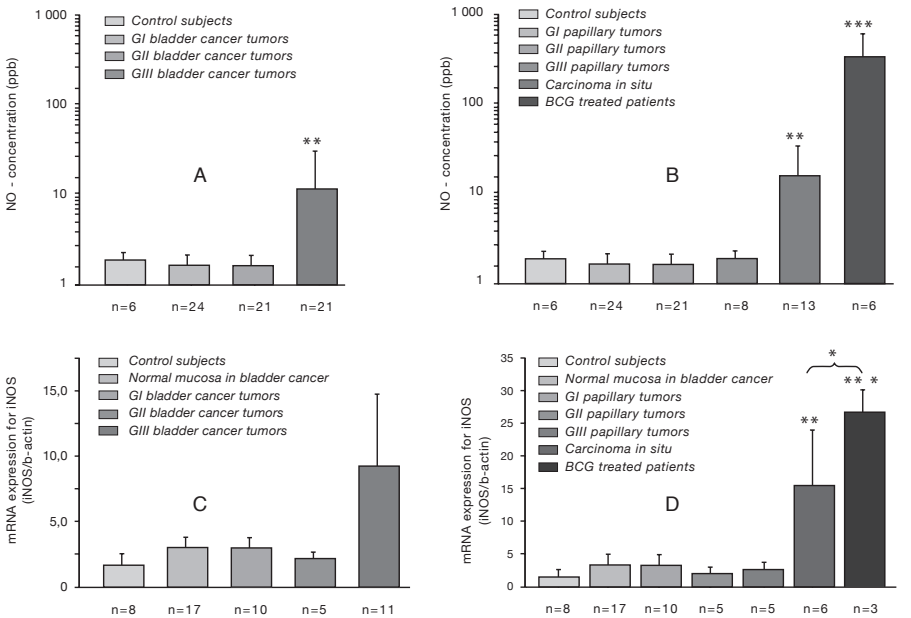

Fig 9. *(A) There was a significantly higher level of endogenously formed NO in patients with GIII tumours compared to GI–II tumours and control subjects. (B) Patients with CIS lesions (alone or concomitant with a GIII papillary tumour) had significantly higher levels of NO compared to patients with papillary bladder tumours regardless of grade (GI–GIII). There was a significantly higher level of NO in patients treated with BCG as compared to all other groups. (C) iNOS gene expression did not differ in biopsies from bladder tumours of different grade (GI–GIII), normal bladder mucosa in patients with bladder tumours and control subjects. (D) There was a significantly higher level of iNOS mRNA in biopsies from CIS lesions as compared to biopsies from papillary bladder tumours of different grade (GI–GIII), normal mucosa in bladder cancer patients and control subjects. In biopsies from BCG treated bladder cancer patients there was a significantly increased iNOS gene expression as compared to all other groups. *p < 0.05, **p < 0.01, ***p < 0.001.*

Three patients treated with BCG for bladder cancer were also investigated for iNOS mRNA and protein expression as well as for endogenously

formed NO. BCG treated patients showed markedly increased levels of NO (716 ± 409 ppb) and mRNA expression (27.4 x 10^{-5} ± 5.7 x 10^{-5}) in the bladder, (Fig 9). Biopsies were also examined for iNOS protein expression using Western Blot. This demonstrated higher iNOS protein expression in biopsies from CIS lesions and patients treated with BCG, thus confirming the PCR results.

4.3 NO in BCG treatment for bladder cancer *(paper III)*

In this paper we studied 11 patients with urinary bladder cancer who had received a six-week induction treatment with BCG and 11 tumour free control subjects. Luminal NO was measured in all patients (386 ± 245 ppb) and control subjects (2 ± 1 ppb) and there was a significant difference between the two groups, $p<0.001$ (Fig 10). With real-time PCR mRNA levels for iNOS were investigated and showed detectable levels in both control subjects and BCG treated patients but there was a ten-fold increase in iNOS mRNA expression in the BCG treated patients compared to control subjects (Fig 10).

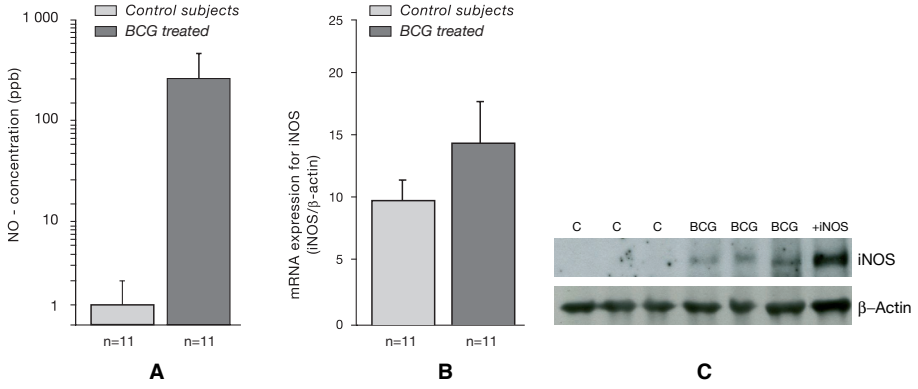

Fig 10. *(A) Endogenously formed NO in urinary bladders from patients with BCG treated bladder cancer and control subjects. There was a significantly higher level of NO in patients treated with BCG as compared to control subjects ($p<0.001$). (B) iNOS transcriptional expression in biopsies from BCG treated bladder cancer patients and control subjects as measured by real time PCR. In biopsies from BCG treated bladder cancer patients there was a significantly increased iNOS gene expression as compared to control subjects ($p<0.003$). (C) Protein expression of iNOS in biopsies taken from patients with BCG treated bladder cancer and control subjects. iNOS protein expression was found in cancer patients who had received BCG treatment (BCG). LPS and INFγ stimulated RAW cells served as positive control for iNOS.*

iNOS protein expression was evaluated with Western Blot and iNOS protein was found in the BCG treated patients (Fig 10). These data are well in line with

our the results regarding NO levels and iNOS expression in BCG treated patients published in paper **II**.

Furthermore, we used immunohistochemistry to study the location of iNOS within the bladder wall. We found strong immunolabeling for iNOS in the urothelium of patients treated with BCG compared to control subjects. The iNOS immunostaining was most prominent in the umbrella cells but also in the cell layer closest to the basal membrane (Fig 11). In BCG treated patients iNOS immunoreactivity was also found in inflammatory cells in the submucosa of the bladder wall. The inflammatory cells were located in submucosal clusters but were also found individually close to the basal membrane of the urothelium (Fig 11).

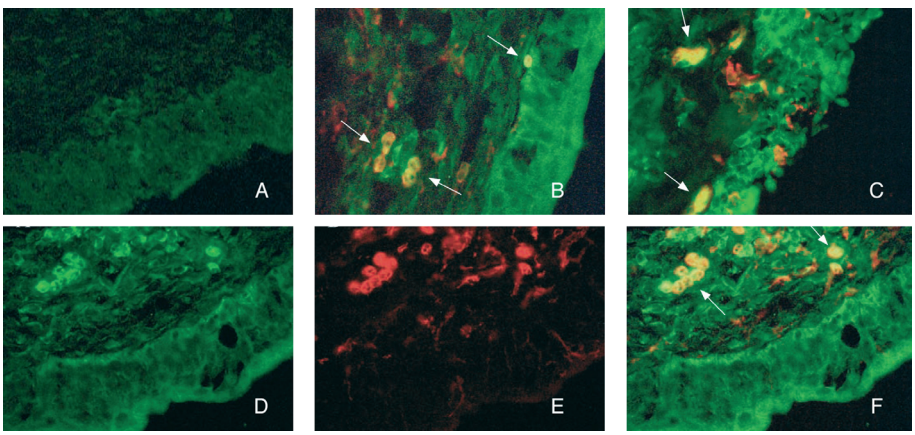

Fig 11. *Immunohistochemistry for iNOS in biopsies from a control subject (A) and patients treated with BCG (B-D). iNOS-like immunoreactivity (green fluorescence) was found in the urothelial layer and was stronger in the BCG treated (B-D) patients. Double immunolabeling of iNOS and the macrophage marker CD16 (red fluorescence) in the consecutive bladder sections from a patient who has received BCG treatment (D-F). Arrows show co-localisation of iNOS and macrophages in the bladder mucosa (F). Macrophages were located in clusters within the submucosa (B and F) but were also found underlying the basal membrane of the urothelium (B), as indicated by arrows. In biopsies where the urothelium was detached the urothelium showed strong iNOS immunolabeling and iNOS expressing macrophages within the urothelium (C).*

4.4 NOS polymorphisms and BCG treatment (paper IV)

In this study we investigated whether polymorphisms in the iNOS/NOS2 and eNOS/NOS3 genes may influence outcome after BCG treatment in patients with high risk NMIBC, e.g. CIS, TaGIII and T1 tumours.

For the iNOS/NOS2 (CCTTT)n microsatellite promoter (-2.6kb) polymorphism we found no significant differences in cancer specific survival

between BCG treated and non BCG treated patients for those having a long set of repeats, (L=13 or more, L-carriers), p=0.593 (Fig 12). In non-L-carriers cancer specific survival was significantly improved for those who had received BCG, p=0.027, with a lower risk for cancer specific death (HR:0.26; CI:0.05-1.21; p=0.086, Fig 12 and Table 2). Regarding disease progression BCG seemed to reduce the risk, but this was not enhanced by the polymorphism.

		HR (95% CI)	p Value	Adjusted HR (95% CI)	p Value
Ca specific death		**NOS2 (CCTTT)n microsatellite promoter (-2.6kb)**			
L-carriers	BCG treated	0.74 (0.24-2.26)	0.596	0.79 (0.26-2.45)	0.794
Not L-carriers	BCG treated	0.21 (0.05-0.97)	**0.045**	0.26 (0.05-1.21)	0.086
Stage progression					
L-carriers	BCG treated	0.46 (0.17-1.27)	0.132	0.53 (0.18-1.52)	0.234
Not L-carriers	BCG treated	0.37 (0.12-1.18)	0.093	0.40 (0.13-1.28)	0.122
Ca specific death		**NOS3 -786T>C (rs2070744)**			
TT	BCG treated	0.10 (0.01-0.79)	**0.029**	0.08 (0.01-0.675)	**0.020**
CT/CC	BCG treated	0.92 (0.33-2.58)	0.869	1.13 (0.39-3.27)	0.818
Stage progression					
TT	BCG treated	0.14 (0.03-0.61)	**0.009**	0.14 (0.03-0.63)	**0.011**
CT/CC	BCG treated	0.94 (0.36-2.48)	0.903	1.12 (0.41-3.09)	0.823
Ca specific death		**NOS3 Glu298Asp (rs1799983)**			
GG	BCG treated	0.29 (0.07-1.20)	0.087	0.29 (0.07-1.23)	0.092
GT/TT	BCG treated	0.65 (0.18-2.30)	0.505	1.03 (0.28-3.82)	0.959
Stage progression					
GG	BCG treated	0.09 (0.02-0.43)	**0.003**	0.07 (0.01-0.38)	**0.002**
GT/TT	BCG treated	0.99 (0.30-3.26)	0.986	1.47 (0.39-5.57)	0.565

Table 2. *Cancer specific death and disease progression after BCG-treatment.*

For the eNOS/NOS3 promoter polymorphism -786T>C (rs2070744) patients with the TT genotype responded better to BCG and had a lower HR for the risk of cancer specific death if treated with BCG (HR:0.08; CI:0.01-0.68; p=0.020, Fig 12 and Table 2). Also the risk for disease progression was lower in this genotype with a HR:0.14; CI:0.03-0.63; p=0.011 (Fig 12 and Table 2). In patients with the CT and TT genotype no advantage regarding cancer specific death and disease progression was seen after BCG treatment.

For the eNOS/NOS3 intragenic polymorphism Glu298Asp (rs1799983) GT and TT genotypes showed no difference in cancer specific death and disease progression between those who had received BCG and those who had not. However GG genotype patients had a lower hazard ratio for stage progression if given BCG (HR:0.07; CI:0.01-0.38; p=0.002, Fig 12 and Table 2). The same

was seen for cancer specific death (HR:0.29; CI:0.07-1.23; p=0.092, Fig 12 and Table 2).

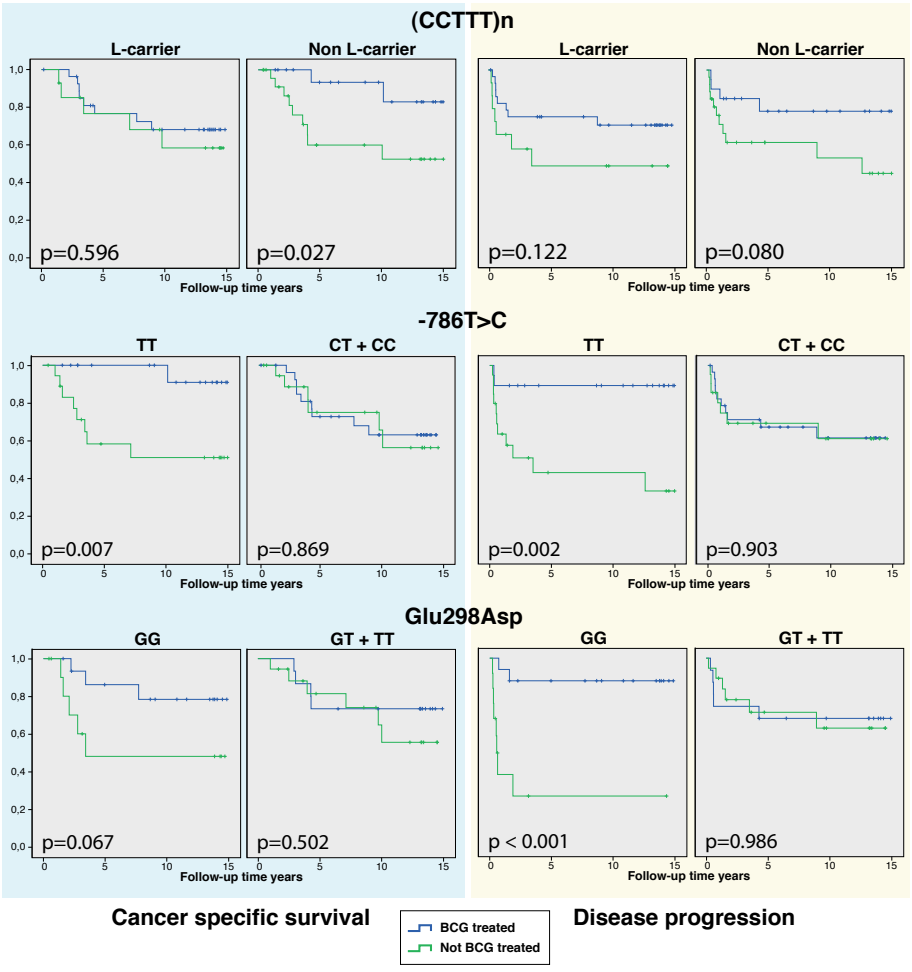

Fig 12. *Kaplan-Meier analyses for cancer specific survival and disease progression for the NOS2 (CCTTT)n polymorphism, the NOS3 -786T>C polymorphism and the NOS3 Glu298Asp polymorphism.*
For the NOS2 (CCTTT)n polymorphism pair wise comparisons showed a significant difference in cancer specific survival after BCG treatment in the non-L-carrier group (p=0.027), but not in the L-carrier group (p=0.593). Disease progression was decreased after BCG treatment but the (CCTTT)n polymorphism did not influence the outcome after BCG treatment.
For the NOS3 -786T>C polymorphism pair wise comparisons showed a significant difference in cancer specific survival and disease progression after BCG treatment in the TT-group, p=0.007 and p=0.002 respectively.
For the NOS3 Glu298Asp polymorphism pair wise comparisons showed a significant difference in disease progression after BCG treatment in the GG-group, p<0.001.

DISCUSSION

Nitric oxide has a variety of functions, both as a signalling molecule and as an effector molecule in host defence reactions and in immune regulatory processes. High levels of NO are seen in inflammatory diseases and NO may have a role in tumour biology. The present thesis mainly focuses on the role of NO in BPS/IC pathogenesis and the role for NO in bladder cancer biology and its potentially anti-tumour effects following BCG treatment.

5.1 No in Bladder pain syndrome / Interstitial cystitis

Already in 1996 Lundberg *et al*., (11) showed that NO formation is increased in the urinary bladder in patients with inflammatory diseases and in 2004 Logadottir *et al*., (89) concluded that measuring luminal bladder NO could discriminate between patients with the classic form of BPS/IC and those with the non-ulcerous form. One of the aims with this thesis was to determine the source of NO and its site of production in the bladder mucosa in patients with BPS/IC.

Bladder biopsies from patients with BPS/IC and control subjects were investigated for iNOS expression with real-time PCR and Western Blot. We found that iNOS mRNA expression was increased in BPS/IC patients and that protein expression for iNOS was detected in the BPS/IC patients. In addition, this corresponded to the elevated levels of endogenously formed NO seen in these patients, a finding which in all likelihood is related to the aforementioned iNOS mRNA and protein increase. In biopsies from patients with classic BPS/IC the immunoreactivity for iNOS was higher in the urothelium compared to control subjects suggesting the urothelial cells as a source of the elevated NO levels seen in these patients. In addition, strong iNOS immunolabeling was also found in immunocompetent cells in the bladder wall, both in the submucosa but also within the urothelial layer, which may imply that these cells may also contribute to the high levels of NO found in the bladder in patients with BPS/IC. These observations are well in line with findings reported by others demonstrating an increased NOS activity in the urothelium and iNOS staining in urothelial cells and inflammatory cells in the bladder during inflammation, in both animal models and humans (129, 161-163).

Despite previous efforts researchers have not as yet been able to fully elucidate

the aetiology or pathogenesis of this complex disease. In the light of the high levels of NO found in patients with classic BPS/IC it is possible that NO may mediate some of the pathophysiological events seen in BPS/IC. NO may be harmful when produced in excess for a prolonged time due to possible tissue damage caused by increased formation of free radicals such as peroxynitrite (164). One important factor in BPS/IC pathogenesis is the presumed increase in urothelial permeability (58, 165). Excessive iNOS production has been suggested to cause barrier dysfunction due to an increase in epithelial permeability in several other tissues (166-169). Tight junctions play an important role in epithelial permeability as they hold adjacent cells together and act as semi permeable elements. Tight junctions are essential for the formation of epithelial sheets, which form an important barrier against various noxious substances. In biopsies from BPS/IC patients decreased levels of ZO-1 protein, a cytoplasmatic peripheral membrane protein located to tight junctions in human bladders was found (170, 171). Furthermore, several studies support the notion that iNOS dependent NO production causes epithelial barrier dysfunction related to altered expression of the tight junction protein ZO-1 (166, 169). In some studies this adverse effects on barrier function has been attributed peroxinitrite rather than NO itself (172-174).

Another role for NO in BPS/IC pathogenesis is the possibility that augmented iNOS expression in the bladder wall may be involved in bladder fibrosis, which is seen in end- stage classic BPS/IC. It has previously been reported that iNOS expression has been related to increased collagen expression (175-179) and in a rodent model of pathological fibrosis the inhibition of iNOS activity blocked the increase in collagen (180, 181). Furthermore, human bladder smooth muscle cells treated with LPS and inflammatory cytokines expressed collagen type III through an iNOS dependent pathway (182).

In the late nineties therapy with L-argenine was tested in patients with BPS/IC with the rationale that patients with BPS/IC had a lower expression of iNOS in urine pellets (183). It was suggested that administration of L-arginine to patients would increase NO production in the bladder leading to relaxation of the bladder and modulation of afferent firing (184). Several similar studies have, however, revealed conflicting results (185-188) and the positive effects seen in the first study by Smith *et al.*, (184) have not been reproduced. In a study by Korting *et al.*, (185), L-argenine had positive effects only in those with a bladder capacity exceeding

800ml, and Ehrén *et al*., (188) showed no positive effect of orally given L-argenine to patients with classic BPS/IC who already at the start of the study displayed markedly increased NO production. Due to the lack of a proper definition of BPS/IC patients at that time it is likely that patients in some of the studies consisted of both classic and non-ulcerous form of BPS/IC, which could explain some of the diverging results. For patients with classic BPS/IC a more rational approach may be to pharmacologically decrease iNOS activity and NO production. Bearing in mind that NOS inhibitors have anti inflammatory properties in an experimental setting (189, 190), iNOS inhibitors may tentatively display beneficial effects also in clinical practice. Interestingly, some of the already available drugs for treating BPS/IC may exert some of their therapeutic effects through NO/iNOS related mechanisms.

Cyclosporin A is a calcineurin inhibitor with potent immunosuppressive and anti-inflammatory properties (80-82, 191) and has in randomized control studies proven effective in the treatment of BPS/IC (80-82). Calcineurin has been shown to be required for full iNOS expression in macrophages (192) and it has been shown that Cyclosporin A inhibits NO production in macrophages (193-196). A recent study by Hämäläinen *et al*., (197) demonstrated that Cyclosporin A down-regulates NO production by destabilising iNOS mRNA. This is in line with observations by Ehrén *et al*., that have shown that endogenously formed NO, as measured in the urinary bladder, is decreased during Cyclosporin A treatment in patients with classic BPS/IC (personal communication). Also PPS, that can be administrated both orally and intravesically in patients with BPS/IC, has been implicated to affect the NO/NOS pathway. In a study from 2007, Veszelka *et al*., (198) showed that PPS reversed LPS induced barrier impairment and lowered NO levels in brain endothelial cells and that changes in the tight junction protein ZO-1 were also reversed by PPS. Thus, PPS may reverse changes in the ZO-1 protein seen in the bladder of patients with BPS/IC (171) thus improving bladder impermeability an effect that could be elicited via NO/iNOS pathways. DMSO, an intravesical agent for treating BPS/IC, has been attributed a variety of mechanisms through which it might exert its beneficial effects in BPS/IC patients. For one, DMSO may act through modulation of sensory nerves with a stimulation of bladder afferent nerves accompanied by a release of NO and relaxation of the bladder (199, 200). This may represent a putative mechanism of action in patients with non-ulcerous form of BPS/IC but would probably not cause relaxation and

desensitization of nociceptive pathways in classic BPS/IC because of the already markedly increased levels of NO produced in the bladder wall of these patients. DMSO has also been established as a scavenger for intracellular hydroxyl radicals and has also been suggested to be capable of inhibiting peroxynitrite induced DNA stand breakage (201) which is one important way through which peroxinitrite may elicit cytotoxicity (202).

5.2 Nitric oxide in bladder cancer biology

Evidence for NO involvement in tumour biology has been reported from several different forms of cancer both *in vitro* and *in vivo*. However, there are conflicting reports whether NO promotes or inhibits tumour growth. In some studies it appears that NO enhance tumour proliferation and angiogenesis (109, 113, 203) while in others an increased NOS activity is correlated to a diminished metastatic ability (107, 114) or has no apparent effect at all (110). One way in which NO may act tumour promoting has been attributed to its possibility to stimulate angiogenesis (113) which is correlated to tumour invasiveness and metastatic potential (204). These diverging results may be due to differences in the rate of NO formation and the concentration of produced NO. Thomae *et al*., (117) described that low concentrations of NO stimulated endothelial cell growth, whereas high concentrations acted in an inhibitory fashion. This dual effect of NO on cell growth has also been demonstrated in bladder cancer cell lines by Morcos *et al.,* (112).

In this study iNOS expression was found in biopsies from bladder tumours, which is in line with previous reports both *in vitro* (112) but also *in vivo* (111, 115, 118). We found no correlation between the levels of iNOS and tumour grade or stage, although GIII tumours expressed higher levels of mRNA for iNOS. Further stratification revealed that the high levels of iNOS mRNA expression seen in patients with GIII tumours were found in the patients with CIS (alone or concomitant to a papillary GIII tumour). This observation may be related to the enhanced growth capacity of this tumour. CIS is a high-grade (GIII) tumour presenting as a flat lesion, and has a high risk of progressing into invasive cancer (104). This risk is higher than that seen in papillary TaGIII tumours (105) implicating that CIS is a seperate entity of bladder cancer. Alternatively, the higher levels of iNOS mRNA and protein levels, as well as the higher levels of NO produced in patients with CIS, may originate from inflammatory cells in the

mucosa of the bladder as a response to an immune mediated host defence reaction. This is in line with findings by Klotz *et al*., describing that iNOS is localized in invading macrophages and neutrophiles in bladder cancer patients (205). Further investigations with immunohistochemistry to identify the site of NO production would be of inportance in understanding the involvement of NO in CIS of the bladder.

It is plausible that iNOS expression and activity in part is affected by polymorphisms and the iNOS promoter (-2.6 Kb) microsatellite (CCTTT)n polymorphism has been correlated to the development and aggressiveness of bladder cancer, thus further supporting the involvement of NO in bladder tumour biology (154, 155). Patients with a long set of repeats of the (CCTTT)n polymorphism had a lower risk of developing bladder cancer but a higher risk for stage progression and cancer specific death once tumour hade developed (155). It has been suggested that patients with a long set of repeats for this polymorphism have a more active promoter, thus leading to increased NO production (206, 207). Ryk *et al*., suggested that a more active version of iNOS could be beneficial before the development of a bladder tumour since higher NO production would give rise to a more potent host-defence reaction. On the contrary, in patients who develop bladder tumours, higher iNOS activity could promote angiogenesis, cause further aggressiveness and through the formation of reactive nitrogen species repress macrophage activity. In addition, polymorphisms in the eNOS gene have been found to affect bladder cancer development and aggressiveness (Ryk *et al*., manuscript under revision in Nitric Oxide: Biology and Chemistry).

5.3 nitric oxide in bcg treatment for bladder cancer

BCG treatment is considered the most effective intravesical treatment for NMIBC and is golden standard for treating CIS (126). Although major efforts have been made to elucidate its mode of action the exact mechanism through which BCG elicits tumour eradication is not fully understood. As reviewed by Brandau (125), compelling evidence has been put forward suggesting that BCG triggers a strong non-specific inflammatory immune response with T-cell involvement that, together with macrophages, result in cytotoxic effects. Several studies propose that NO also actively mediate some of the anti tumour effects seen after BCG treatment (112, 118).

In this work we have demonstrated elevated levels of NO formation in the

urinary bladder after BCG treatment and that these levels corresponded to elevated levels of iNOS at both transcriptional and protein levels. These findings are in line with previous reports on elevated levels of endogenously formed NO in the bladder after BCG treatment (11, 118) and the presence of iNOS protein and increased iNOS activity in the bladder mucosa following BCG instillations (118, 132). We found that iNOS staining was located to the urothelium, predominantly the umbrella cells, and to immune competent cells e.g. macrophages located in clusters and individually in the submucosa. It is likely that a majority of the NO seen in the urinary bladder after BCG treatment originates from the urothelium and the inflammatory cells in the submucosa.

In vitro studies have demonstrated both tumouricidal and tumor-promoting effects of NO on tumour cells (208, 209) but the massive production seen after BCG treatment is most likely to have deleterious effects on the tumour cells. Jansson *et al*., and Morcos *et al*., (112, 118, 129) showed that the induction of iNOS activity in bladder cancer cells inhibited cell growth and that NO could induce apoptosis in bladder cancer cells.

Several of the cytokines found in urine in BCG treated bladder cancer patients can induce NOS activity (7) and when added to bladder cancer cell cultures these cytokines caused growth arrest and apoptosis, which was not the case when adding a NOS inhibitor together with the cytokine mixture, suggesting that NO/NOS pathways mediated apoptosis (112). This is in line with other studies that show growth arrest and apoptosis induced by increased iNOS activity and NO production following stimulation with cytokines (210).

Macrophages are thought to play an important role in BCG induced cytotoxicity and NO has been implicated to participate in this effect since NO is considered one of the main factors responsible for the cytotoxic activity that macrophages exert on tumour cells (211). Interestingly, BCG was one of the first compounds shown to induce iNOS dependent macrophage tumour cytotoxicity (1). The mechanisms for NO induced cytotoxicity have been attributed to intracellular iron loss with inhibition of mitochondrial respiration (1), inhibition of ribonucleotide reductase activity leading to the inhibition of DNA synthesis (133) and DNA strand breaks caused by peroxynitrite (164).

Even though macrophage derived NO has been established as an important anti-neoplastic mediator the excessive NO production may also suppress

macrophage activity and have deleterious effects on non-tumour surrounding tissue (212). This illustrates the complexity of NO signalling and the fine balance between tumouricidal activity, on one hand, and negative effects on the immune cells, on the other hand.

Despite being the best bladder sparing treatment option for patients with high risk NMIBC a large proportion of patients (30-35%) do not respond to BCG treatment. This effect could possibly be due to an acquired resistance to NO that has been reported in both macrophages and tumour cell lines (213). These studies implicate that a prolonged exposure to low doses of NO later offers protection to a secondary exposure of higher levels of NO.

The high levels of NO seen after BCG treatment in addition to the known cytotoxic effects of NO on bladder tumour cells support the notion that NO might act as an effector molecule in BCG induced tumour eradication. However, the need for molecular markers to identify those at risk for BCG failure is crucial since radical surgery in BCG non-responders may have a less favourable prognosis than those undergoing immediate cystectomy (128). Polymorphisms may be associated to drug metabolism and thus treatment response (138-140) and it is plausible that polymorphisms could also be one factor responsible for the fact that 30-35 % of the BCG treated patients do not respond to treatment. Thus, we studied the influence of iNOS and eNOS polymorphisms on the outcome after BCG treatment. We found that patients homozygous or heterozygous for a long set of iNOS (CCTTT)n repeats had a higher risk for cancer specific death as compared to those who did not. In addition, the eNOS -786T>C polymorphism influenced outcome after BCG treatment, showing a lower risk for cancer specific death and disease progression in patients with the TT genotype. The same was also noted for the eNOS Glu298Asp polymorphism, where the GG genotype responded better to BCG.

For the iNOS (CCTTT)n promoter polymorphism it has been suggested that a long set of repeats give rise to a more active promoter thus leading to increased NO production (206, 207). This could theoretically be prometastatic due to promoted angiogenesis and a repressed macrophage activity, but could also cause resistance to the high levels of NO seen after BCG treatment. On the contrary, the C-allele in the eNOS -786T>C promoter polymorphism is associated with a less active promoter (214, 215) which is also the case for the T-allele in the eNOS intragenic

Glu298Asp polymorphism (216). These findings reflect the differential response frequently encountered when studying NO and its pathways. In this scenario it may reflect the concentration and duration of NO produced by eNOS and iNOS, and that the NO produced from eNOS might be too low to have the ability to cause an acquired resistance against NO and that the effect on BCG treatment response seen in these patients are mediated through other mechanisms.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

- In patients with classic BPS/IC iNOS immune labelling was localized to the urothelium and immune competent cells within the urothelium and submucosa. In addition, iNOS mRNA and protein expression was elevated in patients with classic BPS/IC as compared to control subjects. The endogenously formed NO was significantly higher in BPS/IC patients than in controls.
  These data further support a possible role for NO in the pathogenesis of BPS/IC and that drugs targeting the NO/NOS pathways may in the future be useful in the treatment for this disease.

- Endogenous NO production was elevated in patients with CIS of the bladder, both in primary CIS but also in CIS with a concomitant papillary GIII tumour. Also mRNA expression and protein levels for iNOS was significantly higher in biopsies from CIS lesions as compared to papillary tumours and healthy controls. This could reflect the enhanced growth potential in CIS but could also be caused by an increased host-defence reaction. Further investigation of the site for NO production in CIS lesions need to be done. In addition elevated NO measured at the time of cystoscopy could in the absence of a UTI be a marker for CIS. In these cases cystoscopy with fluorochromes could be used in order to find possible primary or concomitant CIS.

- In BCG treated patients iNOS expression at transcriptional and protein levels are elevated in bladder biopsies. iNOS activity is located in the urothelium and in the immune competent cells in the submucosa. Also luminal NO formation is increased after BCG treatment. This further supports the notion that NO might mediate some of the anti tumour effects exerted by BCG, both directly but also through macrophage induced NO cytotoxicity.

- Polymorphisms in the iNOS and eNOS genes may influence the outcome after BCG treatment. In patients not carrying a long set of (CCTTT)n repeats there was a significantly lower risk for cancer specific death in the BCG treated group while there was no difference found in patients with a long set of (CCTTT)n repeats. For the eNOS -786T>C promoter polymorphism patients with the TT genotype had a lower risk for cancer specific death and disease progression, which was also the case for the GG genotype in the eNOS intragenic Glu298Asp polymorphism. This further supports a possible involvement for NO in bladder cancer biology and in BCG treatment for bladder cancer. In addition, this may have clinical implications in the selection of patients to BCG treatment, allowing those at risk for BCG failure earlier initiation of other treatments, such as cystectomy.

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