NERVE GROWTH FACTOR AND LOWER URINARY TRACT DYSFUNCTION

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“What you have learned is a mere handful; what you haven't learned is the size of the world"

Avvaiyar, 13th century

DECLARATION

The studies described in this thesis were conducted at the Department of Urogynaecology, St Mary's Hospital, Imperial College Healthcare NHS Trust. Ethical approval was obtained from local research ethics committee (REC reference number:11/LO/1029) .

I am the principal investigator of all the studies and was involved in recruiting patients after informed consent, clinical assessment, collecting data, performing ELISA analysis, data analysis and writing up the thesis.

The results presented in this thesis have been produced entirely by my own original research and the work of other researchers has been appropriately referenced.

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ABSTRACT

The polypeptide nerve growth factor (NGF) has been explored extensively over the span of six decades since its detection with amazing discoveries from its neurotrophic action to tissue healing properties. In lower urinary tract there is substantial evidence linking NGF and lower urinary tract dysfunction (LUTD). Over recent years the role of urinary NGF [UrNGF] in diagnosing LUTD as well as monitoring treatment response has been investigated extensively. However, the available studies report conflicting data regarding an association between lower urinary tract symptoms [LUTS] and UrNGF, and there is limited evidence for the validity and reliability of urinary NGF assays.

In a quest to explore the role of UrNGF as a LUTS biomarker, levels were measured in patients with LUTD, prolapse and asymptomatic controls. This thesis hypotheses that measurement of NGF is of no value in LUTD and prolapse. Therefore the aims were to evaluate the diagnostic and discriminant ability of UrNGF measurement in LUTD and to test the reliability of NGF assays. The other objective was to study the association between UrNGF levels and cystoscopic and histology findings of bladder inflammation in women with overactive bladder [OAB] to explore the link between NGF and inflammation. Change in UrNGF levels after cystocele repair was studied, since bladder wall stretching has been postulated as one of the causes for increased NGF levels. Finally UrNGF levels before and after antibiotic

treatment for refractory OAB were measured with the aim to evaluate its role to assess treatment response.

On test retest reliability analysis of 13 samples there was almost perfect reliability with an Intraclass correlation coefficient of 0.889; 95%[C.I=0.676-0.965; p<0.001]. Urinary NGF was significantly but non-specifically increased in symptomatic patients [n=205] when compared to controls [n=31](13.33 vs. 2.05 ng NGF/ g Cr, Mann Whitney test; p <0.001) However ROC analysis, demonstrated poor discriminant ability between either different symptomatic groups or urodynamic groups. Using a cut off of 13.0 ng NGF/ g creatinine the test provides a sensitivity of 81%, but a specificity of only 39 % for overactive bladder.

UrNGF levels were not associated with cystoscopic or histology findings of inflammation and did not improve after anterior repair in women who had an improvement in OAB symptoms. However in the study done to explore the role of urinary NGF as a biomarker to assess treatment response, NGF levels were found to decrease significantly after six weeks of antibiotic therapy in women with refractory OAB symptoms [n=35 patients] (Wilcoxon Signed rank test; p=0.015). This was associated with improvement in OAB symptoms.

UrNGF does not appear to be a good diagnostic biomarker but may have a role as a marker of treatment response, hence may have limited role in assessment of women with LUTD.

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Completion of this MD thesis has been a long journey nevertheless it has given me a great sense of achievement and knowledge, which are irreplaceable. I would like to thank the participants in this study who has made this thesis possible.

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ABBREVIATIONS

AUC	Area under curve
BDNF	Brain derived neurotrophic factor
BOO	Bladder outlet obstruction
BP	Bladder pain
BP/IBS/RBC	Bladder pain/Increased bladder sensation/reduced bladder capacity
BPS	Bladder pain syndrome
C	Control
CGRP	calcitonin gene related peptide
CNS	central nervous system
CP/CPPS	Chronic prostatitis/Chronic pelvic pain syndrome
CRP	C –reactive protein
CVA	Cerebrovascular accident
CVD	Cerebrovascular disease
DO	Detrusor overactivity
DRG	dorsal root ganglia
ELISA	Enzyme linked immunosorbent assay
ESSIC	European Society for the Study of interstitial cystitis
FDV	First desire to void
FSF	First sensation of filling
GDNF	glial-derived neurotrophic factor
HRP	horseradish peroxidase

HRQoL	Health related quality of life measures
IBS	Increased bladder sensation,
IC	Interstitial cystitis
IC/BPS	Interstitial cystitis/ bladder pain syndrome
ICC	Intraclass Correlation Coefficient
ICI	International consultation on incontinence
ICS	International continence society
IDO	Idiopathic Detrusor Overactivity
IOAB	Idiopathic OAB
KHQ	King's health questionnaire
LUTD	Lower urinary tract dysfunction
LUTS	Lower urinary tract dysfunction
MAPK	mitogen-activated protein kinase
MCC	maximum cystometric capacity,
MSU	midstream specimen of urine
MUI	Mixed urinary incontinence,
NDO	Neurogenic DO
ng/g	nano grams/gram
NGF	Nerve growth factor
NGF/Cr	Nerve growth factor/creatinine ratio
NICE	National Institute for health and care excellence
NOAB	Neurogenic Overactive Bladder
NPV	negative predictive values
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
OAB	Overactive bladder
OAB-Q	overactive bladder questionnaire

OD	Optical Density
pAb	Anti-NGF polyclonal antibody
PGE2	Prostaglandin E2
PKC	protein kinase C
POPQ	pelvic organ prolapse quantification
PPBC	Patient perception of bladder condition
PPIUS	Patients' Perception of Intensity of Urgency Scale
PPV	positive predictive value
PRO	patient reported outcomes
PROM	patient reported outcome measures
rhNGF	Recombinant human nerve growth factor
ROC	receiver-operator characteristics
SCI	Spinal cord injury
SUI	Stress Urinary Incontinence
TMB	tetramethylbenzidine
Trk-A	tropomyosine related kinase A receptor
TRPV1	transient receptor potential ion channel
U	Urgency
UDS	urodynamics
UrNGF	Urinary NGF
US	urge sensation
USI	Urodynamic stress urinary incontinence
UTI	urinary tract infection
UUI	Urgency urinary incontinence
VALUE	Value of Urodynamic Evaluation
VAS	Visual analog scale

Publications:

The following publication has resulted from this thesis:

Vijaya G, Cartwright R, Derpapas A, Gallo P, Fernando R, Khullar V **Changes in nerve growth factor level and symptom severity following antibiotic treatment for refractory overactive bladder**. Int Urogynecol J. 2013 **24**(9): 1523-1528.

[Reliability and validity of urinary nerve growth factor measurement in women with lower urinary tract symptoms
Authors: Vijaya G, Cartwright R, Bhide A, Derpapas A, Fernando R, Khullar V. Manuscript submitted to European journal of obstetrics and gynaecology and reproductive biology]

Abstract Presentations at National and International meetings:

The following abstract presentations has resulted from this thesis:

- Can Urinary Nerve growth factor replace urodynamics to diagnose LUTS? - Podium presentation at International urogynaecology association [IUGA] meeting, 2011
- Increased nerve growth factor in overactive bladder :Is it caused by infection? Oral poster Presentation at IUGA meeting, 2011.
- A simplified method of Biomarker normalisation for urinary concentration-Podium presentation at IUGA, 2011

- Can Urinary Nerve growth factor replace urodynamics to diagnose LUTS? - Oral presentation, RCOG Annual scientific meeting, Athens. 2011.
- Urinary nerve growth factor levels are not associated to bladder inflammation-Oral presentation-British society of urogynaecology [BSUG] ARM 2012
- What is the test-retest reliability of urinary neurotrophic factors measured in women with lower urinary tract symptoms (LUTS) BSUG ARM 2012

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Measurement of urinary nerve growth factor is of no value in the assessment of women with lower urinary tract dysfunction or prolapse

CHAPTER 1

Setting

Chapter 1: Setting

The research for this thesis was carried out in the department of urogynaecology, St. Mary's hospital, Imperial college healthcare NHS trust from September 2010 to April 2012.The lab work for NGF ELISA analysis was carried out at Institute of reproductive and developmental biology, Hammersmith campus of Imperial college healthcare NHS trust.

The department of urogynaecology is led by Mr V. Khullar and consisted of two subspecialist consultants, subspecialty trainee, two research fellows, Clinical nurse specialist and nurse practitioner at the time of research. The unit is a tertiary referral centre and often referrals are accepted from all over UK. Each year more than 5000 urogynaecology patients are seen and about 1000 urodynamic investigations are performed. The unit offers a wide range of diagnostic techniques including video cystourethrography, saline cystometry, three-dimensional ultrasonography, urethral pressure profilometry and ambulatory urodynamics. There are eight urodynamic clinics run every week by either nurse specialists or doctors. Weekly pelvic floor clinic is run where endoanal ultrasound and anal manometry is offered routinely to women who had sustained obstetric anal sphincter injuries.

Clinical assessment of patients includes urine analysis, POP-Q examination and use of Patient reported outcome measures such as frequency volume chart, King's health questionnaire, prolapse

Quality of life questionnaire and patient perception of bladder condition.

The department offers treatment for routine and complex conditions of urinary incontinence, vaginal prolapse, recurrent urinary tract infections, bladder pain and pelvic floor injury after childbirth.
The unit works in close liaison with urologists, colorectal surgeons, elderly care physicians, microbiologists, pain team consultants, neurologist, physiotherapist and continence nurses. Weekly multidisciplinary meetings are conducted where management of complex cases are discussed. Monthly research meetings are run to discuss ongoing research projects and research ideas.

The unit is actively involved in various research projects and is usually multidisciplinary involving other disciplines such as microbiology, histopathology, radiology, neurology and physiotherapy. Projects are focused on various aspects including basic science, infection and overactive bladder, biomarkers, imaging and other investigative methods. There is active participation in multicenter international research projects that includes surgical and drug trials. This attracts honorary research fellows from other parts of UK and Europe with an interest to get involved in ongoing research. In addition there are BSc students attached to the unit on a regular basis. The interest and commitment in research is reflected by the high volume of publications in peer reviewed journals and presentations at national and international meetings.

CHAPTER 2
NERVE GROWTH FACTOR

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Introduction

Nerve growth factor (NGF) is a neurotrophic factor essential for the growth, differentiation and maintenance of developing sensory and sympathetic nervous system. Since its detection there has been overwhelming and emerging discoveries supporting its diverse action in human body such as nociceptive and tissue reparative effects. There is a growing interest in developing neurotrophic factors as a biomarker in the diagnosis of lower urinary tract dysfunction (LUTD) since evidence from experimental and human studies have found an association between increased levels of bladder and urine NGF and various bladder dysfunctions. NGF is postulated to play a role in neuroplasticity by mediating inflammation, as well as morphological and functional changes, in sensory and sympathetic neurons innervating the urinary bladder leading to bladder dysfunction. (Schnegelsberg et al. 2010, Steers 2002)

2.1 Structure and biological properties of NGF:

Nerve growth factor [NGF] is a polypeptide discovered during 1950's (Levi-Montalcini and Angeletti 1968) by an Italian developmental biologist, Rita Levi-Montalcini who won the Nobel Prize for it's discovery in 1986. This protein was termed Nerve growth factor (NGF) due to its effect on the growth of the developing sensory and

sympathetic neurons(Levi-Montalcini 1964).NGF belongs to a family of neurotrophins and other members of this group are brain derived neurotrophic factor(BDNF), neurotrophins NT 3 and NT4/5 .

The 26-kDa β subunit of the 130–140 kDa 7S NGF complex is responsible for the biological activity(Fahnestock et al. 1991).The single gene encoding βNGF in humans is located on chromosome 1 (Francke et al. 1983) .It encodes a precursor protein known as prepro-NGF, which is sequentially cleaved into pro-NGF and then active βNGF(Chao 2003). Each βNGF subunit is made up of two identical pairs of antiparallel strands of 118 amino acids held together by monovalent bands (Figure2.1)(McDonald et al. 1991).Cystine knots help in providing rigidity to the strands at one end of the protomer (McDonald and Chao)

The action of NGF is mediated by two receptors tropomyosine related kinase A receptor (Trk-A) (Patapoutian and Reichardt 2001) and the p neurotrophin receptor (p75NTR) (Chao 1994), which are tissue specific. Trk-A binds specifically to NGF and depends upon intrinsic tyrosine kinase activity for its action(Patapoutian and Reichardt 2001). After being secreted by the target organ, NGF is absorbed by sympathetic and small sensory fibers via a high affinity Trk-A receptor and retrogradely transported to the cell body where it exerts its action(Hendry et al. 1974, Thoenen et al. 1988). Activation of tyrosine kinase A induces a cell-signalling cascade involving mitogen-activated protein kinase (MAPK) pathways(Freund-Michel and Frossard 2008, Steers and Tuttle 2006).

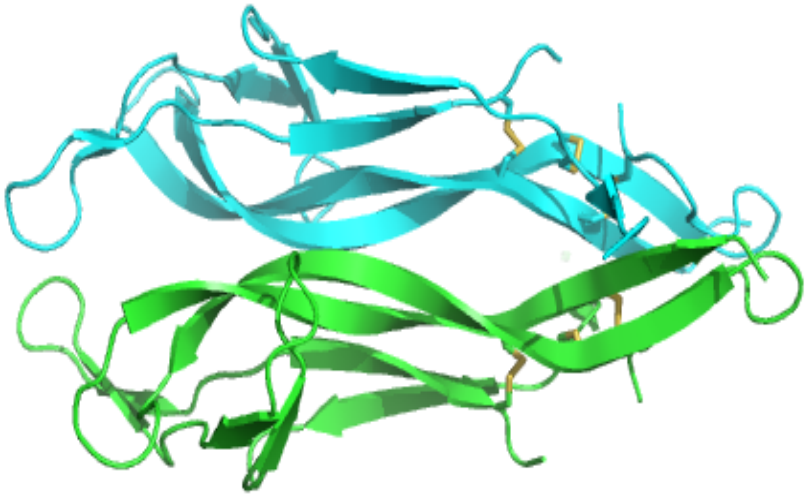


Figure 2.1: NGF dimer (extracted from PDB 1SG1): each subunit is made up of two pairs of non-parallel polypeptide chains

2.2 Sources of NGF:

NGF is synthesized by neuronal and non-neuronal cells (Aloe et al. 1997).NGF is produced by neuronal cells physiologically(Aloe et al. 2002). In the central nervous system (CNS) the highest amount of NGF is produced in the cerebral cortex and hippocampus (Aloe et al. 1997) and there seems to be a reciprocal interaction between cholinergic afferents and NGF producing neurons. The acetylcholine increases the production of NGF, which in turn has an effect on acetylcholine receptors and increase the release of acetylcholine(Knipper et al. 1994).

NGF is produced by variety of other cells outside nervous system such as epithelial cells(Alleva and Santucci 2001) eosinophils (Aloe et al. 1997) lymphocytes(Aloe et al. 1997, Santambrogio et al. 1994) macrophages (Alleva and Santucci 2001), mast cells(Leon et al. 1994),Schwann cells and fibroblasts(McMahon 1996) to name a few.

It has been found in various immune organs including spleen, lymph node and thymus(Aloe et al. 1997).The salivary gland(Nam et al. 2007),vascular smooth muscle(Schaper et al. 2009),cardiomyocytes (Kaye et al. 2000)are only few among the other sources of NGF production.

2.2.1 Source of NGF in bladder:

In bladder NGF is produced by smooth muscle(Lamb et al. 2004, Steers et al. 1991) and bladder epithelium(Lowe et al. 1997, Oddiah et al. 1998, Schnegelsberg et al. 2010) TrkA receptors, which bind to NGF, are expressed in bladder urothelial cells and primary afferents(Allen and Dawbarn 2006, Birder et al. 2007, Steers and Tuttle 2006).

2.3 Functional properties of NGF and its diverse role in systemic disorders:

NGF promotes growth, differentiation and survival of central and peripheral nervous system neurons during fetal life(Levi-Montalcini 1987). In adult life NGF appears to play a role in neuroplasticity by stimulating the regeneration and repair of damaged nerve cells in both central and peripheral nervous system in response to trauma and other insults such as ischemia and degeneration (Sofroniew et al. 2001) . Neural plasticity is the ability of neural circuits to undergo changes in function or organization to compensate for new activity such as injury, disease, change in the environment etc. NGF has a diverse role, which includes its trophic effects on neurons as well as non-neuronal cells(Chaldakov 2011, Sofroniew et al. 2001).

NGF plays a vital role in mediating pain in inflammatory conditions(Anand 1995, Chuang et al. 2001, McMahon 1996, Pezet and McMahon 2006). It serves as a link between inflammation and hyperalgesia. Increased NGF levels in experimental inflammatory conditions are found to be associated with hyperalgesia, which is blocked by anti-NGF antibodies(Woolf et al. 1994). Increased NGF levels in response to noxious stimuli leads to both peripheral sensitization of nociceptors and central sensitization of dorsal horn neurons(Dray 1995, McMahon 1996, Woolf 1996) NGF-mediated modification of gene expression in the dorsal root ganglion during inflammation contributes to persistent pain(Woolf 1996).

NGF may produce hyperalgesia by acting directly on sensory nerve endings or by promoting the synthesis and release of sensory neuropeptides, substance p and calcitonin gene related peptide (CGRP), which facilitates some of the peripheral and central effects of inflammation(Anand 1995, Donnerer et al. 1992, Lindsay and Harmar 1989, McMahon 1996, Schnegelsberg et al. 2010).The phenotype changes produced by NGF during inflammation include the upregulation of growth related molecules, which may lead to a hyper innervation of injured tissue by promoting terminal sprouting(Woolf 1996). NGF might also regulate nociceptive signalling by altering the expression of brain derived neurotrophic factor (BDNF)(Allen and Dawbarn 2006),[QiaoLY 2013] voltage-gated sodium channels as well as membrane ion channels such as transient receptor potential ion channel [TRPV1] that are thought to play a central role in inflammation or tissue injury induced pain and

hypersensitivity(Amaya et al. 2004, Pezet and McMahon 2006, Woolf 1996).

The role of NGF in allergic inflammation is explained by two hypotheses. NGF might have a modulatory role or may represent the product of inflammatory cells (Bonini et al. 1999).NGF is found to be a stimulus for mast cell production(Levi-Montalcini et al. 1996, Marshall et al. 1990, Matsuda et al. 1991) as well as an important mediator of mast cell function in normal and pathological states (Horigome et al. 1993) .Further supporting the role of NGF in inflammation, NGF has been shown to cause release of histamine from mast cells (Horigome et al. 1994)and basophils (Bischoff and Dahinden 1992).

Increased NGF expression has been found in animal models of pancreatitis(Toma et al. 2000)gastric ulcers(Bielefeldt et al. 2003), bowel obstruction(Williams et al. 1993) as well as in patients with inflammatory bowel disease(di Mola et al. 2000) and pancreatitis (Friess et al. 1999). Increased serum NGF levels have been found in a multitude of other disorders such as systemic lupus erythematosus (Bracci-Laudiero et al. 1993), arthritis (Falcini et al. 1996) ,anxiety disorders (Aloe et al. 1997), asthma(Bonini et al. 1999), allergic disorders (Bonini et al. 1999), keratoconjunctivitis (Lambiase et al. 1995), stroke(Stanzani et al. 2001) ,cardiac failure(Singh et al. 2013),renal disorders (Bonofiglio et al.),emotional stress(Laurent et al. 2013) and physical stress(Alleva and Santucci 2001).

In the reproductive system, NGF and other neurotrophic factors was found to play a role in oocyte maturation(Linher-Melville and Li

2013). Increased NGF levels in the peritoneal fluid has been found in patients with endometriosis postulating NGF to be involved in the endometriosis associated pain(Kajitani et al. 2013) .

NGF demonstrates angiogenic properties (Blais et al.) and is found to promote tumour progression in human cholangiocarcinoma (Yue et al. 2013) and stimulate cellular proliferation in epithelial ovarian cancer(Urzua et al. 2012) .NGF receptor expression has been linked with specific subtypes of breast cancer (Tsang et al. 2013).

Decreased levels of NGF have been found in experimentally induced diabetic states(Dey et al. 2013, Tong and Cheng 2005), depressed patients(Diniz et al. 2013)as well as in atherosclerotic coronary walls in clinical studies(Chaldakov 2011).NGF signalling seems to be altered in Alzheimer's disease(Salehi et al. 2004).

Thus NGF seems to play an important role in the pathogenesis of plethora of neurological, inflammatory, allergic, autoimmune, infective and degenerative disorders. NGF is described to have neurotrophic, immunotrophic, nociceptive, epitheliotrophic and metabotrophic functions(Chaldakov 2011).

2.4 Clinical applications of NGF

Potential therapeutic properties of NGF in both central and peripheral nervous system disorders, cardio metabolic disorders as well as diseases of eye and skin ulcers have been explored recently in both experimental and clinical studies(Aloe 2011). There is also

interest in developing Recombinant human nerve growth factor (rhNGF) and recombinant human mAb against NGF.

Anti NGF therapy in mouse models has shown reduction in tumor induced nerve sprouting and cancer pain(Jimenez-Andrade et al. 2011) .It has been shown in experimental and clinical studies that NGF may have profound effects on reparative process and tissue regeneration (Bernabei et al. 1999, Kolostova et al. 2011). Therapeutic potential of NGF in ocular diseases such as glaucoma, corneal ulcers and retinitis pigmentosa has been experimented in animal studies. Topical treatment with NGF eye drops has shown to be effective in the treatment of patients with neurotrophic and herpetic keratitis, and glaucoma(Lambiase et al. 2011) .

NGF therapy in both experimental and clinical studies for Alzheimer's disease has shown encouraging results (Covaceuszach et al. 2009, Tuszynski et al. 2005)

The use of rhNGF in symptomatic diabetic polyneuropathy was found to be effective in phase II trial, however the development of painful side effects was a major drawback(Apfel 2002). AntiNGF monoclonal antibodies has been used in clinical trials as analgesics for varied conditions such as osteo arthritis, lower back pain and interstitial cystitis with favourable and nonfavourable results(Bannwarth and Kostine 2014, Lewin et al. 2014).In a randomised double blinded placebo controlled phase 2 study tanezumab ,a humanised monclonal antibody was used to treat patients with interstitial cystitis.There was a significant reduction in pain and urgency episodes compared to placebo group with commonest adverse effects of headache and paraesthesia(Evans et al. 2011).

Although some of the preliminary studies have shown promising results the safety, mode of administration, dosage etc. of both NGF and anti NGF therapy is yet to be established.

2.5 Evidence from animal studies supporting the role of NGF in LUTD:

The role of NGF in the pathophysiology of LUTD has been explored in animal experiments, which demonstrates a strong link between increased NGF levels and bladder dysfunction. NGF seems to regulate neuroplasticity in micturition pathways. The key results from various studies are summarized below.

2.5.1 Effects of endogenous and exogenous NGF:

Generation of mouse models that overexpress NGF were found to have marked sensory and sympathetic nerve fibre hyperplasia in the submucosa and detrusor smooth muscle and elevated numbers of tissue mast cells. These mice had reduced urinary bladder capacity and an increase in the number and amplitude of nonvoiding bladder contractions Interestingly there was also increased referred [secondary] somatic hypersensitivity in the pelvic area and hind paw, which supports the role of NGF in somatosensory nociception(Schnegelsberg et al. 2010).

Intravesical administration of NGF has been shown to sensitize the bladder afferent fibres (A delta and C fibres)(Dmitrieva and McMahon 1996).It also produced bladder hyperreflexia(Dmitrieva et

al. 1997) and reduced volume threshold for micturition in experimental rodents compared to controls (Chuang et al. 2001).

In another animal experiment viral vectors encoding for NGF were injected into bladder wall of rats .NGF protein levels in the bladder of these animals increased significantly compared to that of controls. Bladder overactivity was also demonstrated in these animals on cystometrogram(Lamb et al. 2004).

Similarly infusion of NGF into the bladder wall of rats was also found to reduce bladder capacity, reduce the intercontraction interval of bladder and increase the amplitude of non-voiding contractions. There was also increased expression of Fos protein in L6-S1 spinal cord, which is an indicator for neuroplasticity and, CGRP immunoreactivity in the lumbosacral cord in response to this experiment (Zvara and Vizzard 2007).

Furthermore intrathecal administration of NGF in animal experiments at L6-S1 level was found to result in bladder overactivity. There was also evidence of increased tetradoxin resistant Na+ currents and reduction in K + current in C fibre bladder afferent neurons which could contribute to the hyperexcitability of bladder afferent neurons and bladder overactivity (Yoshimura et al. 2006).Intraperitoneal injections of NGF in TRP1 knockout mice were found to be associated with significant reduction in bladder overactivity compared to controls, postulating TRP1 to play a major role in the NGF driven bladder overactivity(Frias et al.).

2.5.2 Bladder outlet obstruction and NGF:

Steer et al demonstrated (Steers et al. 1991) about two decades ago that bladder outlet obstruction (BOO) in rodents was associated with

bladder hypertrophy and increased urinary frequency. There was also evidence of hypertrophy of dorsal root ganglia (DRG) that provide sensory afferent fibres to the bladder and increased growth of afferent and efferent neurons. Hypertrophied bladders contained significantly more NGF levels than normal bladders. The rise in the NGF levels occurred before the neuronal hypertrophy and increased urinary frequency. Autoimmunity to NGF was found to reduce the neuronal hypertrophy. Further supporting these findings NGF immune rats did not show increased expression of GAP 43, which is a marker for axonal sprouting, following urethral obstruction. These findings support the link between NGF and neuronal hypertrophy as found in other systems(Steers and Tuttle 2006).

Evidence that increase in bladder NGF levels are specific to bladder pathology also comes from the same study where NGF levels were measured in bladder tissue, abdominal aorta and lung specimens of both normal and obstructed animals. There was a significant difference in NGF levels between the control and obstructed group only in the bladder specimen. The numbers in subgroups are very small, therefore larger studies are needed to provide more evidence for the specificity of measuring bladder NGF levels.

Chronic BOO results in stretching of the urothelium and smooth muscle, which can stimulate NGF production. In vitro experimental models have demonstrated that repetitive stretch stimulation of bladder smooth muscle cells, which mimics the effects of outlet obstruction, resulted in increased expression of a variety of growth factors like NGF(Persson et al. 1995, Yamaguchi 2004).

Relief of obstruction only caused a partial reversal of neuronal

hypertrophy as well as increased NGF level. This incomplete reversal of neuronal plasticity could be the etiology for the incomplete eradication of symptoms after relief of obstruction(Steers and Tuttle 2006). Corroborating these findings, transient receptor potential vanilloid receptor TRPV1 which is a regulator of bladder reflex activity and bladder NGF levels were found to be elevated in animal models with persistent detrusor overactivity (DO) after relief of BOO compared with controls(Kim et al. 2004, Zvara et al. 2002)

2.5.3 Inflammation and NGF:

Experiments done in rodents have shown that chemical inflammation of the bladder is associated with increased NGF levels postulating NGF to be the mediator of sensory disorders associated with inflammation(Oddiah et al. 1998, Vizzard 2000). Beta NGF expression in the bladder was found to be increased also in response to animal models of cystitis induced by bacterial endotoxin lipopolysaccharide and substance P(Saban et al. 2002).

In another animal experiment varying models of inflammation were induced in animal bladders using formalin to reproduce chemical cystitis, lipopolysaccharide to reproduce bacterial cystitis and chromic catgut to reproduce a foreign body irritant. This resulted in increased NGF levels as well as morphological changes in the sensory and motor neurons innervating the bladder(Dupont et al. 2001).Cystitis and NGF overexpression in the bladder triggered bladder hyperactivity(Bielefeldt et al. 2006)as well as mechanical and thermal hypersensitivity in cutaneous referral sites(Guerios et al. 2006).

Cats diagnosed with feline interstitial cystitis (IC) were found to have increased levels of NGF and substance P in the urothelium compared to control animals. The DRG neurons in these cats were also hypertrophied(Birder et al. 2010).

2.5.4 Spinal cord injury and NGF

Spinal cord injury (SCI) in animal experiments was associated with increase in bladder NGF mRNA levels compared to controls. There was also increase in other neurotrophic factors such as BDNF, glial-derived neurotrophic factor (GDNF), neurotrophin-3 and neurotrophin-4 (Vizzard 2000). Increased levels of NGF in the bladder, spinal cord and dorsal root ganglia were associated with bladder hyperreflexia after SCI. Intrathecal administration of NGF antibody in spinal cord injured rats suppressed NGF levels in the L6 to S1 dorsal root ganglia, which contain bladder afferent neurons, and also suppressed bladder hyperreflexia(Seki et al. 2002).

2.5.5 Effect of antibodies to counteract the action of NGF

Antibodies to NGF (Hu et al. 2005, Steers et al. 1991) or to the NGF Trk-A receptor (Dmitrieva and McMahon 1996) seemed to prevent the morphological and functional changes of the nervous system and urinary frequency associated with obstruction or inflammation. NGF neutralizing antibody attenuated the bladder hypertrophy associated with cyclophosphamide induced cystitis (Chung et al. 2010) Down regulation of NGF expression using antisense oligonucleotides was found to suppress bladder overactivity in an experimental study

which provides scope for antiNGF treatment in OAB(Kashyap et al.2013).These data consolidate the evidence linking NGF and lower urinary tract dysfunction.

2.5.6 Effects of treatment for LUTD and NGF

Studies have also explored the effect of treatment for LUTD on NGF levels. Hyaluronic acid that is used for the treatment of IC significantly reduced the production of urinary NGF in response to chemically induced cystitis in rodents(Ho et al. 2011). Botulinum toxin reduced the expression of NGF in the urothelium and detrusor muscle of experimentally induced BOO in rodents and also recovery of overactive bladder (Ha et al. 2011).

2.5.7 Other interesting studies:

Other animal experiments have studied the relationship between NGF and pelvic ischemia, nerve injury and diabetic state. Experimentally induced pelvic ischemia in rabbit models resulted in overactive bladder and was also associated with increased expression of NGF in bladder tissue(Azadzoi et al. 2011).

Pudendal nerve injury resulted in increased bladder NGF levels, overactive bladder and stress urinary incontinence (SUI)(Furuta et al. 2008). It is postulated that pudendal nerve injury induces irritation of afferent pudendal nerves and increase levels of bladder NGF. This in turn sensitizes bladder afferent pathways and induces bladder overactivity. A recent study in rodents has provided intriguing evidence linking increased NGF levels and SUI.SUI induced

by urethrolysis in rodents was found to be associated with a significant increase in urethral NGF levels compared to controls(Ko et al. 2011).

In diabetic rats NGF levels were found to increase rapidly at one week after induction of diabetic state but then declined over the next few weeks(Steinbacher and Nadelhaft 1998).NGF levels were low in bladder tissue of diabetic rats compared to controls, which returned to normal after treatment for diabetes(Tong and Cheng 2005). Goins et al has demonstrated the effectiveness of NGF gene transfer to the bladder and bladder afferent pathways in an animal study and has postulated that this technique might be useful in treating diabetic cystopathy in which decreased NGF transport may be a causative factor(Goins et al. 2001).

Sohrabji F et al hypothesized that oestrogen may regulate neuronal sensitivity of neurotrophins such as NGF since oestrogen was found to upregulate NGF receptor mRNA in sensory neurons(Sohrabji et al. 1994). However this interesting finding has not been investigated widely to study the relationship between oestrogen and NGF in LUTD.

2.6 Evidence from human studies supporting the role of NGF in LUTD

The established link between altered NGF levels and LUTD in experimental studies has been investigated in clinical studies over the past few years. NGF levels have been measured in bladder tissue, urine, serum and prostatic fluid.

2.6.1 Studies done on bladder tissue

Increased bladder tissue NGF levels have been found in patients with idiopathic sensory urgency, chronic cystitis, IC(Lowe et al. 1997) idiopathic DO (Tanner et al. 2000) and Neurogenic DO(Giannantoni et al. 2006). However NGF levels were not increased in patients with urodynamic stress incontinence (USI)(Lowe et al. 1997).

Treatment with Botox in patients was found to decrease NGF levels in patients with neurogenic DO. It is postulated that Botox by reducing the release of acetylcholine and neurotransmitters might reduce the NGF production(Giannantoni et al. 2006). In contrast to the above studies Birder et al did not find an association between increased NGF levels and DO(Birder et al. 2007). The results are summarised on table 2.1.

2.6.2 Studies done in urine, serum and prostatic fluid:

2.6.2.1 Studies done in LUTD by Liu and Kuo group:

Liu and Kuo et al have conducted the majority of studies in patients with LUTD and they have proposed NGF could be used as a diagnostic biomarker as well as to assess treatment response. The studies done by Liu and Kuo et al are summarized on table 2.2 and 2.3 and elaborated below.

Urinary NGF [UrNGF] levels were found to be raised in patients with overactive bladder (OAB), DO, mixed urinary incontinence and denovo DO after continence surgery. UrNGF levels were higher in patients with OAB wet when compared to that of OAB dry. The

UrNGF levels were low in USI and persistent USI after continence surgery(Liu, Chen, et al. 2010, Liu and Kuo 2008). NGF levels were also increased in patients with IC but not in patients with increased bladder sensation (IBS)(Liu, Tyagi, et al. 2010) (Liu and Kuo 2012). UrNGF levels were significantly higher in patients with BOO and OAB. The levels were low in patients with controls, BOO/non OAB and well treated BOO. However it is difficult to draw conclusions from this study since no pre-treatment levels were done (Liu and Kuo 2008).

Patients with urethral, renal stone and urothelial cancer were among other patient groups who were found to have elevated UrNGF levels. Benign tumours and muscle invasive transitional cell carcinoma did not have high UrNGF levels. Patients with bacterial cystitis had raised UrNGF levels, which reduced significantly after antibiotic treatment (Kuo et al. 2010)

UrNGF levels were raised in patients with cerebrovascular accident and also correlated with the severity of the neurological impairment. However in this study the NGF levels did not correlate with the urinary symptoms or urodynamic findings .It was thought that the elevated NGF was due to neurological impairment rather that LUTD(Liu, Liu, et al. 2009).

2.6.2.2 Studies done in LUTD by other researchers:

Other researchers have also explored the role of NGF in LUTD and the key studies are summarised on table 2.4. Increased level of urinary NGF has been demonstrated in patients with IC (Jacobs et al. 2010, Okragly et al. 1999), sensory urgency(Yokoyama et al. 2008),OAB(Antunes-Lopes et al. 2011), Neurogenic Overactive

Bladder [NOAB](Jacobs et al. 2010), neurogenic DO due to SCI(Yokoyama et al. 2008),BOO(Yokoyama et al. 2008)and bladder cancer(Okragly et al. 1999). Contrary to other studies UrNGF levels were not increased in patients with idiopathic DO without BOO and neurogenic DO due to cerebrovascular disease(Yokoyama et al. 2008). A study done by Kim et al found an increased level of urinary NGF in patients with OAB but these levels did not correlate with urodynamics parameters(Kim et al. 2006).

In contrast to the evidence from animal studies where bacterial cystitis produced increased NGF levels, there was no difference in the UrNGF levels between UTI patients and controls(Okragly et al. 1999).

Increased NGF levels have been found in prostatic fluid of patients with chronic prostatitis /chronic pelvic pain syndrome (CP/CPPS). There was also a correlation between the increased NGF levels and symptom severity(Miller et al. 2002). The NGF levels decreased significantly in patients who responded to treatment of chronic prostatitis. Therefore the authors proposed that UrNGF levels could be used as a biomarker for the diagnosis of CP/CPPS, as well as for the assessment of successful treatment outcome of CP/CPPS(Watanabe et al. 2011).

2.6.2.3 Studies related to urine sample collection:

Few studies have explored the relationship between the UrNGF levels and sample collection. Liu et al studied the association between UrNGF levels and sensations during filling. Urinary NGF levels were very low at first sensation of filling (FSF) and were significantly

higher at urge sensation in normal controls. Patients with OAB had significantly higher urinary NGF/Cr levels both at FSF and urge sensation when compared to controls. The difference in urinary NGF/Cr levels between FSF and urge sensation in OAB patients, however, was not significant. The NGF levels were thought to increase at urge to void physiologically in asymptomatic controls but in OAB patients were said to increase pathologically at small bladder volume(Liu and Kuo 2009) . Interestingly in another study urinary NGF/Cr levels were very low when the bladder was not distended and significantly elevated with a full bladder in patients with IC/BPS (Interstitial cystitis/bladder pain syndrome)(Liu, Tyagi, et al. 2009) . UrNGF levels were also found to significantly increase after natural filling but not after artificial filling with normal saline in OAB patients(Kuo et al. 2010). Antunes-Lopes and his co workers have shown that Ur NGF levels are stable regardless of the time of collection during the day in healthy volunteers (Antunes-Lopes et al. 2013) .

2.6.2.4 Studies related to NGF levels and patient characteristics:

Liu et al did not find any significant correlation between NGF levels and body mass index. However there was a significant correlation between NGF levels and age. There was no significant difference between the menopausal state and NGF levels in OAB wet patients but in OAB dry patients the NGF levels were higher in premenopausal age group(Liu, Chen, et al. 2011) . Study done by Antunes–Lopes at al in healthy volunteers showed that there were no significant

difference in the NGF levels between men and women (Antunes-Lopes et al. 2013).

2.6.2.5 Studies done to assess treatment response:

NGF levels were found to decrease after antimuscarinic treatment in patients who responded to treatment (Antunes-Lopes et al. 2013) but not in non-responders. Persistent high urinary NGF/Creatinine level [NGF/Cr] was thought to be due to either very high NGF production or persistent inflammation in the urothelium and suburothelium. Changes in the urinary NGF levels were associated with the changes in the urgency severity scale.(Liu, Chancellor, et al. 2009)Patients who responded to treatment for IC and who had an improvement in visual analogue scale (VAS) for pain of ≥2 had significantly lower NGF/Cr levels than nonresponders who had a VAS improvement of <2 (Liu, Tyagi, et al. 2009). Patients who responded to Botox treatment had reduced NGF levels compared to baseline levels. Patients who did not respond to Botox had significantly high NGF levels. The NGF/CR levels also correlated with the urgency severity scale (Liu, Chancellor, et al. 2009). NGF levels seem to correlate with symptom severity and treatment response. However, since these studies were not placebo controlled, results should be interpreted with caution.

Serum and urinary NGF levels were found to be high in patients who were refractory to antimuscarinics and there was also a correlation between serum and urinary NGF levels. The authors postulated that systemic inflammation might exist in some of the OAB patients which contributed to raised serum NGF levels(Liu, Lin, et al. 2011). Serum

NGF levels have been shown to decrease after Botox treatment in patients with idiopathic OAB(Knippschild et al. 2012). However serum NGF levels can be affected by systemic conditions since serum NGF levels is increased in a variety of conditions including asthma, allergic conditions, keratoconjunctivitis, stress etc. It can also be raised in asymptomatic healthy individuals(Lang et al. 2003).

In studies done by Liu et al urinary NGF levels were low (0.05 or less) in up to 30% of patients with OAB dry, BOO or OAB wet. It was postulated by the authors that that the cause of OAB in these patients might have originated from pathways other than NGF(Liu, Chancellor, et al. 2009).

To summarise there is some evidence in human studies supporting the link between NGF and bladder dysfunction, however there are certain conflicting data. Liu et al (Liu, Chancellor, et al. 2009)have found an association between increased NGF levels and DO, however other studies show conflicting data(Birder et al. 2007, Kim et al. 2006, Yokoyama et al. 2008). Liu et al (Liu, Tyagi, et al. 2010)did not find an association between increased NGF levels and IBS but studies done by others did find an association between raised NGF levels and sensory urgency(Lowe et al. 1997, Yokoyama et al. 2008). Urinary NGF levels have not been observed to be elevated in women with urodynamic stress urinary incontinence (Liu et al. 2008), however a recent study in a rat model has provided interesting evidence linking increased NGF levels and rat model of SUI (Ko et al. 2011)Care should be taken to interpret the findings since the studies are designed differently with heterogonous study groups and are not adequately powered.

Abbreviations used in tables:

OAB-Overactive bladder, SUI: Stress Urinary Incontinence, MUI: Mixed urinary incontinence,

USI: urodynamic stress urinary incontinence, BDNF: Brain derived neurotrophic factor

C: Control, CP/CPPS: Chronic prostatitis/Chronic pelvic pain syndrome

IDO: Idiopathic Detrusor Overactivity, NDO: Neurogenic DO, DO: Detrusor overactivity,

BOO: Bladder outlet obstruction, IOAB: Idiopathic OAB, NOAB: Neurogenic Overactive Bladder

IC: Interstitial cystitis, IC/PBS: Interstitial cystitis/Painful bladder syndrome

IBS:Increased bladder sensation, UUI: Urgency urinary incontinence

SCI: Spinal cord injury, CVD: Cerebrovascular disease, CVA: Cerebrovascular accident

FSF: First sensation of filling, US: urge sensation, FDV: First desire to void

MCC: maximum cystometric capacity, VAS: Visual analog scale

PGE2: Prostoglandin E2, UTI: urinary tract infection

Table 2.1:NGF Studies in Bladder tissue

Author &Year	Study group [sample size]	Finding	Drawbacks
E.M Lowe 1997	IC, Sensory urgency, Chronic cystitis [4 each] SUI-controls	Increased NGF levels in all 3 groups including sensory urgency but no significant difference among groups	Small numbers and SUI-controls
R.Tanner 2000	DO: 7; controls: 11	NGF content was significantly higher in DO tissues	Small numbers; control group: cancer patients
Giannantoni 2006	NDO: 23 [13 male and 10 female] *baseline, 1 and 3 months post Botox injection*	Post Botox: significant decrease in NGF bladder tissue content	
Birder 2007	DO: 12 Controls: 15	NGF was not significantly associated with DO	NGF levels decreased with storage. 7 patients had supra pontine lesions

Table 2.2: Summary of urinary NGF studies [Liu and Kuo group]

Author/Year	Patient Groups And Sample Size	Results	Drawbacks/Interesting Points
Liu 2008	C=40; IBS=23; OAB dry =54; OAB wet =80 (UDS in 52 and DO =40) *OAB wet: 1uui/day*	NGF levels increased in OAB wet more than dry and low in controls and IBS	NGF levels were low in a certain percentage of patients with OAB IBS – no urgency, but frequency Centrifuged within 1 hour
Liu 2009	35 controls, 39 OAB *Urine samples were collected at the first sensation of bladder filling (FSF) and at urge sensation (US)*	NGF levels in normal controls were very low at FSF and were significantly higher at US. Patients with OAB had significantly higher urinary NGF levels at FSF compared to controls. The difference in urinary NGF levels between FSF and US in OAB patients, however, was not significant	Some patients with OAB did not have a high urinary NGF level
Liu 2008	DO=26; USI=17; MUI=21; USI after continence surgery=6; De novo DO =15; controls=31	NGF levels were low in the controls, pure USI and persistent USI after anti-incontinence surgery. The urinary NGF levels were significantly higher in women with USI and DO, pure DO and de novo DO	Only 17 had USI
Liu 2009 [Pre and post Botox]	38 controls.143 (IDO: 40 failed treatment; 66 untreated, 37 well treated); 100 –NDO (16 well treated; 59 untreated; 25 failed treatment)	Increased in IDO and NDO and who have not responded to treatment and un treated but decreases after Botox treatment. NGF levels were significantly higher in patients who had a higher urgency severity score	Lack of controls
Liu 2008	38 controls; BOO=153(21=no OAB; 25=OAB; 47=DO; OAB treated=60)	NGF levels are low in controls and BOO with non-OAB and treated [medical trt] OAB. High in BOO and OAB and DO	Wide standard deviation No pre-treatment levels but conclusion that treatment decreases NGF levels were made
Liu 2009	40=IC; 54=OAB; [23 –DO; 31 IBS] 27=controls	NGF levels higher in patients with DO and IC but not in IBS and controls. PGE2: no significant difference in subgroups.no correlation between PGE2 and NGF in any of the subgroups. Based on a urinary NGF/Cr threshold of 0.015,the sensitivity and specificity of diagnosing IC/BPS was 75% and 65.5%, respectively	
Liu 2009	28 controls; 122 patients IC	NGF levels high in patients with IC and decreased in patients who responded to treatment associated with improvement in VAS.	Only in 58 patients pre-treatment levels obtained
Liu 2012 [Serum and urinary NGF]*	28 controls; 30 patients IC	Urinary and serum NGF levels high in patients with IC, but no correlation between urinary and serum NGF levels	No correlation between serum NGF levels and clinical features

*Serum and urinary NGF levels measured

Table 2.3: Summary of urinary NGF studies [Liu and Kuo group] continued

Author/Year	Patient Groups And Sample Size	Diagnostic criteria	Results	Drawbacks/Interesting Points
Liu 2009	C=40 CVA= 93		NGF levels correlated well with the severity of neurological impairment, NGF levels were not correlated with age, location of CVA, multiplicity of CVA, duration of CVA, urodynamic findings or the presence of UUI.	no significant difference in NGF levels among the symptomatic or urodynamic subgroups.
Liu 2009	38-C, 70 –OAB *[Baseline, 1, 2 and 3 months after antimuscarinics and 1 month after discontinuing treatment]*	Urgency or urgency incontinence at least 1 per day were diagnosed to have OAB.	OAB -significantly higher baseline urinary NGF levels than the controls. NGF levels were significantly reduced at 3 months in responders but not in the non-responders. However, after discontinuing antimuscarinic treatment for 1 month, the NGF level was elevated in 23 responders and in five non-responders	No significant difference in NGF levels between the age groups 30% had zero NGF levels Limitation: lack of a control arm
Liu 2011	113-OAB dry; 106-OAB wet; 84 controls	OAB-dry (i.e. urgency at least once per day without urgency incontinence) or OAB-wet (i.e. at least one episode of UUI per 3days	Mean age of the controls was significantly less than OAB women. No significant correlation between NGF and age or BMI in women with OAB. However in the overall subjects NGF levels significantly correlated with age but not with BMI .no significant difference in NGF levels was found between pre-menopausal and post-menopausal women with OAB-wet NGF levels high in OAB wet and OAB dry compared to that of controls. Threshold value of urinary NGF/Cr level of 0.085 provided a sensitivity of 84.9% and specificity of 84.5% for differentiation of OAB-wet and controls .The area below the curve was 0.766 for urinary NGF/Cr, for differentiation of overall OAB patients and controls	Patients were requested to drink 1000 ml of water to create a strong desire to void. A higher NGF level was noted in pre-menopausal than postmenopausal women with OAB-dry.
Liu 2011 Serum and urine NGF*	34 [17 OAB dry; 17 OAB wet]; 31 –c *Baseline and 3 months after antimuscarinics*	Failed antimuscarinic therapy 3 episodes of urgency/urgency incontinence within 3 days in patients diagnosed as OAB.	Serum and urinary NGF levels were significantly elevated in OAB compared to the controls. Serum NGF levels significantly correlated with urinary NGF in OAB patients. The serum and urinary NGF levels remained unchanged in OAB patients after failed solifenacin therapy.	There was no significant difference of serum NGF levels between OAB-dry and OAB-wet. Patients were on tolterodine when baseline levels were taken

*Serum and urinary NGF levels measure

Table 2.4: Summary of NGF studies in LUTD [other researchers]

Author/Year	Patient Groups And Sample Size	Results	Drawbacks/Interesting Points
Okragly1999 UrNGF	4 IC 6 past h/o bladder cancer 7 UTI 7 C	Increase in Trytpase and increase in 3 neurotrophic factors [neurotrophin-3, nerve growth factor, glial cell line-derived neurotrophic factor] in IC and cancer No sig. difference in chemokine levels	No difference between C & UTI Acid treatment of urine prior to analysis
Kim 2006 UrNGF	65 OAB 20 C	NGF, PGE2, PGF2 increased in OAB compared to controls Only PGE 2 but not NGF correlated positively with FDV and MCC	DO in 35% remaining diagnosis not mentioned Centrifuged at 5000 rpm Freeze at -20^0 c No creatinine correction
Yokoyama 2008 UrNGF	13 IDO without BOO 6 IOAB without DO (sensory urgency) 16 BOO, 16 NDO 32 C	NGF levels in patients with NDO due to SCI, BOO and sensory urgency were significantly higher compared with those of controls. However, the levels of urinary NGF were not statistically significant between patients with IDO without BOO, NDO due to CVD and patients with normal cystometric findings	Controls- urodynamics prior to prolapse The levels of urinary NGF in patients with sensory urgency were significantly higher than those of controls. In contrast, the levels of urinary NGF of patients with idiopathic DO and neurogenic DO due to CVD were not significantly elevated. Urine centrifuged within 3 hours at 5000rpm Acid treatment prior to analysis Analysis: average of duplicate
Jacobs 2010 UrNGF	NOAB = 13, IOAB= 17, IC/PBS = 8, Prostate cancer = 7, Active bladder cancer = 4 H/o prostate cancer status post robot-assisted laparoscopic prostatectomy = 6; nephrolithiasis = 4 C = 13	Urinary NGF levels were significantly elevated in patients with NOAB and IC/PBS and approached significance in patients with nephrolithiasis compared to controls	Some controls had SUI Details of diagnosis of OAB dry and wet not given UDS done only in 3/4th of IOAB & NOAB NGF undetectable in 13 controls and 24 OAB. Cancer patients NGF not increased 92%of NOAB and 87% of OAB were on treatment Centrifuged at 2400 rpm
Watanabe 2010 Prostatic secretion	20 CP/CPPS 4 C *[Levels measured at baseline and 8 week after trt]*	NGF level in patients with CP/CPPS correlated directly with pain severity. Successful treatment significantly decreased NGF levels in responders	
Antunes-Lopes [2013] UrNGF	C=40 OAB=37 *[Post antimuscarinic trt].*	NGF and BDNF decreased after antimuscarinic treatment There was no circadian variation to urinary NGF/Cr levels	Reduction in number of urgency episodes per week correlated with BDNF but not with NGF.
Knippschild 2012 Serum NGF	IOAB =26 *[Before and after Botox treatment]*	NGF level decreased after Botox in IOAB patients	

2.7 Mechanisms underlying the role of NGF in the pathophysiology of LUTD

Evidence from experimental and human studies links raised NGF levels and bladder dysfunction such as BOO, IC/BPS, OAB, SUI, bladder inflammation, SCI, nerve denervation, UTI, urinary tract stone, urothelial tumour and prostatitis. Antibodies to NGF or the receptor prevents the neural plasticity associated with NGF action.

NGF has been implicated as a chemical mediator in bladder dysfunction such as BOO and inflammation resulting in bladder overactivity (Steers 2002, Steers and Tuttle 2006, Vizzard 2000). In response to BOO, inflammation and SCI the bladder smooth muscle and urothelium produce increased levels of NGF(Dupont et al. 2001, Oddiah et al. 1998, Steers et al. 1991, Vizzard 2000).

After being secreted by target organ [smooth muscle cells or urothelium], NGF is absorbed by sympathetic and small sensory fibers via a high affinity Trk-A receptor and retrogradely transported to the cell body where it exerts its action(Hendry et al. 1974, Thoenen et al. 1988). Activation of tyrosine kinase A induces a cell-signalling cascade involving MAPK pathways(Freund-Michel and Frossard 2008, Steers and Tuttle 2006). Increased NGF levels results in reduced threshold or increased excitability of bladder afferent fibres(Dmitrieva and McMahon 1996, Yoshimura and de Groat 1999) and enhanced spinal reflex(Steers and Tuttle 2006). Change in

sodium channel properties is thought to be the responsible mechanism for the alteration in conductance of afferent nerves(Steers and Tuttle 2006, Yoshimura et al. 2006). Interaction between NGF and TRPV1 has shown to be crucial for the development of bladder overactivity and chronic inflammatory pain (Frias et al.).

NGF could also play a role in altered pain signalling in inflammatory and painful conditions such as IC and prostatitis. NGF might indirectly modulate hyperalgesia by increasing the expression of sensory neuropeptides, such as substance P, CGRP and other neurotransmitters.(Schnegelsberg et al. 2010) Substance P and CGRP, which are released peripherally, and centrally following the activation of sensory afferents can initiate local neuroinflammatory responses and enhance sensory neuron excitability(Schnegelsberg et al. 2010). Consistent with these mechanisms, exogenous delivery of NGF to the detrusor is associated with increased Fos protein, substance P and CGRP expression in central micturition pathways(Zvara and Vizzard 2007). Mechanisms involved in NGF associated bladder overactivity are summarized in figure 2.2

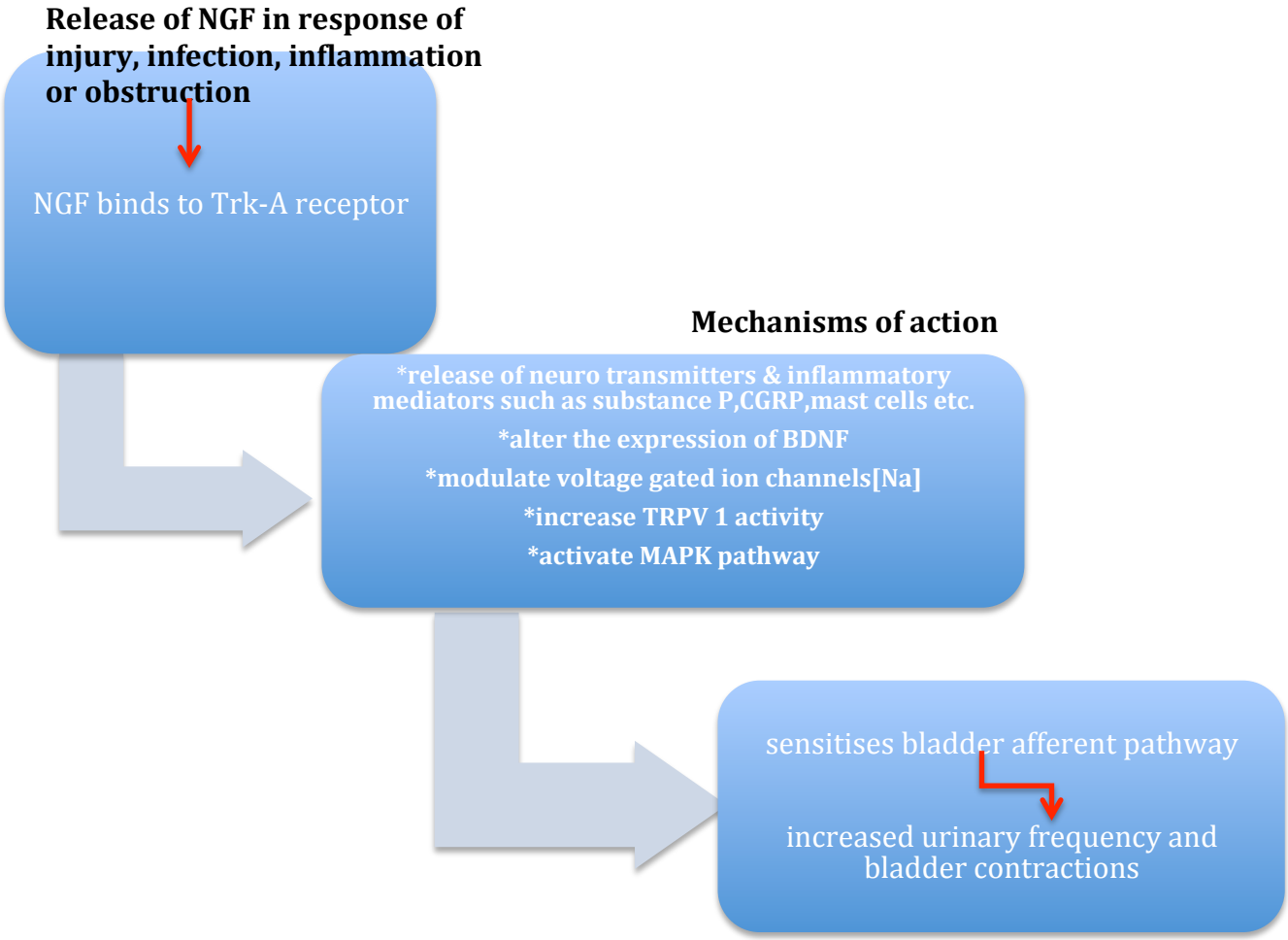


Figure 2.2: Possible mechanisms involved in NGF associated bladder overactivity

2.8 Other serum and urinary biomarkers studied in patients with LUTD

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention " by the Biomarkers and surrogate endpoint Working

Group (Naylor 2003). It can be a substance e.g. protein, enzyme, hormone or gene that is measured in blood, urine or bodily fluid or can be an imaging biomarker. It can be used for screening, diagnosis, risk stratification, measure disease severity, monitor disease progression, predict treatment response and monitor response to therapy.

Urinary NGF is the widely studied biomarker both in experimental and clinical studies. Few other urinary and serum biomarkers have been studied in LUTD but the evidence available is very sparse.

BDNF is a neurotrophin with functional properties similar to NGF. Researchers have assessed urinary levels of BDNF in OAB patients and found an increased level of BDNF compared to controls(Antunes-Lopes et al. 2011, Wang et al. 2014). Urinary BDNF was also found to be elevated in IC/BPS patients and to significantly reduce after botulinum toxin administration to the bladder trigone(Pinto et al. 2010)but not after treatment with hyaluronic acid(Jiang et al. 2014). Preliminary studies although few in number are promising. But large studies are needed to evaluate its role further as a biomarker in the assessment of patients with LUTD.

Few studies have been done on inflammatory markers such as prostaglandins, cytokines, chemokines and CRP to assess their role as biomarkers in LUTD.
A study done by Kim et al in patients with OAB demonstrated raised levels of urinary NGF, prostaglandins PGE2 and PGF2 compared to

controls(Kim et al. 2006). However a study done by Liu et al did not find urinary PGE2 to be significantly different between patients with OAB, IC and controls (Liu, Tyagi, et al. 2010). Prostaglandins E2 also did not decrease after successful treatment with anticholinergics in patients with OAB(Cho et al. 2013) .The available evidence for utilizing prostaglandin, as a biomarker is not convincing.

ATP and its relationship with LUTS has been explored and increased levels in the urine have been found in in patients with interstitial cystitis(Sun et al. 2001) and patients with OAB in an another small case control study.(Silva-Ramos et al. 2013) Elevated levels of a selective subset of chemokines and cytokines have been demonstrated in the urine of OAB patients compared to controls. (Ghoniem et al. 2011, Tyagi et al. 2010) However the studies involve a very small number of patients, therefore much larger studies are needed to explore the role of measurement of urinary ATP and cytokines as diagnostic biomarkers.

In a recent study pro-inflammatory cytokines/chemokine (IL-1β, IL-6, TNF-α, and IL-8A), CRP and NGF were found to be significantly increased in the sera of patients with IC/BPS compared to controls(Jiang et al. 2013) .Other studies have also demonstrated raised serum CRP levels in patients with OAB and IC (Chung et al. 2011, Hsiao et al. 2012). However serum CRP levels can be influenced by systemic conditions therefore the feasibility of using CRP and serum NGF as potential biomarker needs to be interpreted with caution.

Although various biomarkers such as prostaglandins, cytokines, CRP etc. have been explored for their role in the assessment of LUTD, the neurotrophins NGF and BDNF seem to have the most promising evidence. However a lot of work is needed to evaluate the role of urinary NGF for its appropriateness of use in clinical practice.

2.9 Conclusion:

NGF since its discovery in 1950's has been studied considerably in experimental and human studies. In lower urinary tract, NGF is increased in various conditions of bladder dysfunction and it seems to correlate with the severity of symptoms and also shown to decrease after successful treatment of overactive bladder. It is not clear whether NGF might be one of the causative factors in the pathophysiology of overactive bladder or whether it might be a consequence of overactive bladder. The concept of using NGF, as a biomarker of LUTD is appealing but research into converting urinary NGF measurement as a bedside tool is far from complete.

CHAPTER 3

ASSESSMENT TOOLS COMMONLY USED IN LOWER URINARY TRACT DYSFUNCTION

CHAPTER 3: ASSESSMENT TOOLS COMMONLY USED IN LOWER URINARY TRACT DYSFUNCTION [LUTD]

3.1 Assessment tools:

Lower urinary tract symptoms comprise a wide range of symptoms and various assessment tools are used in clinical practice to define the diagnosis, exclude other pathologies and to guide the correct management. The most commonly used assessment tools are discussed here**.**

3.1.1 Patient reported outcome measures:

Patient self completed questionnaires or patient reported outcome measures [PROM] (Comperat et al.2006) are used as an objective assessment of patient's subjective experience of symptoms(D.Staskin 2009) . There are several PROMs available which address different concepts such as Health related quality of life measures [HRQoL], patient satisfaction, symptom bother, impact of urgency and screening. At the international consultation on incontinence [ICI], the international continence society committee has put forward standardized grades of recommendations for the various questionnaires.

Assessment of patient symptoms by clinicians tends to be inaccurate and irreproducible, hence it is essential to use PROM in the evaluation of patients with lower urinary tract symptoms [LUTS]. PROMs are useful in guiding the clinician to manage the patient's condition since it provides information about the severity of

symptoms form a patient's perspective and its impact on health related quality of life. It helps patients to contribute to their treatment. They are very often used in clinical trials to evaluate treatment efficacy.

The disadvantages of some of the questionnaires are their lengthiness and difficulty in interpreting results such as King's health questionnaire [KHQ](Kelleher et al 1997) and overactive bladder questionnaire [OAB-Q](Coyne et al 2002). Questionnaires such as the urogenital distress inventory have limited information due to its specific content. Single reported outcome measures tend to be too focused and fail to provide an overall reflection of patient's condition. PROMs such as visual analog scales are not validated, can be very subjective and lack specificity for patients with LUTS(Khullar 2012) . In addition PROMs may not correlate well with other clinical outcome measures such as urodynamics(Albo et al. 2007). Hence care should be taken in choosing a PROM for clinical evaluation, which is appropriate to the patient group.

3.1.2 Bladder diaries:

Bladder diaries are used in clinical practice as well as in clinical trials as an objective tool to evaluate patient's lower urinary tract symptoms. They range from simple micturition time charts, Frequency-volume charts to more detailed Bladder diaries and the extent of information recorded in each format varies. The information recorded in these diaries could include time of each void, voided volumes, fluid intake, incontinence episodes, pad usage and

degree of urgency/incontinence. The bladder diaries are recorded for a minimum of one day to a maximum of 14 days.

Bladder diaries are prospective assessment tools that reduces recall error and has the advantage of assessing incontinence in the patient's own environment and under actual daily life conditions(Locher et al. 2001).

From the data recorded the clinician can obtain information about the patient's diurnal and nocturnal frequency, functional bladder capacity, severity of urgency/leakage, diagnose fluid restriction, nocturnal polyuria and verify patient's symptoms. Bladder diaries can also be used to monitor effects of treatment and are commonly used in clinical trials to assess treatment efficacy.

A recent review of the published literature on bladder diaries confirmed that there was limited evidence regarding the validation of dairy content and format (Bright et al. 2011).However in a recent cross sectional prospective study the 3 day bladder diary showed good feasibility, reliability and moderate validity to be used in the assessment of LUTS in women.(Jimenez-Cidre et al. 2013). However there was low agreement between the urodynamic variables and in addition bladder diary was found to under diagnose stress urinary incontinence.

Bladder diaries can reflect patient's symptoms and severity but tend to have poor discriminatory value in differentiating the type of incontinence. It also has the potential effect of changing the monitored behaviour(Locher et al. 2001) .One of the other main problems with bladder diaries is patient compliance (Khullar 2012) especially with diaries of longer duration.

3.1.3 Urodynamic assessment:

Conventional urodynamics is an objective test, which involves artificial filling of bladder and simultaneous recording of abdominal and intravesical pressure that helps in studying the detrusor function. The test commonly includes free flow study, dual channel filling cystometry and pressure flow study.

Urodynamics is the only objective test that aims to reproduce patients' symptoms as well as determining the cause for their LUTS. It can help in predicting the outcome of planned treatment or surgery and also in understanding the reasons for previous failed continence surgery(G.Hosker 2009) .The evaluation helps the clinician to counsel patients appropriately and manage patient's expectations before any invasive treatment. It can also help the clinician to predict consequences of LUT dysfunction on the upper renal tract as a result of high detrusor pressures(G.Hosker 2009).

However urodynamics is an invasive test with a small risk of infection(Foon et al. 2012).

There is no consensus among experts regarding the indications for urodynamics. Urodynamics has not shown to be cost effective in patients with pure stress urinary incontinence (Weber and Walters 2000).The NICE recommendations are to perform urodynamics prior to surgery only in patients with mixed urinary incontinence or voiding difficulties or anterior compartment prolapse or when previous surgery has failed(NICE 2013). A recent systemic review found that valsalva leak point pressures during urodynamics may

help in predicting the outcome of midurethral sling surgery (Kawasaki et al 2012]. However a multicentre trial [VALUE] did not find urodynamics prior to SUI surgery in patients with predominant SUI symptoms to correlate with treatment success.(Zimmern et al.2014)

A number of studies have reported up to 15% test retest variation as well as clinically relevant inter-rater/observer variation(G.Hosker 2009) so it is recommended that clinicians take this into account during their interpretation. Not all patients with OAB symptoms have DO on urodynamics and not all patients with DO have OAB symptoms(Digesu et al. 2003, Hashim and Abrams 2006). Moreover up to 30 % of asymptomatic patients can have DO on urodynamics (Hashim and Abrams 2006), which raises concerns not only on the reliability of subjective reporting of symptoms but also the credibility of urodynamics.

3.2 The rationale for use of a urinary biomarker in LUTD

Overactive bladder is characterized by urinary urgency with or without urgency urinary incontinence usually with urinary frequency and nocturia. (Haylen B.T et al 2010). Urgency is the key symptom and the degree of urgency is measured subjectively using an urgency severity scale(Abrams 2005, Nixon et al. 2005). However patients might find it difficult to understand and differentiate urgency and urge to void as well as difficult to grade the urgency. Patient reported symptoms that are commonly used in clinical practice for diagnosis and to evaluate efficacy of treatment can be inconsistent and biased due to their subjective nature. In addition there is considerable

overlapping of symptoms in various lower urinary tract disorders such as overactive bladder, IC/BPS and BOO which makes the correct diagnosis difficult.

Patient reported outcome measures as mentioned above have their shortcomings in spite of being used widely as objective assessment tools.

Urodynamics is considered to be the best available objective test in evaluating LUTD, however there are a few studies that raise concerns about its reliability and to add it is an expensive and invasive test.

Therefore the concept of developing a urinary biomarker, which is easily accessible, affordable, noninvasive, reliable and superior or comparable to the current diagnostic tools in LUTD, is appealing.

3.3 NGF as a potential urinary biomarker

NGF measurements have been done on bladder biopsies as well as urine samples in experimental and human studies. It is postulated that NGF in urothelium and detrusor muscle is secreted into urine. Urine collection is easily accessible compared to bladder biopsies. NGF measurements in urine are simple compared to NGF measurements in bladder biopsies, which are very complex.

Urinary NGF levels are quantified using enzyme linked immunosorbent assay (ELISA), which is non-invasive and more affordable when compared to UDS. Measurement of urinary NGF levels can be affected by various factors since there is room for error in urine sample handling, sample processing, centrifugation steps and ELISA analysis. The test retest variability of ELISA analysis has not been reported yet.

The role of urinary NGF as a diagnostic biomarker needs further exploration since the reports regarding sensitivity and specificity of NGF assays seem to be varied. Using a cut off value of more than 0.05pg/ml, the sensitivity of a urinary NGF/Cr level in the diagnosis of overactive bladder was 67.9% and the specificity was 93.8%(Liu and Kuo 2008). However a study done by Antunes-Lopes et al reported lower values of sensitivity and specificity for NGF/creatinine ratio [>200pg/mg] with an area under the curve in receiver-operator characteristics (ROC) analysis of only 0.68(Antunes-Lopes et al. 2011). Diagnostic accuracy of a test is usually assessed by comparing with a reference standard and in LUTD urodynamics is the best available objective test. The study done so far to compare NGF levels and various urodynamic diagnoses was of a very small sample size(Liu and Kuo 2008).

In all the studies done so far there are no uniform inclusion criteria e.g. two different classifications have been used to classify patients as OAB wet by the same group of researchers(Liu, Chen, et al. 2011, Liu and Kuo 2008). Patients used as controls can range from asymptomatic patients to patients with USI or bladder cancer (Lowe et al. 1997), which could bias the results since some studies have shown an association between increased NGF levels and SUI(Furuta et al. 2008, Ko et al. 2011). There are also some inconsistent data regarding NGF levels in DO, SUI and IBS in the available evidence as stated earlier in chapter 2

In vitro experimental models have demonstrated that repetitive stretch stimulation of bladder smooth muscle cells which mimic

bladder outlet obstruction result in increased NGF levels(Persson et al. 1995). Bladder outlet obstruction in men is associated with increased NGF levels(Liu and Kuo 2008).
Bladder outlet obstruction is uncommon in women however anterior wall prolapse /cystocele is common and the relationship between anterior vaginal wall prolapse and NGF levels have not been studied so far. It is possible that anterior vaginal wall stretching in prolapse results in raised NGF levels and hence results in overactive bladder symptoms. If this is true then this has an implication of measuring UrNGF levels in women with anterior vaginal wall prolapse.

The available evidence regarding the role of Ur NGF, as a biomarker in LUTD is inconsistent and sparse, therefore based on the available and the absence of evidence the following hypotheses were formulated on which the research studies were based on.

Hypothesis 1:

NGF in urine sample is quantified using Enzyme linked immunosorbent assay [ELISA] .The test-retest reliability of currently used urinary NGF ELISA assays has not yet been reported yet. Therefore the hypothesis for the first study was as follows:

Enzyme linked immunosorbent assay is not a reliable technique to measure NGF levels in urine

Hypothesis 2:

The validity of urinary NGF as a diagnostic biomarker in women with LUTD is still unclear due to conflicting reports in the literature. The following hypothesis was made for the second project and urinary NGF levels were compared with various LUTD and urodynamic diagnoses to evaluate the validity of urinary NGF measurement:

Urinary NGF level measurement is not a valid test in assessment of women with LUTD with poor sensitivity, specificity, positive and negative predictive values and has no discriminatory ability to differentiate the different symptomatic and urodynamic groups

Hypothesis 3:

Nerve growth factor (NGF) is one of the neurotrophic factors, which is considered as a key regulator of neurogenic inflammation in several tissues including the bladder. Increased urinary NGF levels in OAB might be associated with severity of bladder inflammation and may be used as a non-invasive biomarker to predict the inflammatory status of bladder if it correlates with cystoscopy and histology findings. This speculation led to the hypothesis for third study:

Urinary NGF levels are not associated with cystoscopic and histology findings of inflammation in women with refractory OAB and cannot be used as a non-invasive biomarker to predict the inflammatory status of bladder.

Hypothesis 4:

Women with anterior vaginal wall prolapse frequently suffer with overactive bladder symptoms. Overactive bladder symptoms have been shown to improve significantly after anterior colporrhaphy. If increased NGF levels are contributing to the overactive bladder symptoms then NGF levels should decrease after anterior repair in women who have had an improvement in their overactive bladder symptoms. On this postulation the hypothesis was made for the fourth study as follows:

NGF levels do not change in women who have had improvement in their overactive bladder symptoms after anterior colporrhaphy.

Hypothesis 5:

NGF has been suggested as a biomarker to assess treatment response for overactive bladder and few studies have demonstrated to this effect. There is emerging evidence linking infection to overactive bladder and use of antibiotics to treat symptoms of refractory overactive bladder. The role of urinary NGF to assess response to antibiotic therapy in patients with refractory OAB was explored in the final study and the hypothesis was as follows:

Urinary NGF level measurement does not change in response to antibiotic therapy for refractory OAB and cannot be used to assess treatment response.

CHAPTER 4

NGF ASSAY AND RELIABILITY OF NGF ASSAY

Hypothesis 1: Enzyme linked immunosorbent assay is not a reliable technique to measure NGF levels in urine

CHAPTER 4: NGF ASSAY AND RELIABILITY OF NGF ASSAY

Investigational studies performed so far to quantify NGF in urine samples have used sandwich ELISA kits. The measurement of urinary NGF concentration in this project was performed by Enzyme linked immunosorbent assay [ELISA] using the NGF Emax® ImmunoAssay System (Promega, Madison, WI, USA). This immune assay system is designed for sensitive and specific detection of NGF in tissue culture supernatants or tissue extracts in an antibody sandwich format.

The steps involved in the NGF assay including the sample collection, storage and the ELISA technique are detailed in this chapter with an assessment of the reliability and discussion of this technique.

4.1 Technique for analysis of NGF levels in urine

4.1.1 Sample collection and handling:

There have been few studies pertaining to the collection of urine samples and NGF levels. Urinary NGF levels were found to be very low at first sensation of filling (FSF) and were significantly higher at the sensation of urge in normal controls in a study conducted by Liu and co workers. Patients with OAB had significantly higher urinary NGF/Cr levels both at FSF and urge sensation when compared to controls. The difference in urinary NGF/Cr levels between FSF and urge sensation in OAB patients, however, was not significant. The NGF levels were thought to increase at urge to void physiologically in asymptomatic controls but in OAB patients were said to increase

pathologically at small bladder volume(Liu and Kuo 2009) . Interestingly in another study urinary NGF/Cr levels were very low when the bladder was not distended and significantly elevated with a full bladder in patients with IC/BPS (Interstitial cystitis/bladder pain syndrome)(Liu et al. 2009) . But in most of the studies urine samples have been collected from participants when they reported a comfortably full bladder(Antunes-Lopes et al. 2013, Liu et al. 2010). NGF levels were also found to significantly increase after natural filling but not after artificial filling with normal saline in OAB patients(Kuo et al. 2010). Study done by Antunes–Lopes et al in healthy volunteers showed there was no significant difference in the NGF levels irrespective of time of the day of urine sampling(Antunes-Lopes et al. 2013).

Therefore in this project urine was collected from participants prior to urodynamics or cystoscopy or during their clinic appointment when they had a comfortably full bladder. Participants were requested to provide a midstream sample of urine. A urine dipstick analysis was done to rule out urine infection prior to further urine sample handling.

All participants were given an information sheet about the study and a written consent was obtained. Women were recruited following ethical approval (REC reference number:11/LO/1029) into this study from October 2011 to March 2012.

4.1.2 Centrifugation:

Collected urine samples [about 1.5-2 ml] were immediately refrigerated or placed in ice and centrifuged at 3000rpm at 4 ^{0}C for

10 minutes within 20 minutes of collection [Figure 4.1]. Urine was centrifuged immediately since the room temperature and exposure to air can induce chemical reactions in the urine and protein can become unstable at room temperature.

Figure 4.1: Centrifuge

4.1.3 Storage:

The centrifuged supernatant urine was pipetted and stored at -80^{0}C in ependorfs until further processing.

4.1.4 Analysis:

In this study the NGF assays was performed in batches between 3-4 months after collection of the urine samples. Protein degradation has been said to take place with prolonged storage. A small study done by Birder et al showed that NGF concentration in bladder tissue had a trend to decrease after prolonged storage(Birder et al. 2007) .Assays were repeated at storage intervals of 13,22 and 58 days. The storage

time was speculated to have influenced the variability of NGF results however it didn't seem to affect the conclusions drawn from the study.

The urine samples were allowed to defrost naturally and then urinary NGF levels were measured by enzyme linked immunosorbent assay (ELISA) using the NGF Emax Immunoassay System (Promega, Madison, WI, USA). A standardized protocol was followed as advised by the manufacturers on the technical bulletin of NGF Emax Immunoassay System, Promega. The system uses an antibody sandwich format for detection of free NGF in the range of 3.9-250pg/ml. The manufactures quote less than 3% cross reactivity with other structurally related growth factors Human Recombinant Brain Derived Neurotrophic Factor, Neurotrophin-3 (rhNT-3) and Neurotrophin-4 (rhNT-4).

The ELISA analysis [Figure 4.2] is detailed in the following steps:

4.1.4.1 Step 1:

10**µ**l Anti-NGF polyclonal antibody (pAb) was mixed with 10ml carbonate coating buffer (pH 9.7). This anti- NGF pAb was coated onto a 96 well micro titre plate [100µl/well] and incubated overnight at 4 ^{0}C. This will prevent other proteins in the sample adsorbed to the ELISA plate surface. Each ELISA plate has eight rows and 12 columns [Figure 3]

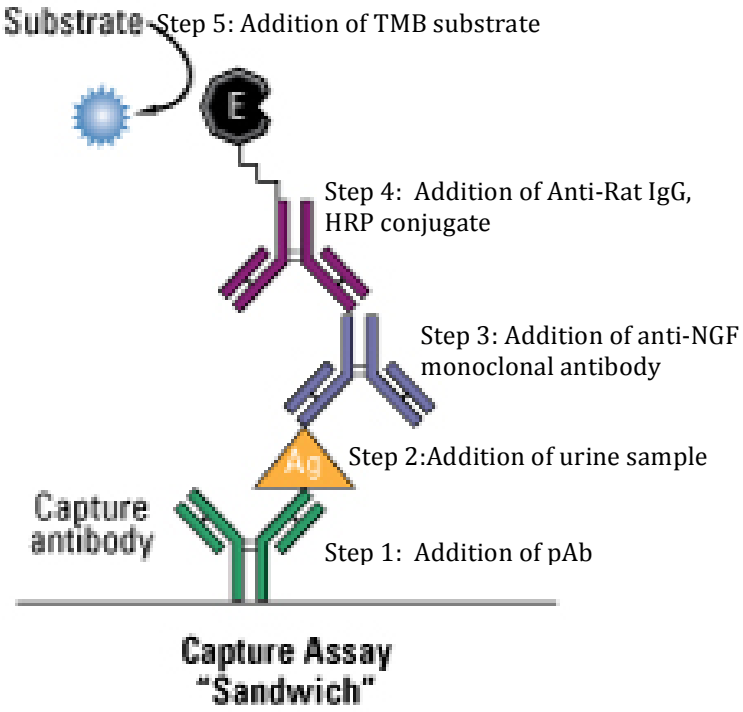


Figure 4.2:Elisa Analysis

4.1.4.2 Step 2:

a] Block & Sample Buffer was prepared to the correct dilution by mixing with deionized water. The plate was washed and then 200**µ**l was added to each well and incubated for 1 hour at room temperature. This was done to block non-specific binding.

b] The plates were washed once and 100 **µ**l urine samples were added to the wells, except for the last two columns [Figure 4.3] .The soluble NGF in urine sample is immobilized on the solid surface of the wells with the anti- NGF pAb.

The NGF Standard was diluted with the NGF Block & Sample Buffer to produce the recommended dilution. Last two columns are designated to prepare the standard curve and provide a reference

standard.200µl of diluted NGF standard was added to row A, columns 11 and 12 of a 96-well plate and six 1:2 serial dilutions [100 **µ**l each well] were performed .In the last row NGF standard is not added. This will produce a final concentration ranging from 0-250pg/ml of NGF in the last two columns, which will act as reference standard.

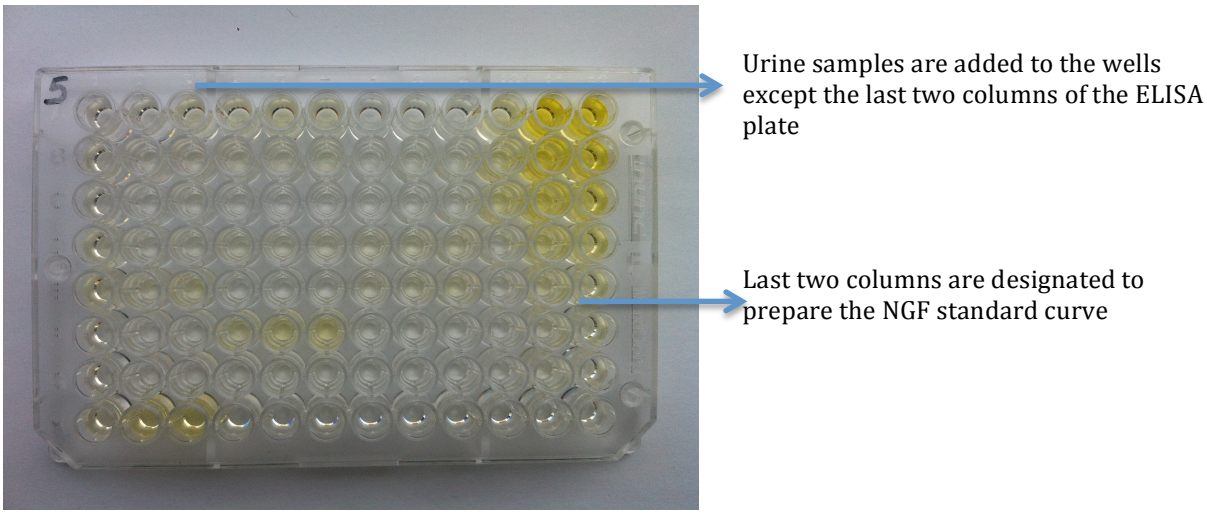


Figure 4. 3: ELISA plate with NGF standard in the last two columns

The plates were incubated with shaking for 6 hours at room temperature and then washed 5 times. [Figure4. 4]

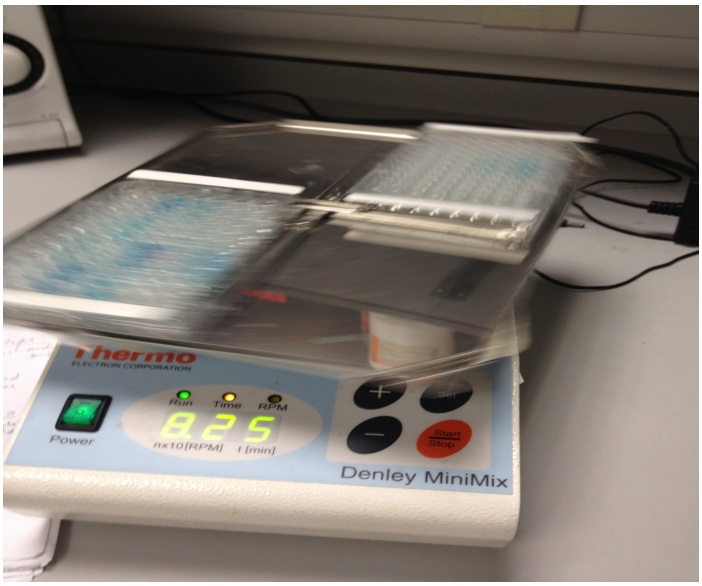


Figure 4.4: Plate shaker with ELISA plates

4.1.4.3 Step 3:

Anti- NGF monoclonal antibody was mixed with NGF Block & Sample Buffer to produce the recommended dilution and 100µl/well was added which binds to the captured NGF, and incubated overnight again at 4°C.

4.1.4.4 Step 4:

The following day the plates were washed 5 times. Anti-Rat IgG, horseradish peroxidase [HRP] conjugate was mixed with NGF Block & Sample Buffer to produce the recommended dilution and 100µl was added to each well to detect the amount of specifically bound monoclonal antibody.
The plates were incubated with shaking for 2.5 hours at room temperature; and then washed 5 times to remove the unbound conjugate.

4.1.4.5 Step 5:

And the final step is addition of 100µ of [tetramethylbenzidine] TMB at room temperature to each well. TMB is a chromogenic substrate, which changes the colour of the solution to blue in proportion to the amount of bound beta NGF in the sample [Figure 4. 5] .The plate is incubated with shaking for 10 minutes and the colour reaction is stopped with 100µl/well 1N hydrochloric acid and this changes the blue to yellow colour [Figure 4.6].

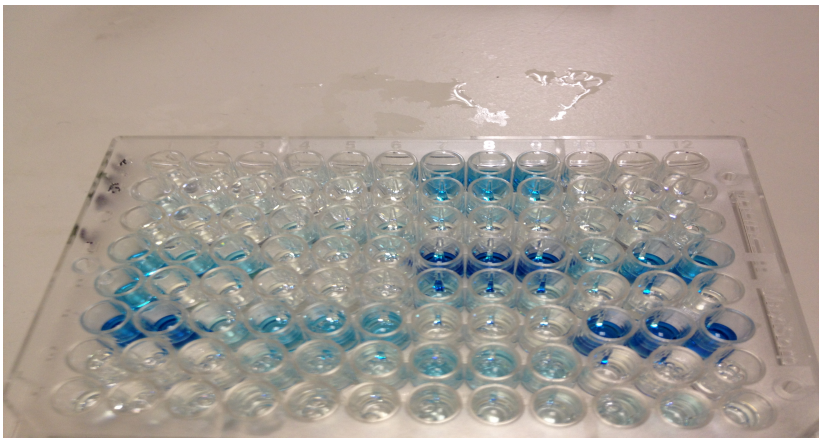


Figure 4.5: Addition of TMB changes the sample to blue colour in proportion to amount of bound NGF

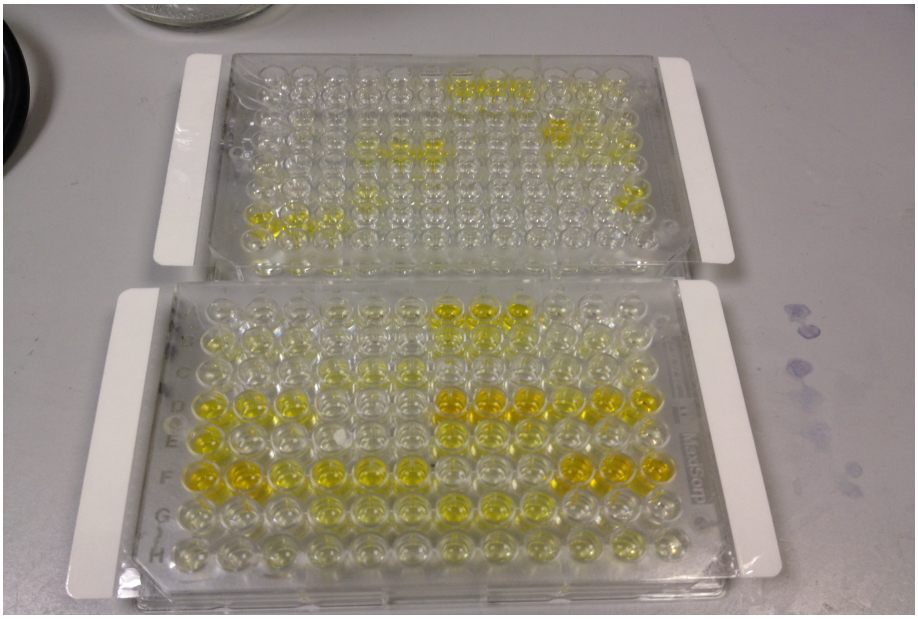


Figure 4. 6: Addition of hydrochloric acid stops the colour reaction and changes the blue to yellow colour

4.1.5 Quantification of NGF:

Intensity of colour absorbance was measured at 450nm using a spectrophotometer, OptiMax micro plate reader (Molecular Devices, Sunnyvale, CA, USA) within 30 minutes of stopping the colour reaction [Figure 4.7]. The spectrophotometer is employed to measure the amount of light the sample absorbs at a particular

wavelength, which is proportional to the concentration of the substance.

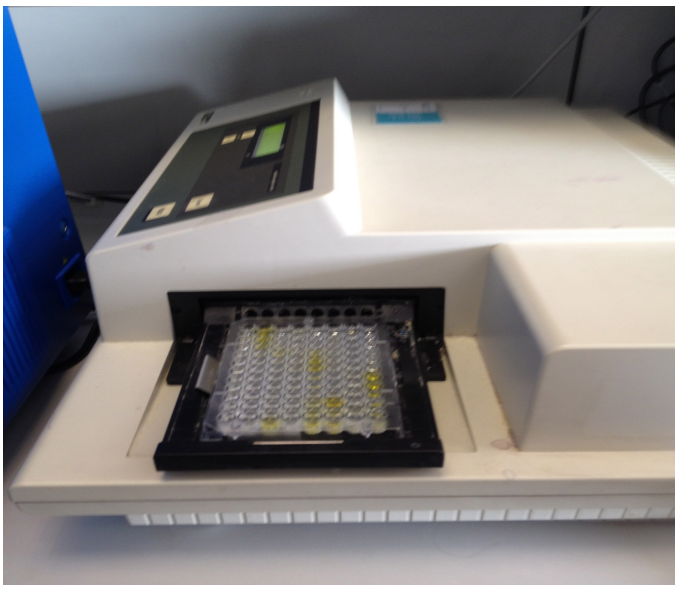


Figure 4.7: Spectrophotometer, which measures the optical density of the samples

A linear NGF standard curve [figure 4.8] was created from the known concentrations of the NGF solutions produced by serial dilution and their corresponding optical density.

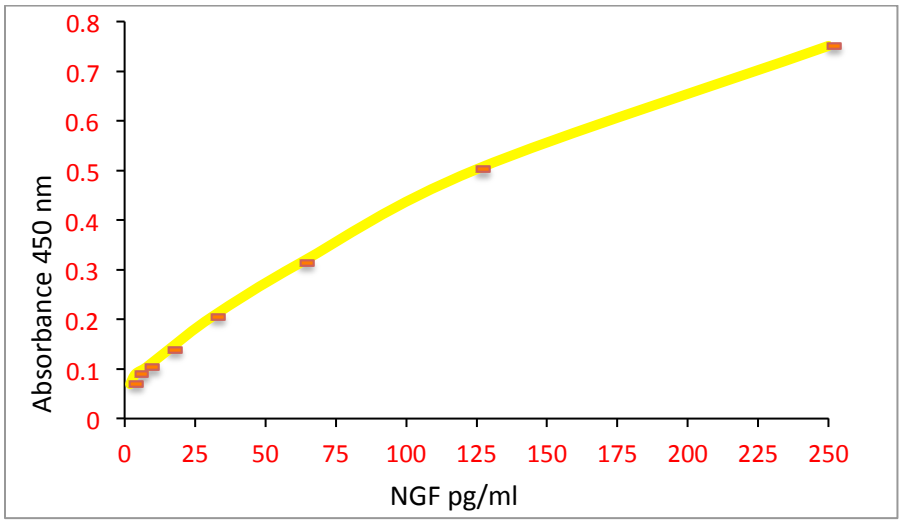


Figure 4.8:NGF standard curve

The standard curve demonstrates a direct relationship between Optical Density (OD) and NGF concentration

The concentration of NGF in the urine samples was extrapolated

from the NGF standard curve. This was done by using curve fitting analysis. In SPSS, curve fitting analysis was used to check different regressions of the average optical density of the last two columns against the different known NGF standard concentrations. Cubic model provided the best fit (r^2=1) and using cubic equation the NGF concentration in the urine samples was calculated using their optical densities. Values below zero were transformed to 0.1

For each urine sample assays were done in triplicates and an average was taken as done by other researchers(Kuo et al. 2010).

4.2 Creatinine correction:

An aliquot of whole urine was also used to measure urine creatinine. Urine creatinine was quantified in the lab by modified Jaffe method, which is a colorimetric method used for measurement of urine creatinine. The total urinary NGF levels were further normalized to the concentration of urinary creatinine (NGF/Cr) to take into account the variation in urine osmolality. Urinary creatinine is usually used to correct for variations in concentration for proteomic studies e.g. protein creatinine ratio for diagnosis of preeclampsia(Tyagi et al. 2010).

4.3 Units of measurement:

NGF/Cr concentrations were multiplied by factor of 10^9 and the values were expressed in ng/g units. In the studies reported by Liu and co workers NGF/Cr concentration is expressed in pg/ml.

4.4 General considerations regarding storage and analysis:

A standardised protocol was followed as suggested by the manufacturers. The materials used for NGF quantification such as ELISA plates, plate shaker, plate reader etc. were as per recommendations.

Improper storage of reagents can make the solutions unstable. The reagents in the assay were stored at -20 ^{0} C until analysis. Once thawed the reagents were stored at 4^{0} C and were used within three months. The diluted reagents were used only on the day of dilution. The reagents were kept on ice while carrying out the experiments. The urine sample was defrosted thoroughly in natural conditions before use.

Care was taken while transferring the samples, antibodies and reagents to the wells in the ELISA plate with a pipette since pipettes can scratch the surface of the wells and dislodge the coated antibodies. Extreme care was taken to accurately dilute the reagents and maintain pH of coating buffer as per manufacturers instructions as well as while adding the reagents and samples to the ELISA plate by using calibrated pipettes. The pipette tips were changed after using each reagent.

An automatic plate washer was used to wash the plates as necessary. The plates were sealed with plate sealer to avoid exposure to air during incubation and washing. A timer was used to maintain the specific time advised by technical bulletin for incubation, washing plates, shaking plates and for colour development.

4.5 Cost issues:

The cost for one ELISA plate is £105and 26 samples can be analysed in one plate. In addition to the cost of the ELISA kit, the cost of the consumables and lab facilities has to be into account.

4.6 Pitfalls in NGF ELISA analysis:

Enzyme linked immunosorbent assay [ELISA] is a laborious -3 day process and needs to be done in batches with no rapid turn over. The procedure needs technical expertise. Urine creatinine need to be measured to correct for urine concentration.

There is room for error in ELISA analysis at every single step such as urine sample handling, sample processing, centrifugation steps and ELISA analysis, hence manufacturer's instructions needs to be strictly adhered to. Obtaining a perfect linear NGF standard curve is important since this will invariably affect the quantification of NGF level in the urine samples.

4.7 TEST RETEST RELIABILITY

4.7.1 Introduction:

The test-retest reliability of currently available urinary NGF assays by ELISA has not yet been reported in human studies. Reliability studies are important because they provide information about the amount of error in-built in any measurement, which influences the validity of the measurements(Kottner et al. 2011). Assessment

outcomes cannot be meaningfully reproduced or interpreted if the tests are unreliable. An ideal test has to be reproducible over time as well have satisfactory inter and intraobserver reliability.

The aim of this study was to assess the intraobserver reliability of urinary nerve growth factor (NGF) measurement by enzyme-linked immunosorbent assay (ELISA) analysis.

4.7.2 Method:

The urine samples of 13 symptomatic patients who reported lower urinary tract symptoms were chosen randomly. NGF assays were performed by ELISA using the NGF Emax® ImmunoAssay System (Promega, Madison, WI, USA) and the NGF assays were repeated four weeks later. The assays were performed using the manufacturer's technical bulletin as described previously in this chapter.

The same two researchers conducted the test and the retest analysis. The researchers were blinded to patient's symptoms. The two researchers performing the NGF assays obtained training by expert lab technicians and research associates regarding every step involved in the NGF assays. The data were not normally distributed using a one-sample Kolmogorov-Smirnov test with a p value of 0.997;therefore non-parametric tests were used.

Median values of the two measurements were compared using Wilcoxon signed Rank test. Spearman correlation was run to assess the correlation between the two measurements.

Test retest analysis of NGF measurements was conducted using Intraclass Correlation Coefficient [ICC] to assess the consistency of measurements on repeating it on two occasions .The Bland–Altman

plot (difference vs. mean plot) was used to show the degree of agreement between the measurements. In this plot the differences between the two measurements are plotted against the averages of the two measurements. Horizontal lines are drawn at the mean difference and at the limits of agreement, which are defined as the mean difference ±1.96 times the standard deviation of the differences. This range of values defines the 95 % limits of agreement, which indicates an interval within which 95 % of differences between measurements by the two methods are expected to lie(Bland and Altman 1999, Bland and Altman 1986). Analyses were carried out using SPSS version 20, Chicago, IL, USA.

4.7.3 Results:

There was no significant difference between the median values of the two measurements; *p* =0.116[Wilcoxon signed Rank test] .The median values and the interquartile ranges are summarised on table 4.1.

Table 4.1: Median values and results of
Values are expressed in pg/ml and standard deviation is shown in brackets

	Median value 1 [Interquartile range]	Median value 2 [Interquartile range]	Z value	*P*
NGF pg/ml	10.66[4.32-22.37]	6.96[2.86-18.64]	-1.572	0.116

There was a strong positive correlation between the two measurements rho=0.879; p<0.001(Spearman correlation test). On test retest reliability analysis of 13 samples there was almost perfect reliability with an Intraclass correlation coefficient of 0.889; 95% C.I=0.676-0.965;p<0.001. On Bland Altman plot, all the values were within limits of agreement, see figure 4.9.

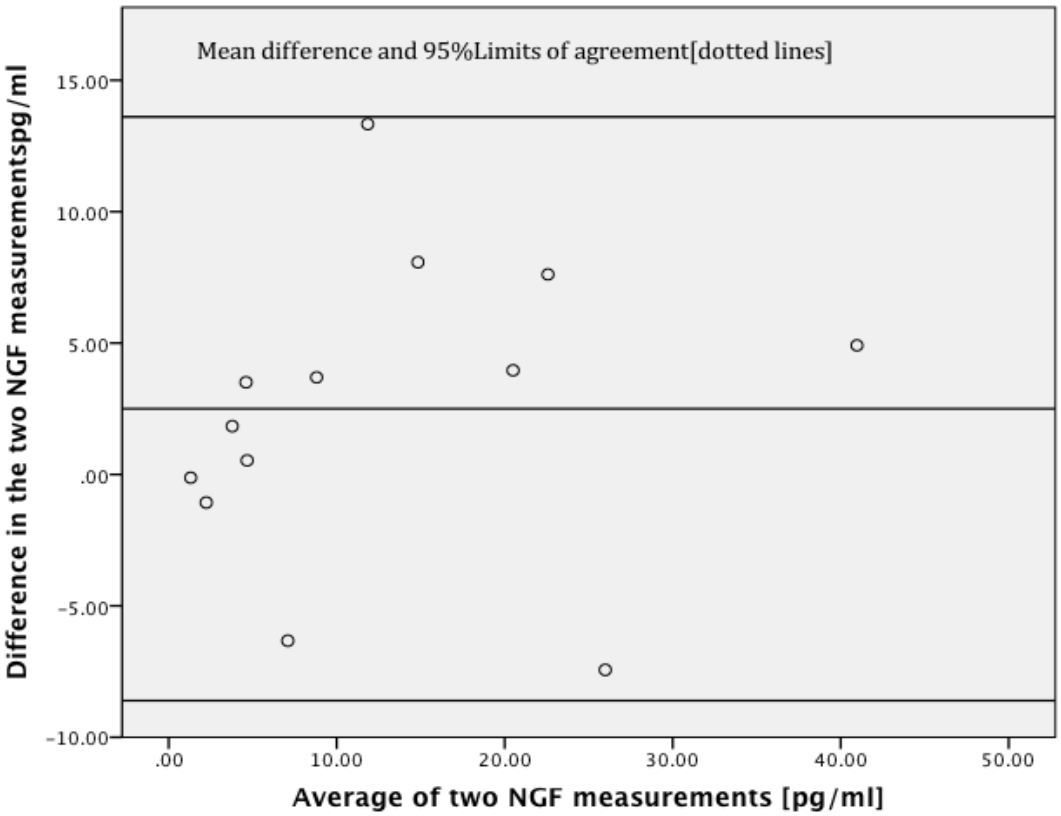


Figure 4.9: Bland Altman plot with 95%limits of agreement.

Mean NGF difference=2.5016[±5.668]

Limits of agreement: mean difference ±1.96 times the standard deviation of the differences

4.7.4 Discussion:

This study is the first to report test retest intra –observer reliability of urinary NGF assays, which offers evidence that it is a reliable test with an ICC of 0.889. Test-retest assessments were done within a short time interval of four weeks and these measurements represent the reproducibility only within this particular time interval. Measurements can be affected by prolonged intervals due to uncontrolled variables such as degeneration of protein. Further studies with large numbers need to be done at various time intervals to account for variations in the assays. In this study only intra observer reliability has been assessed and it is important to also test for interobserver reliability in future studies.

CHAPTER 5

VALIDITY OF URINARY NGF MEASUREMENT IN WOMEN WITH LOWER URINARY TRACT SYMPTOMS

Hypothesis: Urinary NGF level measurement is not a valid test in assessment of women with LUTD with poor sensitivity, specificity, positive and negative predictive values and has no discriminatory ability to differentiate the different symptomatic and urodynamic groups

CHAPTER 5: VALIDITY OF URINARY NGF MEASUREMENT IN WOMEN WITH LOWER URINARY TRACT SYMPTOMS

5.1 Introduction:

Over recent years there has been a growing interest in developing a biomarker, which could be an objective measure of patient reported symptoms in diagnosing and assessing treatment outcome for lower urinary tract symptoms [LUTS]. Current diagnostic strategies employ various tools including self-completed symptom scales, quality of life questionnaires (D.Staskin 2009, Khullar 2012, Shy and Fletcher 2013), bladder diaries (Al Afraa et al. 2012), pelvic floor ultrasound (Derpapas et al. 2011), and urodynamics (Massey and Abrams 1985). Although there is good evidence of validity for patient reported measures (D.Staskin 2009) there remains concern that patients may not themselves easily distinguish between symptoms (Digesu et al. 2008), and that such scales may not provide adequate discrimination between pathophysiological complexes with overlapping symptoms such as overactive bladder (OAB), bladder pain syndrome (BPS) and dysfunctional voiding.

Urodynamics is commonly used for objective diagnosis, but despite decades of development, there remain concerns about both the reliability of urodynamic diagnoses (Renganathan et al. 2009) and the frequent mismatch between symptoms and cystometry (Digesu et al. 2003, Hashim and Abrams 2006). Urodynamics is also an expensive and moderately invasive test; therefore it is reasonable to develop an affordable, and non-invasive diagnostic tool, which can be

used in the diagnostic work up of patients with lower urinary tract dysfunction [LUTD]. A urinary biomarker that effectively discriminated between different types of bladder dysfunction would be valuable in directing management.

Measurement of urinary NGF concentrations has potential both as a diagnostic biomarker for LUTD, as well as for the assessment of therapeutic outcome in patients with OAB (Liu et al. 2008, Liu, Chen, et al. 2010). However, available studies report conflicting data regarding an association between LUTS and urinary NGF, and there is limited evidence for the validity, reliability and feasibility of urinary NGF assays in this population. Although increased urinary NGF concentrations have been reported in women with detrusor overactivity [DO] other researchers have not found the same association(Birder et al. 2007, Kim et al. 2006, Yokoyama et al. 2008). Conflicting reports have found either no association between NGF concentrations and increased bladder sensation (Liu, Tyagi, et al. 2010), or highly elevated NGF in women with sensory urgency(Lowe et al. 1997, Yokoyama et al. 2008). Urinary NGF levels have not been observed to be elevated in women with urodynamic stress urinary incontinence (Liu et al. 2008), however a recent study in a rat model has provided interesting evidence linking increased NGF levels and rat model of SUI (Ko et al. 2011). The validity of urinary NGF as a diagnostic biomarker in women with LUTD is still unclear.

The aim of this study was to compare urinary NGF concentration with different LUTD and urodynamic diagnoses and to evaluate the

validity of urinary NGF measurement in patients with LUTD and healthy volunteers. Although urodynamics has its shortcomings it is the best objective test available for evaluating LUTD, therefore it was used as a reference standard to compare the validity of urinary NGF measurements.

5.2 Materials and methods:

Women were recruited from a tertiary urogynaecology referral centre following ethical approval (REC reference number: 11/LO/1029) into this case control study from October 2011 to March 2012.

5.2.1 Inclusion and exclusion criteria:

Women above the age of 18 who were undergoing urodynamics to investigate their LUTS were included in the study. Women were excluded if they had a history of neurological dysfunction, bladder cancer, bladder or renal calculi, previous continence surgery, current use of antimuscarinics at the time of recruitment, presence of voiding dysfunction, vaginal prolapse [≥ Stage II on POPQ] or UTI. Asymptomatic volunteers and patients from general gynaecology clinics who did not have any LUTS on their KHQ served as controls.

5.2.2 Assessment:

All eligible symptomatic women completed a three-day frequency-volume chart, a King's Health Questionnaire (KHQ)(Kelleher et al. 1997), and underwent urodynamics in accordance to ICS guidelines.

5.2.3 Urodynamic assessment:

Dual channel cystometry was performed with each woman supine and the bladder filled through a 10Ffilling catheter; a fluid-filled 4.5F catheter was used to measure the intravesical and abdominal pressures. The bladder was filled with room-temperature saline at 100 mL/min. The filling catheter was removed when the patient developed a strong desire to void or 500 mL had been infused into the bladder. Provocative manoeuvres were used with each woman in a standing position. Women were asked to cough 1, 3, and 5 times with maximal effort and then listen to running water and wash their hands in cold water. Finally, they were seated for a pressure-flow study that was performed in private. Urodynamics was carried out by certified urodynamicists.

5.2.4 NGF assays:

A midstream specimen of urine was collected from symptomatic patients after obtaining their consent, prior to urodynamics and immediately centrifuged at 3000 rpm at 4 ^{0} C for 10 minutes. An aliquot of whole urine was sent to the lab for measurement of urine creatinine. The centrifuged supernatant fluid was then frozen at -80^{0} C. Once defrosted the measurement of urinary NGF concentration was performed by ELISA using the NGF Emax® ImmunoAssay System (Promega, Madison, WI, USA) as described previously in chapter 4.

The total urinary NGF concentration was further normalized to the concentration of urinary creatinine (NGF/Cr). Urine samples from asymptomatic volunteers were also processed to measure the NGF

concentration. The NGF assays were done between 3-4 months after collection of the urine samples. The researchers conducting the NGF assays were blinded to the patient symptoms and urodynamic diagnoses.

5.2.5 Group stratification in symptomatic patients:

Women were classified into symptomatic groups based on patient reported symptoms on the KHQ, bladder diary and confirmed by self-reported symptoms. Some patients were assigned to more than one group due to overlapping of symptoms. Women who reported moderate or severe urinary urgency with or without urgency urinary incontinence were classified as having overactive bladder. They were classified as OAB wet if they reported at least one episode of urinary incontinence on their 3-day frequency volume chart. However both OAB dry and OAB wet were considered as one group for analysis due to small number of patients in the OAB dry group. Patients who reported moderate or severe urinary incontinence with physical activity e.g. coughing running etc. were classified as the SUI group Patients were classified as having Bladder pain syndrome [BPS] if they reported to have bladder pain for at least for six months duration and accompanied by either urinary urgency or frequency(van de Merwe et al. 2008).

5.2.6 Statistics:

Sample size calculation: To achieve an 80% power and a significance of .05 for a 0.60 standard deviations difference in NGF level between groups, 28 subjects were needed in each group of cases

and controls. Shapiro-Wilk test was used to test the normality of NGF/Cr values and the values were not normally distributed. Therefore non-parametric tests were used for statistical analysis.

Independent sample T test was used to compare the age between the asymptomatic group and symptomatic group. Spearman correlation test was used to assess the relationship between age, voided volume and NGF levels in the study population. Kruskal –Wallis Test was used to compare the NGF levels between the different ethnic groups in the symptomatic group.

Urinary NGF concentrations were compared between these symptom subgroups as well as between the symptomatic groups and controls using Mann Whitney U test. Urinary NGF concentrations were compared between groups of women with different urodynamic diagnoses using the Kruskal Wallis test. Cumulative odds ordinal logistic regression was run to determine the association between increased NGF levels and the severity of symptoms such as frequency, nocturia, urgency, urgency urinary incontinence [UUI], stress urinary incontinence [SUI] and bladder pain [BP] as reported on KHQ. Odds ratios were also calculated after adjusting for age. Univariate and multivariate logistic regression was performed to predict the presence of urinary urgency; UUI and OAB using NGF level as predictor. A multivariate logistic regression were performed to ascertain the effects of urodynamic diagnosis and age on the likelihood of participants having raised NGF of more than 13 ng NGF / g Cr.

The validity of urinary NGF concentration was further evaluated using the receiver operating characteristic curve to determine the diagnostic performance of measuring urinary NGF/Cr concentration in differentiating the symptomatic and urodynamic groups. Sensitivity, specificity, positive predictive value [PPV] and negative predictive values] NPV] of diagnosing were calculated using a cut off value for NGF levels. All analyses were carried out using SPSS version 20, Chicago, IL, USA).

5.3 Results:

5.3.1 Demographics of the study population

A total of 236 women were recruited. Of these, 205 women were symptomatic with LUTS and had undergone urodynamics and the remaining 31 women were asymptomatic. The demographics of the study group is summarised on table 5.1.

There was a significant difference in the mean age between the asymptomatic group and symptomatic group (36.5[±11.6] vs. 52.8 [±13.9] Independent sample T test; p <0.001). There was a poor positive correlation between the age and NGF concentration in the entire study population including asymptomatic subjects [Spearman correlation rho= 0.259;p<0.001]. The median parity of the symptomatic group was 2 and the mean BMI was 25.2.

There was no significant difference in the NGF levels between the different ethnic groups in the symptomatic group [Kruskal –Wallis Test; p =0.217]. There were 101 Caucasians, 23 Middle East, 21 Afro Caribbean, 12 Asians, 3 Chinese and 2 were mixed race.

Voided volume at the collection of urine sample was documented for 117 patients. The mean voided volume was 314.14 ± 250ml. There was no correlation between NGF levels and the voided volume [Spearman correlation rho= 0.045;*p*=0.629].

	Controls [n=31]	Symptomatic patients [n=205]
Mean Age	36.5(±11.6)*	52.8(±13.9)*
Median Parity	NA	2.0(0 to 3)#
Mean BMI	NA	25.2
Mean day time frequency	6.01[(±1.03)*	8.07(±3.93)*
Median Nocturia	0	2.0(1.0 to 3.0)#
Ethnic groups	NA	Documented in 162 101 Caucasians, 23 Middle East, 21 Afro Caribbean, 12 Asians, 3 Chinese and 2 were mixed race

Table: 5.1 Baseline demographics and clinical characteristics

***Values are expressed as: mean (standard deviation)**

 # Values are expressed as median (25^{th}-75^{th} interquartile ranges)

NA: not available

5.3.2 NGF levels and association with patient reported urinary symptoms:

5.3.2.1 Comparison between controls and symptomatic groups:

The median creatinine corrected NGF concentration in the asymptomatic group of women was significantly different from the

median creatinine corrected NGF concentration in the symptomatic patients with LUTS (2.05 vs.13.33 ng NGF/ g Cr Mann Whitney test, Z value-3.899; p <0.001) Figure 5. 1. There was a similar finding in the median NGF/Cr concentrations between the controls and the different individual symptomatic groups [Table 5.2].

Symptom Groups	n	Median ng NGF/ g Cr [Interquartile range]	p (comparison with controls)
Control	31	2.05[0.1-11.88]	
LUTS	205	13.33[2.98-43.69]	<0.001
OAB	165	14.21[3.50-52.73]	<0.001
SUI	104	13.97[2.31-53.53]	<0.001
BPS	54	11.04[3.37-34.84]	<0.002

Table 5.2: Comparison of NGF/Cr concentrations between controls and symptomatic groups.

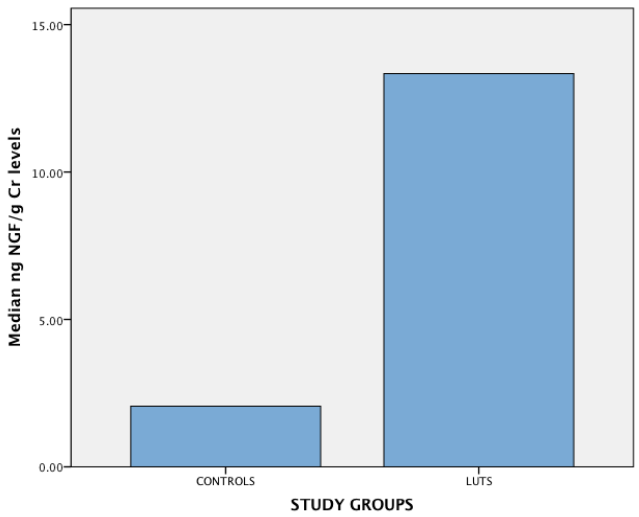


Figure 5.1:Median NGF levels in controls [2.05 ng NGF/g Cr] and patients with LUTS [13.33 ng NGF/g Cr]

5.3.2.2 Comparison among different symptomatic groups:

There was a significant difference in NGF/Cr concentration between the women who reported OAB symptoms and the remaining subjects who did not have OAB symptoms (Mann-Whitney test, p <0.001) Figure 5.2. However there was no statistically significant difference in urinary NGF/Cr concentration between the other symptom groups [figure 5.3] as shown on table 5.3. There was also a significant difference in median NGF levels between groups based on presence or absence of individual symptoms such as urgency or UUI [p<0.001] but not in groups based on presence of SUI or BP [p=>.05 Mann Whitney U test].

	Symptom groups	n	Median ng NGF/ g Cr [Interquartile range]	P value
Group1	Non OAB	71	5.78[0.1-18.80]	<0.001
	OAB	165	14.21[3.50-52.73]	
Group2	Non SUI	132	10.60[1.90-28.92]	0.152
	SUI	104	13.97[2.31-53.53]	
Group3	Non BPS	182	11.29[1.89-35.95]	0.876
	BPS	54	11.04[3.37-34.84]	

Table 5.3: Comparison of NGF/Cr concentrations among different symptom groups.

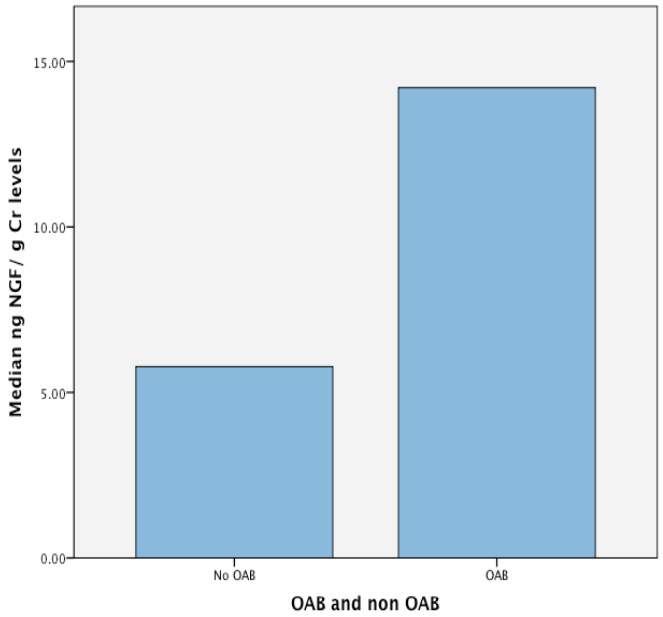


Figure 5.2:Median NGF levels in Non OAB [5.78 ng NGF/g Cr] and subjects with OAB [14.21 ng NGF/g Cr]

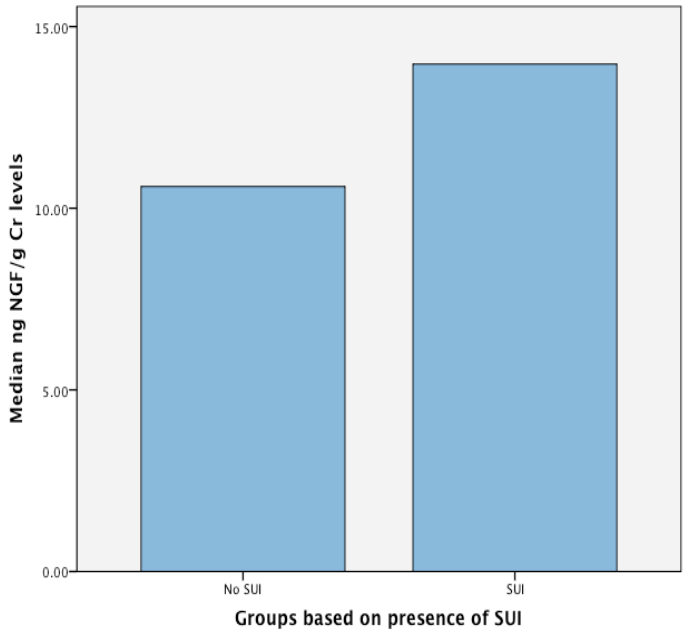


Figure 5.3:Median NGF levels in Non SUI [10.60 ng NGF/g Cr] and subjects with SUI [13.97 ng NGF/g Cr]

On univariate logistic regression analysis NGF levels were not significant predictors of predicting the presence of urgency.

Conversely when logistic regression was performed to ascertain the effects of NGF levels on the likelihood of participants having UUI,

NGF was a good predictor of patients having UUI or not with an odds ratio [exp B] of 1.005;CI=1.000 to 1.011, p = .038. This means that for every unit increase in NGF value the patients are 1.005 more likely to have UUI than who do not have a raised NGF value.

However when multivariate logistic regression was performed to assess the effects of age and NGF on predicting participants having UUI, NGF was not a good predictor of patients having UUI or not [highlighted in red on table 5.4].

A multivariate logistic regression was also performed to ascertain the effects of age and NGF levels on the likelihood of participants having OAB. NGF was a good predictor of patients having OAB or not. Although the adjusted odds ratio was statistically significant [highlighted in red on table 5.4] the value was only 1.006.The Wald Chi square χ^2 values and adjusted odds ratios are summarized on table 5. 4].

	B	Standard error	Wald Chi-square	df	***p***	**Adjusted Odds ratio [Exp B]**	95% confidence intervals Upper bound	Lower bound
UUI	.005	.003	3.518	1	**.061**	**1.005**	1.000	1.010
OAB	.005	.003	3.933	1	**.047**	**1.006**	1.000	1.011

Table 5.4: Association between NGF levels and UUI symptom and OAB group

5.3.2.3 Diagnostic ability of urinary NGF levels to differentiate symptomatic groups:

When ROC analysis was employed to determine the diagnostic accuracy of measuring urinary NGF/Cr concentration in differentiating the symptomatic groups and controls the corresponding Areas Under the Curve were high, ranging from 0.70 to 0.74. However this wasn't the same when urinary NGF/Cr concentration measurement was evaluated using ROC analysis to discriminate various symptomatic groups. The Areas under the ROC curve, SE and 95%CI for measurement of urinary NGF /Cr concentration in the different symptomatic groups are summarised on table 5.5. Using 13.0 ng/g as a cut off for urinary NGF/Cr concentration the test provides a sensitivity of 80.7% and specificity of only 39.4%, PPV of 53.33% and NPV of 70.42% in diagnosing OAB among patients with LUTS. In about 18%of OAB group the median NGF levels were below 2.01ng/g.

Symptoms groups	n	AUC	Standard error	95% confidence interval	p
Non OAB OAB	71 165	0.66	.04	0.58-0. 73	<0.001
Non SUI SUI	132 104	0.55	.04	0.48-0. 63	0.152
Non BPS BPS	182 54	0.51	.04	0.42-0.59	0.876

Table 5.5: The areas under the ROC curve (Yamauchi et al.), SE and 95%CI for measurement of urinary NGF concentration in the different symptomatic groups

5.3.3.4 NGF levels and severity of symptoms:

The severity of symptoms as reported on KHQ are summarised on table 5.6. On ordinal regression analysis, there was no statistical significant association between NGF values and the different severity scales of urinary symptoms. The Wald χ^2 values and the adjusted odds ratio for age are summarised and key results are highlighted in red on table 5.7.

Symptom	Nil n	Mild n	Moderate n	Severe n
Frequency	11	24	57	62
Nocturia	16	37	53	48
Urgency	15	32	46	61
UUI	35	28	43	48
SUI	36	27	33	58
BP	81	27	30	16

Table 5.6: Severity of urinary symptoms based on KHQ

	Wald Chi- square	df	***p***	**Adjusted Odds ratio [Exp B]**	95% confidence intervals Upper bound	Lower bound
Frequency	.108	1	.743	1.000	.997	1.002
Nocturia	.084	1	.771	1.000	.997	1.002
Urgency	.009	1	.925	1.000	.998	1.003
UUI	.033	1	.856	1.000	.997	1.002
SUI	.580	1	.446	1.001	.998	1.004
BP	.886	1	.346	.999	.996	1.001

Table 5.7:Association between NGF values and severity of symptoms

5.3.3 NGF levels and association with urodynamic diagnoses:

On comparing the median NGF/Cr concentration between the different Urodynamic diagnoses there was no significant difference (Kruskal Wallis test, p >0.05), as shown in table 5.8 below. On multivariate regression analysis of urodynamic diagnoses and age, UDS diagnosis of DO was not a good predictor of patients having high NGF of more than 13 ng NGF / g Cr.The logistic regression model was not statistically significant χ^2 (4) = 7.921, p = .095.

Urodynamic Groups	n	Median ng NGF/ g Cr	IQR	p
Detrusor overactivity [DO]	74	13.84	4.02-46.26	0.237
Urodynamic stress urinary incontinence [USI]	31	20.45	1.91-82.6	
Mixed urinary incontinence [MUI]	39	21.13	3.23-82.22	
Bladder pain/increased bladder sensation/reduced bladder capacity [BP/IBS/RBC]	40	8.84	2.46-24.15	

Table 5.8: Comparison of NGF/Cr concentrations between different urodynamic groups

5.3.3.1 Diagnostic ability of urinary NGF levels to differentiate urodynamic groups:

On applying ROC analysis to determine the diagnostic performance of measuring urinary NGF/Cr concentration in differentiating the different urodynamic groups the corresponding AUC's were poor

[<0.60]. The areas under the ROC curve, SE and 95%CI for measurement of urinary NGF/Cr concentration in the different Urodynamic groups are summarised on table 5.9. Using 13.0 ng/g as a cut off for urinary NGF/Cr concentration to diagnose DO provided a sensitivity of 65 % and a specificity of only 44%.

Urodynamic Groups	n	AUC	Standard error	95% confidence interval	p
NO DO	110	0.51	.04	0.43-0.59	0.83
DO	74				
NO USI	153	0.49	.06	0.36-0.61	0.840
USI	31				
NO MUI	145	0.57	.05	0.47-0. 68	0.161
MUI	39				
NO BP/IBS/RBC	144	0.59	.05	0.50-0. 68	0.067
BP/IBS/RBC	40				

Table 5. 9: The areas under the ROC curve, SE and 95%CI for measurement of urinary NGF/Cr concentration in the different Urodynamic groups

5.4 Discussion:

Nerve growth factor is a neurotrophic factor essential for the growth, differentiation and maintenance of developing sensory and

sympathetic nervous system. NGF has been implicated as a chemical mediator in bladder dysfunction being released by urothelium and smooth muscle in bladder dysfunction(Jacobs et al. 2010, Lowe et al. 1997, Oddiah et al. 1998, Okragly et al. 1999, Steers and Tuttle 2006) Increased levels of NGF could lead to reduced thresholds or increased excitability of bladder afferent fibres(Dmitrieva and McMahon 1996, Yoshimura and de Groat 1999) and an enhanced spinal reflex (Steers and Tuttle 2006). Change in sodium channel properties is thought to be the responsible mechanism for the alteration in conductance of afferent nerves (Steers and Tuttle 2006, Yoshimura et al. 2006), thereby resulting in OAB. It has been proposed that urinary NGF could be used as a diagnostic biomarker in LUTD since there is substantial evidence linking NGF and LUTD from both experimental and human studies. Over the past few years the role of urinary NGF as a biomarker has been explored in various studies with conflicting data.

The results of this study support previous evidence that urinary NGF/Cr concentration is increased in patients with lower urinary tract symptoms especially OAB (Liu, Chen, et al. 2010) since there was a significant difference in the Urinary NGF/Cr concentration between controls and patients with LUTS [Table 5.2] as well as patients with OAB and non OAB .However this was not the same when other symptomatic groups or Urodynamic groups were compared [Figure 5.4]Applying ROC analysis demonstrated poor discriminant ability of measuring NGF/Cr concentration in different symptomatic groups [Table 5.5] as well as in different Urodynamic groups [Table 5.9] except the OAB and non OAB group.

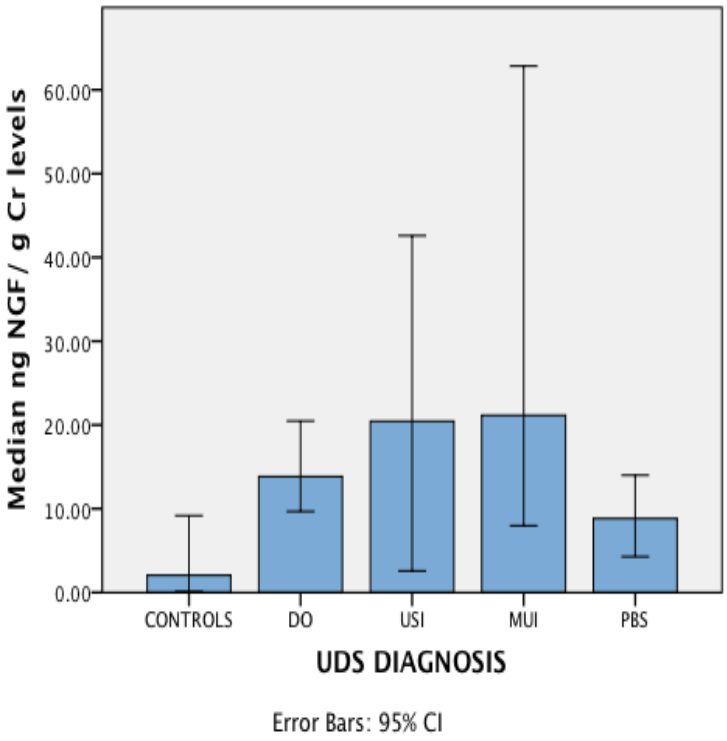


Figure 5.4: Median NGF values in the different urodynamic groups

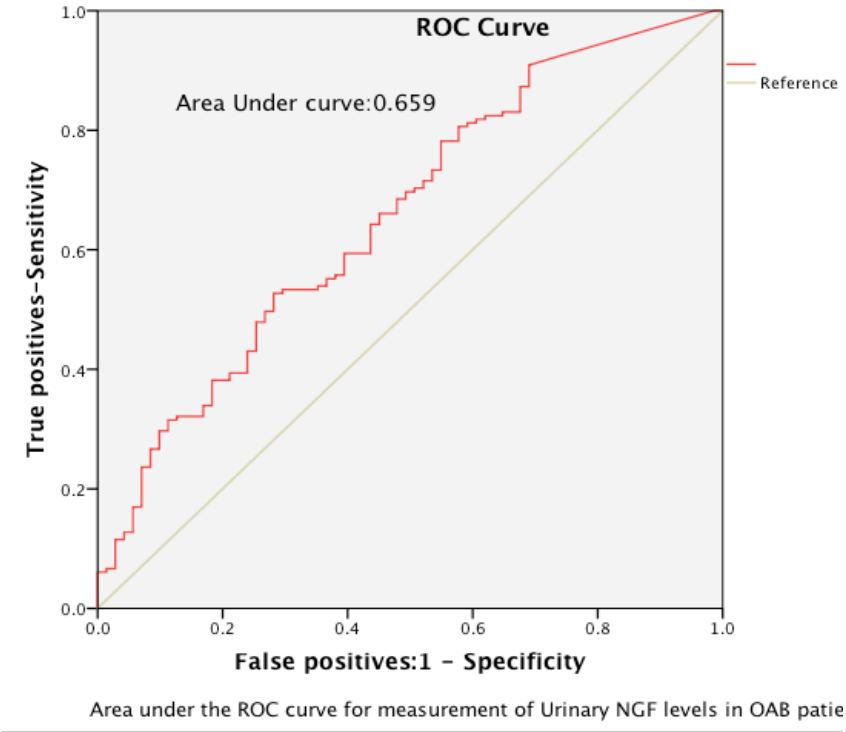


Figure 5.5: Area under the ROC curve for measurement of NGF levels in OAB patients

Contrary to previous evidence (Liu et al. 2008) this study shows significantly increased levels of urinary NGF in patients with SUI when compared to controls [Table 5.2] that support the evidence found in a recent animal study(Ko et al. 2011) . Overexpression of NGF was found in the urethra and neuronal centres after transabdominal urethrolysis to induce stress urinary incontinence in rats (Ko et al. 2011). It has been postulated that loss of supportive tissues or induction of smooth muscle atrophy by transabdominal urethrolysis might stimulate the neuronal voiding centers leading to overexpression of NGF.

On ROC analysis of urinary NGF concentration, areas under the curve for differentiation of various symptomatic groups and controls were high ranging from 0.70 to 0.74, which substantiates results from other research groups (Liu et al. 2011). Similarly urinary NGF/Cr concentration was significantly higher in patients with OAB symptoms when compared to individuals with no OAB symptoms and was found to a good predictor of subjects with OAB on multivariate regression analysis [Table 5.4]. However the same was not seen in patients with SUI and patients with no SUI symptoms [Table 5.3].

When applying ROC analysis to assess the discriminant ability of measurement of urinary NGF/Cr concentration the AUC was poor in different symptomatic groups except the OAB and non OAB group where AUC value was nearly 0.7 [Figure 5.5]. These results show that although Urinary NGF/Cr concentration is certainly increased in

patients with LUTS especially in OAB, it cannot be used as a discriminatory tool.

Using 13.0 ng/g as a cut off for urinary NGF/Cr concentration the test provides a sensitivity of 81% and specificity of less than 40 % in diagnosing OAB in patients with LUTS. This corroborates the results from the study done by Antunes-Lopes and group who reports low values of sensitivity and specificity for NGF/creatinine ratio with an area under the curve in ROC analysis of only 0.68(Antunes-Lopes et al. 2011). However Liu and co workers have reported a sensitivity of 84.9% and specificity of 84.5% for differentiation of OAB-wet and controls using a threshold value of 0.085 for urinary NGF/Cr concentration. However patients in that study were requested to drink a litre of fluid before the test, which might have had an effect on the NGF concentration since distension and stretching of the bladder has shown to increase the NGF levels(Liu et al. 2011) .

There was no significant association between NGF levels and severity of symptoms. This is probably due to increased levels of NGF in all the lower urinary tract symptomatic groups and considerable overlapping of lower urinary tract symptoms in the various LUTD. This finding supports the lack of specificity and poor discriminatory power of NGF as a biomarker

The diagnostic accuracy of urinary NGF as a biomarker was further explored by using urodynamics as the reference standard since it is the best available objective test to investigate patients with LUTS

(Martin et al. 2006). The results of this study show raised levels in

USI and increased bladder sensation contrary to results found by Liu and co workers (Liu et al. 2008, Liu, Tyagi, et al. 2010) however the number of patients in their urodynamic study groups was small. In this study there was no significant difference in the NGF concentration between the different Urodynamic diagnoses [table5. 8] and corresponding areas under the curve indicating poor test performance (AUC 0.49-0.59). Using 13.0 ng/g as a cut off for urinary NGF/Cr concentration to diagnose DO provided a poor specificity of only 44%. This further supports the poor discriminant ability of urinary NGF/Cr concentration in patients with LUTS.

Confounding factors such as systemic disorders and certain drugs that can increase the NGF level have not been accounted for, which is one of the limitations of this study. The control group is small, however on sample size collection to reach a statistical significance of .05 only 28 patients were needed in each group which was achieved. The strengths of this study include large sample size especially of the subgroups with adequate power for clinically relevant associations. The results from this study have added new information with regards to the criterion validity of urinary NGF measurement, which helps in bridging the gaps in the evaluation of urinary NGF as a biomarker as well as corroborating and refuting some of the available evidence.

To conclude the results of our study show that although Urinary NGF/Cr concentration is certainly increased in patients with LUTS

especially in OAB, it cannot be used as a discriminatory tool. The limitations of urinary NGF assays seem to be low specificity, lack of discrimination power and wide standard deviations. It is difficult to establish normal ranges for NGF because NGF levels can be undetectable in OAB patients. Since urinary NGF levels are significantly increased in patients with OAB they may have a role in clinical trials as well as be used to monitor therapeutic interventions for OAB in clinical practice.

CHAPTER 6:

IS URINARY NGF LEVEL ASSOCIATED WITH CYSTOSCOPY OR HISTOLOGY FINDINGS OF BLADDER INFLAMMATION?

Hypothesis 3: Urinary NGF levels are not associated with cystoscopic and histology findings of inflammation in women with refractory OAB and cannot be used as a non-invasive biomarker to predict the inflammatory status of the bladder.

CHAPTER 6: IS URINARY NGF LEVEL ASSOCIATED WITH CYSTOSCOPY OR HISTOLOGY FINDINGS OF BLADDER INFLAMMATION?

6.1 Introduction:

Overactive bladder (OAB) has a multi-factorial aetiology. Recently it has been suggested that urothelial inflammation plays an important role in the pathogenesis of OAB (Birder et al. 2007). The urothelium is said to play an essential role in bladder function due to its sensory and signaling properties and ability to release various chemical mediators and transmitters, which can modulate the afferent nerves(Birder et al. 2010). Supporting this hypothesis, animal and clinical studies on inflammatory biomarkers have provided evidence for the role of cytokines, prostaglandins and nerve growth factor in the development of OAB(Kim et al. 2006, Steers and Tuttle 2006, Tyagi et al. 2010, Zvara and Vizzard 2007). Chronic cystitis has been found in around 60-90%of patients on histopathological examination of bladder biopsies with refractory OAB (Apostolidis et al. 2008, Digesu et al. 2013), which corroborates these data further.

A recent animal study has demonstrated that tolterodine has no effect on detrusor activity in rats with chemical cystitis compared to a sham group(Jin et al. 2011). About 42-80% of patients with OAB are refractory to anticholinergic treatment(Payne and Kelleher 2007) . Experimental studies have shown that anti-inflammatory treatment might be beneficial in improving the overactive bladder(Rahnama'i et

al. 2013, Takagi-Matsumoto et al. 2004). There is also emerging evidence from animal and clinical studies that subclinical bacterial infection can produce chronic cystitis/OAB symptoms and that antibiotics might be effective in reducing refractory OAB symptoms(Anderson et al. 2003, Khasriya et al. 2010, Latthe et al. 2008, Walsh and Moore 2011). So there is a clear need for more research towards understanding the pathogenesis of OAB, which will help us to direct the treatment especially in the refractory OAB group of women.

If urothelial inflammation is one of the causes for refractory overactive bladder it will be useful to assess if the urothelial inflammation resolves after novel treatments like antibiotics and anti-inflammatory drugs. Cystoscopic inspection of bladder mucosa and histopathological examination of bladder biopsies has been proposed as a method of assessing urothelial inflammation(Digesu et al. 2013). However this assessment is costly, invasive and has a number of risks associated with the procedure such as infection, bladder perforation, pain and haematuria. A non-invasive biomarker would therefore be useful to provide diagnostic information about the inflammatory status of the bladder and it could be useful in assessing the therapeutic response.

Nerve growth factor (NGF) is one of the neurotrophic factors, which is considered as a key regulator of neurogenic inflammation in several tissues(Levi-Montalcini et al. 1996) including the bladder(Steers 2002). Increased NGF levels are found in OAB,

detrusor overactivity (DO), interstitial cystitis and varying models of bladder inflammation in both animal and human studies (Liu et al. 2010, Steers 2002, Steers et al. 1991). Increased urinary NGF levels in OAB might therefore be associated with severity of bladder inflammation and may be used as a non-invasive biomarker to predict the inflammatory status of bladder if it correlates with cystoscopy and histology findings.

Therefore the aim of this study was to evaluate the relationship between urinary Nerve Growth Factor levels and cystoscopic and histology findings of bladder inflammation in women with refractory OAB.

6.2 Methodology:

Women with OAB symptoms who were resistant to conservative management (e.g. lifestyle modifications, bladder retraining and physiotherapy) and two or more anticholinergics were recruited into this cross sectional study. All women were studied with vaginal examination using a pelvic organ prolapse quantification system (POP-Q) bladder diary, urinalysis, Kings Health Questionnaire, Patient perception of bladder condition [PPBC], Patients' Perception of Intensity of Urgency Scale [PPIUS] urodynamics and kidney–ureter–bladder ultrasound scans as per the unit protocol. Women were excluded if they had a neurological condition, pelvic organ prolapse, voiding dysfunction, urinary tract infection, bladder pain, bladder cancer or calculi. All participants were counselled, consented and investigated with a rigid cystoscopy, hydrodistension and bladder biopsy under general anaesthesia. Only those women with

detrusor overactivity on urodynamics and cystoscopic evidence of bladder inflammation and histological confirmation of chronic cystitis were included in the study. Women were excluded if there was a cystoscopic appearance of interstitial cystitis as described by the European Society for the Study of interstitial cystitis (ESSIC) e.g. glomerulation grades 2–3, and Hunner's lesion (van de Merwe et al. 2008).

Participants provided clean catch mid-stream urine samples prior to undergoing rigid cystoscopy. Urine samples from asymptomatic volunteers from general gynaecology clinics who did not complain of any lower urinary tract symptoms were also collected and processed to measure the NGF levels.

Urine samples were immediately centrifuged at 3000 rpm at 4 ^{0}C for 10 minutes. An aliquot of whole urine was also used to measure urine creatinine. The centrifuged supernatant fluid was then frozen at -80^{0} C until further processing. Once defrosted the measurement of urinary NGF concentration was performed by ELISA using the NGF Emax® ImmunoAssay System (Promega, Madison, WI, USA) as described on chapter 4. The total urinary NGF levels were further normalized to the concentration of urinary creatinine (NGF/Cr level).

Rigid cystoscopy with 70-degree cystoscope was performed only in symptomatic women under general anaesthesia after emptying the bladder. The bladder was distended with water and cystoscopic findings of presence or absence of trigonitis, erythema (vascularity), trabeculations, glomerulations, diverticulum, hunner's ulcer and

space occupying lesions were recorded. Presence of erythema [figures 6.1- 6.3] was classified as mild if increased vascularity was present in < 1 quadrant, as moderate if present in >1 quadrant (< 50% bladder); and severe if present >1 quadrant (>50% bladder). The classification of severity of vascularity was adapted from the Denson and colleagues classification of bladder glomerulation(Denson et al. 2000) . The severity of bladder trabeculation was classified using the modified grading system of El Din et al, as 0 (none), 1 (slight), 2 (moderate), 3 (severe), and 4 (severe, with diverticula)(el Din et al. 1996)[figures 6. 4 & 6.5].

The bladder was drained, refilled and examined for refill haemorrhages. Finally deep bladder biopsy was taken using 0 degree cystoscope and cold cup biopsy forceps from the most erythematous area. The biopsy site was diathermised and the biopsy specimen was sent to the histopathology lab. The procedure was performed either by the two urogynaecologists or subspecialty trainee in the unit.

Each biopsy was rated for cystitis (mild, moderate, severe) by a histopathologist blinded to clinical data. The bladder histology was classified according to a reproducible technique which has been previously described(Thilagarajah et al. 1997).

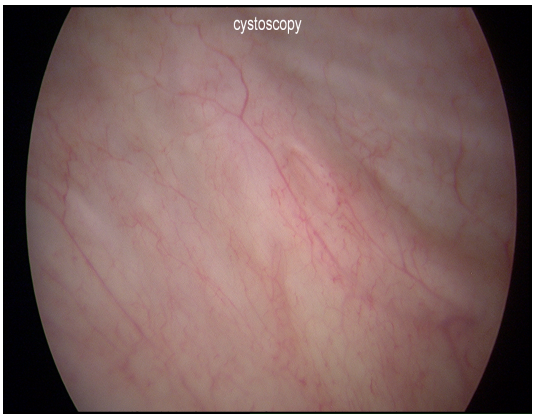
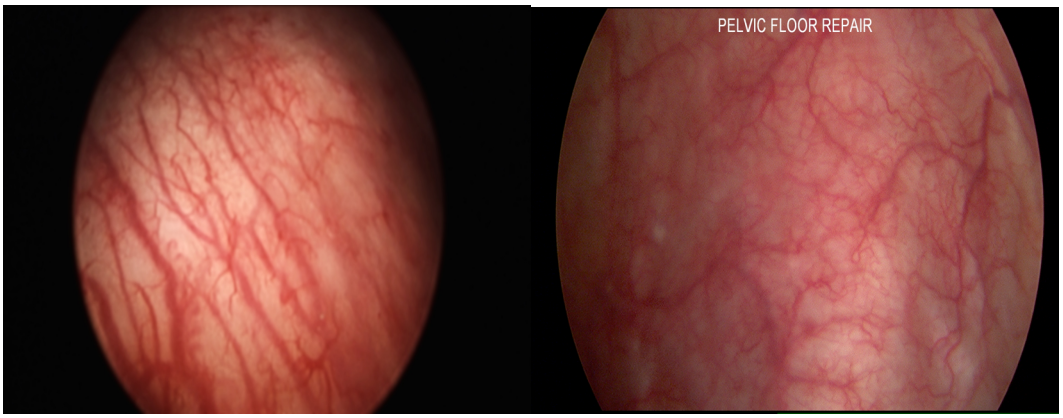


Figure 6.1:Normal appearance of bladder mucosa



Figures 6.2 & 6. 3: severe erythema

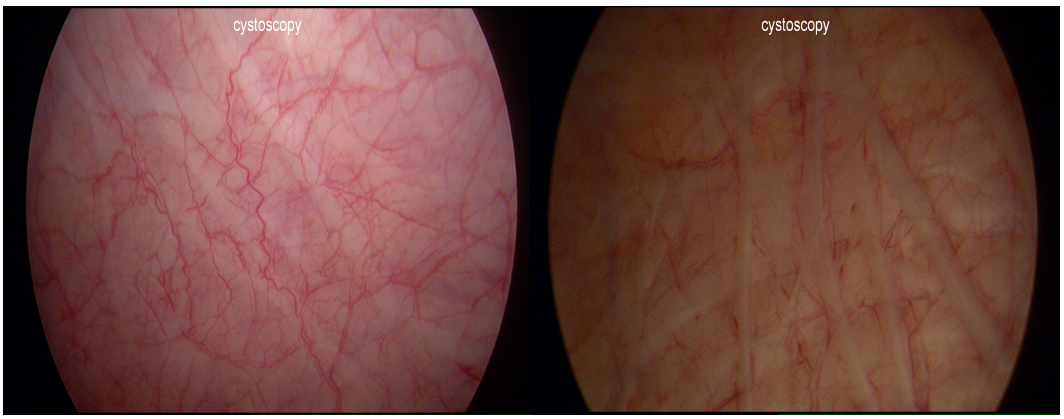


Figure 6.4: Mild trabeculation

Figure 6. 5:Moderate trabeculation

Statistics: No sample size calculation could be carried out as this type of study has not been carried out previously and therefore this study is to be regarded as a pilot study.

The NGF data was non-parametric and therefore the median urinary NGF levels were compared with a control group of asymptomatic women using the Mann Whitney U test. Spearman correlation test was used to assess the correlation between urinary NGF levels and age. Cumulative odds ordinal logistic regression was run to determine the association between increased NGF levels and the severity of trigonitis, erythema and trabeculation on cystoscopy as well as grades of cystitis on histology. Odds ratio was also calculated after adjusting for age. All analyses were carried out using SPSS version 20, Chicago, IL, USA.

6.3 Results:

In total 76 patients were recruited and 9 patients were excluded due to reasons summarised on table 6.1. In addition MSU samples were collected from 31 asymptomatic women who served as controls.

n	Reason for exclusion
4	Not enough tissue for histology
2	Normal on histology
1	Bladder tumour
2	Signs of interstitial cystitis 1-presence of hunner's ulcer 1-detrusor mastocytosis > 28 mast cells/mm^2 on histology

Table 6.1: Reasons for exclusion from study

The demographics of the study group are summarised on the table 6.2. There was a significant difference in the mean age between the asymptomatic group and symptomatic group (36.5[±11.6] vs. 53.3 [±14.7] Independent sample T test; p <0.001). However there was a very weak correlation between the age and NGF concentration in the symptomatic group [Spearman correlation rho= 0.350]. The median creatinine corrected NGF concentration in the asymptomatic group of women was significantly different from the median creatinine corrected NGF concentration in the symptomatic groups (2.05 vs.10.58 ng NGF/ g Cr Mann Whitney test, Z value-3.267; p <0.001) [figure 6.6].

Mean Age	53.3(±14.7)*
Mean duration of symptoms (months)	26 (±7.3)*
Median Parity	2.0(0 to 3)#
Median Number of antimuscarinics tried	3.0(2.0 to 4.0)#
Mean day time frequency	9.10(±2.88)*
Median Nocturia	2.0(1.0 to 3.0)#
PPBC scores	5.0 (4.0 to 6.0)#
PPIUS scores	4.0(2.0 to 5.0)#

Table: 6.2 Baseline demographics and clinical characteristics of patients.

***Values are expressed as: mean (standard deviation)**

 # Values are expressed as median (25^{th}-75^{th} interquartile ranges)

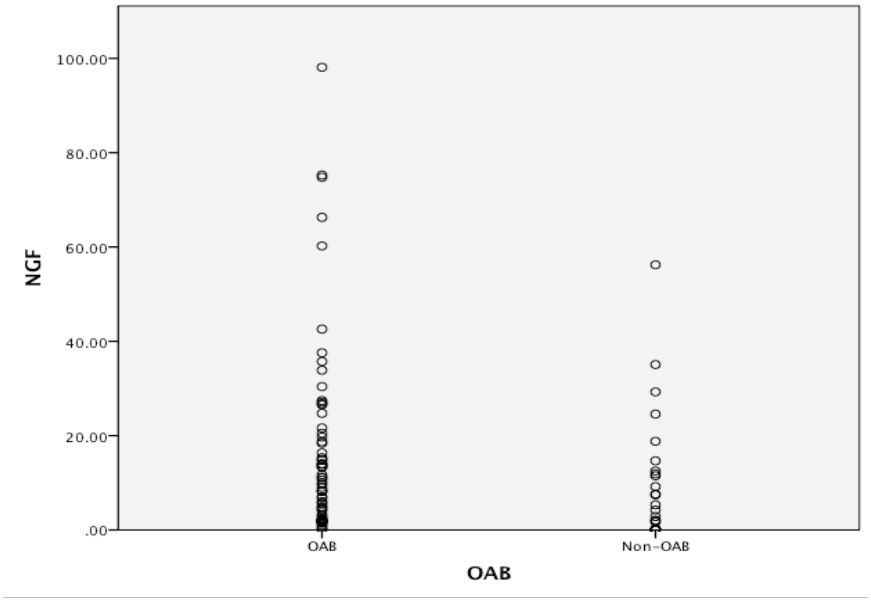


Figure 6.6: Scatter plot showing ng NGF/ g Cr values in OAB and non-OAB groups.

Median ng NGF/ g Cr values [25th – 75 th interquartile ranges]

OAB group – 10.58[2.87-20.48]; Non-OAB group- 2.05[0.1-11.88]

All the symptomatic patients had detrusor overactivity on urodynamics and of these seven patients had both urodynamic stress urinary incontinence and detrusor overactivity. The cystoscopy findings are summarised on table 6.3. On histopathological examination there was mild chronic cystitis in 54[80.6%], moderate chronic cystitis in 11[16.4%] and severe chronic cystitis in two [3%] patients.

	Nil n [%]	Mild: n [%]	Moderate n [%]	Severe n[%]
Trigonitis	8[11.9%]	10[14.9%]	21[31.3%]	28[41.8%]
Erythema	3[4.5]	19[28.4%]	31[46.3%]	14[20.9%]
Trabeculation	-	28[41.8%]	31[46.3%]	8[11.9%]

Table 6. 3:Cystoscopy findings

On ordinal regression analysis there was no statistical significant association between NGF values and the different severity scales of trigonitis, trabeculation, erythema and cystitis. On adjusting for age the odds ratio did not change significantly. The Wald χ^2 values and the odds ratio are summarised on table 6.4 with key values highlighted in red.

	Wald Chi-square	df	***p***	**Odds ratio [Exp B]**	Adjusted Odds ratio* [Exp B]	*P**
Trigonitis	2.443	1	**.118**	**.982**	.991	.427
Erythema	.463	1	**.144**	**.979**	.977	.471
Trabeculation	.872	1	**.350**	**.989**	.992	.546
Cystitis on histology	.554	1	**.457**	**.983**	.986	.408

Table 6.4: Association between NGF values and cystoscopy and histology findings based on their severity scale

***Odds ratio adjusted for age and *p* value**

6.4 Discussion:

NGF is produced and utilized by neuronal and several non-neuronal cell types including immune cells, inflammatory cells, epithelial cells, and smooth muscle cell(Aloe et al. 1997, Birder et al. 2010). Numerous inflammatory cytokines, including interleukin (IL)-1, tumour necrosis factor (TNF)-α and IL-6, can induce NGF production in non-neuronal cell types, such as fibroblasts, endothelial cells and glial cells(Birder et al. 2010). Interestingly NGF has been shown to

increase the expression of sensory neuropeptides such as substance P, CGRP and other neuropeptides (Schnegelsberg et al. 2010) which are released peripherally and centrally and can initiate local neuroinflammatory responses as well as enhance sensory neuron excitability (Zvara and Vizzard 2007).

Experiments done in rodents have shown that chemical inflammation induced using formalin, cyclophosphamide or foreign body irritant such as chromic catgut and bacterial cystitis induced using lipopolysaccharide is associated with increased NGF levels and morphological changes in the sensory and motor neurons innervating the bladder as well as bladder overactivity(Dupont et al. 2001, Hu et al. 2005). The use of NGF sequestering protein REN 1820 in rodents with chemical induced cystitis reduced the bladder overactivity(Hu et al. 2005).Human studies have demonstrated that serum NGF, urinary NGF levels as well as serum C-reactive protein, which are inflammatory markers, are significantly elevated in patients with OAB compared with controls(Chung et al. 2011, Liu et al. 2011). Serum NGF levels also highly correlated with urinary NGF levels. These data from human studies seem to support the evidence in experimental studies linking NGF, inflammation and overactive bladder.

In order to explore this link further this current study was conducted to assess if there is an association between NGF levels and cystoscopy or histology findings in women with OAB and DO. There was a statistical significant difference in the NGF levels between OAB

patients and non-OAB patients. All patients were found to have some degree of trabeculation and at least 90% of patients had erythema and trigonitis. All patients with OAB had evidence of chronic cystitis with the majority (81%)showing mild chronic cystitis, which is similar to previous studies of patients with refractory OAB. However there was no statistical significant association between NGF values and the different severity scales of trigonitis, trabeculation, erythema and cystitis which questions whether the visual appearance of the bladder is a valid method of assessing bladder inflammation or the severity of the cases was similar such that the tools could not be used to discriminate between the different patients.

Only patients with a diagnosis of detrusor overactivity and refractory OAB symptoms were included in this study in order to reduce bias. Patients with refractory OAB and DO were studied since it was suspected that these patients might have a higher degree of inflammation. Unfortunately without a group of OAB patients who responded to conventional treatment to compare we do not have data to support this hypothesis, which is one of the drawbacks of this study.

This study exploring the role of NGF as an inflammatory biomarker did not find an association between the increased NGF levels and the severity of cystoscopy findings. This could be because urinary NGF may be a poor biomarker of bladder inflammation or cystoscopy findings itself may be poor indicators of bladder inflammation. In addition the rating of cystoscopy findings could be subjective which

could have influenced the results. The role of cystoscopy and biopsy is of doubtful significance in female urinary incontinence and is not routinely recommended (Tubaro 2009). However a recent study has shown that bladder trabeculations were significantly associated with DO and UUI when compared to patients with no trabeculations (Liang et al. 2013). In another study the presence of trabeculations and increased vascularity was present in around 70% of patients with chronic cystitis and refractory OAB and was also found to have a positive predictive value of over 80% for chronic cystitis (Digesu et al. 2013).

There was evidence of chronic cystitis in all patients of this present study with refractory OAB symptoms which supports the findings found in experimental studies. Chronic cystitis induced in rodents resulted in increased NGF levels and bladder overactivity (Dmitrieva et al. 1997) as well as varying degrees of oedema, vasodilatation and inflammatory cell infiltrates on histological analysis of bladder wall (Dupont et al. 2001, Hu et al. 2003). Similar to the findings in the animal models histological evidence of bladder inflammation has been found in patients with neurogenic DO as well as idiopathic OAB on histology(Comperat et al. 2006, Loran et al. 2007).

There was no association between the increased NGF levels and the severity of cystitis in this study. This could be because urinary NGF may be a poor biomarker of bladder inflammation. Bladder biopsy of a small area may not be representative since a previous study did not find any correlation between the severity of cystoscopy and histology

findings (Denson et al. 2000). Limitations of this study include lack of control group and also unequal numbers in the subgroups in addition to those discussed above.

The significantly increased NGF levels in the symptomatic group compared to controls could be explained by the presence of overactive bladder. However these increased NGF values did not add to the prediction of severity in erythema, trigonitis or trabeculation on cystoscopy or cystitis on histology therefore urinary NGF should not be considered as a surrogate marker of bladder inflammation.

CHAPTER 7

DO URINARY NGF LEVELS ALTER IN WOMEN WHO HAVE HAD IMPROVEMENT IN THEIR OAB SYMPTOMS AFTER ANTERIOR COLPORRHAPHY FOR VAGINAL PROLAPSE?

Hypothesis: NGF levels do not change in women who have had an improvement in their overactive bladder symptoms after anterior colporrhaphy.

CHAPTER 7: DO URINARY NGF LEVELS ALTER IN WOMEN WHO HAVE HAD IMPROVEMENT IN THEIR OAB SYMPTOMS AFTER ANTERIOR COLPORRHAPHY FOR VAGINAL PROLAPSE?

7.1 Introduction:

Urinary Nerve growth factor (NGF) levels are significantly higher in patients with bladder outlet obstruction (BOO) compared to controls (Liu and Kuo 2008). Steers et al demonstrated about two decades ago that bladder outlet obstruction in rodents was associated with bladder hypertrophy and increased urinary frequency. There was also evidence of hypertrophy of dorsal root ganglia that provide sensory afferent fibres to the bladder and increased growth of afferent and efferent neurons. Hypertrophied bladders contained significantly more NGF levels than normal bladders. The rise in the NGF levels occurred before the neuronal hypertrophy and increased urinary frequency. Corroborating these findings, autoimmunity to NGF was found to reduce the neuronal hypertrophy (Steers, Kolbeck et al. 1991).

Chronic BOO results in stretching of the urothelium and smooth muscle, which can stimulate NGF production. In vitro experimental models on rats have demonstrated that repetitive stretch stimulation of bladder smooth muscle cells, which mimics the effects of outlet obstruction, resulted in increased expression of a variety of growth factors like NGF (Persson, Sando et al. 1995, Yamaguchi 2004). This increased NGF production is postulated to partly contribute to

enhanced spinal reflex and overactive bladder [OAB]. Bladder outlet obstruction is uncommon in women however anterior wall prolapse/cystocele is common and the relationship between anterior vaginal wall prolapse and NGF levels have not been studied so far.

Women with anterior vaginal wall prolapse frequently suffer with overactive bladder symptoms. Prevalence of OAB in patients with pelvic organ prolapse ranges widely from 16%-88% and prevalence of detrusor overactivity ranges from 10-50% (de Boer, Salvatore et al. 2010). Several mechanisms are thought to play a role in the pathophysiology of OAB in patients with prolapse including obstructive, myogenic and neurogenic factors(de Boer, Salvatore et al. 2010, Patil and Duckett 2010).

Overactive bladder symptoms have been shown to improve significantly after anterior colporrhaphy. Postoperatively urinary frequency, urgency and urge incontinence disappeared in 60, 70 and 82% of women respectively (p value < 0.001). Improvement of OAB was not seen in all cases(Digesu, Salvatore et al. 2007). Similarly in other studies, anterior repair was associated with 49% reduction in urgency urinary incontinence (Fletcher, Haverkorn et al. 2010)resolution of urgency in 55% of patients and improvement in 19% of patients(Duckett and Chakani 2013) .A recent study has found an improvement in urinary frequency and urgency as high as 85-89% in patients who underwent apical and or anterior repair(Miranne, Lopes et al. 2013) .

It was hypothesized that patients with anterior vaginal wall prolapse

have increased NGF levels compared to controls since BOO and bladder wall stretching have been postulated as causes for increased NGF levels. It was also postulated that if increased NGF levels are contributing to the overactive bladder symptoms then NGF levels should decrease after anterior repair in women who have had an improvement in their overactive bladder symptoms.

Therefore the objective was to assess whether the NGF levels change in women who have had improvement in their overactive bladder symptoms after anterior colporrhaphy.

7.2 Methods

Women were recruited following ethical approval (REC reference number: 11/LO/1029) into this prospective cohort study.

7.2.1 Inclusion and exclusion criteria:

Women over the age of 18 years who underwent anterior repair for symptomatic anterior vaginal wall prolapse were recruited into this study.

Women with history of neurological dysfunction, bladder pain syndrome, bladder cancer/calculi and previous anterior repair or continence surgery were excluded from the study. Women with urinary tract infection during preoperative and follow up assessment were also excluded from the study.

7.2.2 Study design:

Study participants were seen pre operatively and six to eight weeks following anterior repair. A clean catch midstream specimen of urine

(MSU) was collected with a full bladder at baseline and at follow up to measure the urinary NGF levels. Assessment of symptom improvement was done using validated questionnaires at baseline and six weeks after surgery. To establish normative ranges MSU samples were also obtained from asymptomatic subjects who did not suffer with overactive bladder symptoms or vaginal prolapse. These patients were recruited from general gynaecology clinics.

Either one of the two consultant urogynaecologists or the subspecialist trainee in urogynaecology performed the prolapse surgery. Urodynamics was carried out by certified urodynamicists. The researchers conducting the NGF assays were blinded to the patient symptoms pre and post prolapse surgery.

7.2.3 Methods of assessment:

POP Q quantification system was used for assessment of stage of prolapse(Bump, Mattiasson et al. 1996). Objective and subjective assessment was performed at baseline and at six to eight weeks following prolapse surgery using a three-day bladder diary, King's Health Questionnaire and Patients' Perception of Bladder Condition questionnaire (PPBC) (Coyne, Matza et al. 2006).

The PPBC is a reproducible validated single item questionnaire that assesses the patients' subjective impression of their urinary problems. The questionnaire has a six point scale with responses ranging from ''no problems at all'' to ''many severe problems''. The changes in PPBC score from baseline to post surgery were stratified into four groups: deterioration (difference in scores is positive), no change (difference in scores is 0), minor improvement (difference in

scores is negative in magnitude of 1), and major improvement (difference in scores is negative in magnitude of 2 or more) as previously described by Coyne et al.

Women who reported moderate or severe urinary urgency with or without urgency urinary incontinence were classified as having overactive bladder. All these women had undergone urodynamics at baseline prior to surgery using a standardised protocol according to ICS guidelines (Haylen, de Ridder et al. 2010)

7.2.4 NGF assay:

Midstream specimens of urine was collected at baseline and at follow up for measurement of urinary NGF levels. Urine was immediately centrifuged at 3000 rpm at 4 ^{0}C for 10 minutes. About 3 ml of urine was also sent to measure urine creatinine. The centrifuged supernatant urine was then frozen at -80^0 C and used to measure the urinary NGF levels by ELISA using the NGF E max Immuno assay system (Promega Madison WI) as described in chapter 4. The total urinary NGF levels were further normalized to the concentration of urinary creatinine (NGF/Cr level).

7.2.5 Statistical methods:

Mann Whitney U test was used to compare the NGF levels between patients with prolapse and asymptomatic patients. Urinary NGF levels were compared before and after anterior repair using Wilcoxon signed rank test. . The daytime frequency, nocturia and PPBC scores were compared before and after treatment using

Student's t test or Wilson signed rank test as appropriate. Mann Whitney U test was used to compare the NGF levels after anterior repair between the group of women who had no improvement in their PPBC scores and the group who had an improvement. This was a pilot study; hence sample size calculation was not carried out. All analyses were carried out using SPSS version 20, Chicago, IL, USA).

7.3 Results

A total of 30 women were recruited into the study. Six women were excluded due to reasons summarised on table 7.1. Four patients were excluded due to active infection or post op pain, which might influence the NGF levels.

Reason for exclusion	n
Vault Infection	1
Pain	2
Lost for follow up/late follow up	2
Urinary tract infection	1

Table 7.1: Reasons for exclusion from study

The demographics of study participants [n=24] at baseline are summarised on table 7.2. The preoperative median NGF/Cr levels were much higher than found in asymptomatic women without prolapse [26.07ng/g [7.63-182.04] vs. 2.05ng/g [0.1-11.88] p<. 05]. Fourteen patients underwent anterior and posterior repair; 2 patients underwent anterior, posterior repair and sacrospinous fixation and 8 patients underwent vaginal hysterectomy, anterior and posterior repair.

Mean Age	55.37[±11.6] *
Median parity	3[2-4] #
Mean BMI	26.3[±1.25]*kg/m^2
POP Q Stage II or III prolapse	n=23
POP Q Stage I prolapse	n=1
Daytime frequency	10.5[±2.45] *
Nocturia	1.82[±1.28] *
PPBC score	4.0[2.25-4.75] #
Detrusor overactivity on urodynamics	n=15
Voiding dysfunction	n=0

Table 7.2: Baseline demographics and clinical characteristics of patients

***Values are expressed as: mean (standard deviation)**

 # Values are expressed as median (25th-75th interquartile ranges)

There was no significant difference in the median NGF/Cr levels before and after anterior repair; *p* value > 0.05,Wilcoxon signed rank test [Table 7.3].

	Median [interquartile range] ng/g	*P*
Preoperative NGF/Cr levels	26.07[7.63-182.04]	0.14
Postoperative NGF/Cr levels	120.99[17.43-250.71]	

Table 7.3: Median NGF/Cr levels before and after anterior repair

Sixty six per cent [n=16] of patients had either minor or major improvement in their OAB symptoms after anterior repair as assessed by PPBC scores. There was a statistical significant improvement in the daytime frequency, nocturia and PPBC scores

after anterior repair in the group of patients who had improvement in their OAB symptoms [Table 7.4]. There was no significant difference between the median NGF levels [Table 7.5] after anterior repair between the group of women who had no improvement in their PPBC scores and the group who did [Mann Whitney U test]. The mean difference in the NGF levels before and after surgery was also not significantly different between women who did or did not have an improvement in their overactive bladder symptoms as assessed by PPBC [Mann Whitney U test, p=0.213].

	Pre-op	Postop	*p*
Daytime frequency	10.5(±2.45) *	5.8(±2.19)*	<0.001
Nocturia	1.82(±1.28)*	0.85((±0.93)*	<0.001
PPBC scores	4.0[2.25-4.75] #	2.0(1.0 to 3.0)#	<0.001

Table 7.4. OAB symptoms at baseline and at follow up post surgery

***Values are expressed as: mean (standard deviation)**

 # Values are expressed as median (25th-75th interquartile ranges)

	Median [Interquartile range] ng/g	*P*
Group who had improvement in their OAB symptoms [n=16]	152.56[48.68-308.78]	0.17
Group who had no improvement in their OAB symptoms [n=8]	29.60[11.95-102.89]	

Table 7.5: Comparison of postoperative Median NGF/Cr levels between groups who did or did not have an improvement in their OAB symptoms

7.4 Discussion:

The median NGF levels are high in women with anterior vaginal wall prolapse compared to asymptomatic subjects. However there was no significant difference between preoperative and postoperative NGF levels after the anterior repair. There was also no significant difference in the NGF levels between the groups who did and did not have improvement of their overactive bladder symptoms after the anterior repair. Contrary to what was postulated the urinary NGF levels were high after the anterior repair.

High urinary NGF levels in this study cohort could be due to bladder wall stretching as found in animal experiments(Persson, Sando et al. 1995). Bladder outlet obstruction led to morphological and physiological changes, which includes bladder and neuronal hypertrophy and enhanced spinal reflexes(Steers, Kolbeck et al. 1991) .This was associated with increased NGF production, which precedes the neuronal hypertrophy suggesting a link between NGF and bladder overactivity. The mechanism has been investigated further in vitro models by applying mechanical stretch to cultured smooth muscle cells and this resulted in increased expression of nerve growth factor and other specific proteins (Persson, Sando et al. 1995, Yamaguchi 2004). Stretch-activated ion channels (SACs) and protein kinase C (PKC) act as sensors to mechanical stretch stimulus(Yamaguchi 2004) and PKC is thought to play a role in controlling the synthesis of NGF(Persson, Sando et al. 1995). Stretching of bladder wall in cystocele is postulated to stimulate the stretch receptors, which results in secretion of chemical mediators

and detrusor overactivity (de Boer, Salvatore et al. 2010).

We postulated that if increased NGF levels occur as a result of bladder wall stretching and probably the cause for overactive bladder then the levels should fall after anterior repair accompanied by improvement in the overactive bladder symptoms. However contrary to the hypothesis the urinary levels were high at 6-8 weeks after anterior repair as well as in the group who had improvement in their OAB symptoms, which could be explained by more than one theory. Relief of obstruction in animal studies only caused a partial reversal of neuronal hypertrophy as well as increased NGF level in animal studies in spite of reduction in urinary frequency(Steers, Kolbeck et al. 1991). This incomplete reversal of neuronal plasticity could be the aetiology for the elevated levels in urinary NGF after anterior repair in women who have had improvement in their overactive bladder symptom. There could be other neurotrophic factors as ciliary neurotrophic factor; brain-derived neurotrophic factor and basic fibroblast growth factor responsible for neural plasticity and NGF may not be the only factor contributing to neurotrophic changes (Steers, Kolbeck et al 1991).

Although there was no significant difference between preop and post op values the post op NGF levels were high. It is possible that surgery to the vaginal wall supporting the bladder might itself be a trigger for production of NGF although there is no breech to bladder epithelium due to alteration in the structural milieu. So measuring urinary NGF levels post surgery may not be reliable .It is possible that NGF

measurements were done too early following surgery and the optimal time for measuring NGF levels after an intervention needs to be explored further.

Improvement in OAB symptoms following anterior repair was found in two thirds of this study population, which is similar to other studies. Improvement of voiding function is postulated to be one of the reasons for OAB resolution after anterior repair, but none of the study cohorts had voiding dysfunction on pressure flow studies(Basu and Duckett 2009).

The drawbacks of this study include small numbers with unequal numbers in the subgroups .The striking findings of the study results are the wide ranges in the NGF levels. These prevent any changes being statistically significant and would also mean that large numbers are required in any interventional study to detect a change.

The study has added some valuable information that Urinary NGF levels are high in women with anterior vaginal wall prolapse and OAB symptoms. However it is not possible to attribute these increased NGF levels to either bladder wall stretching or presence of OAB. Measurement of urinary NGF levels after a surgical intervention or presence of prolapse may not be reliable and has to be interpreted with caution.

CHAPTER 8

CHANGES IN NGF LEVEL AND SYMPTOM SEVERITY FOLLOWING ANTIBIOTIC TREATMENT FOR REFRACTORY OAB

Hypothesis: Urinary NGF level measurement does not change in response to antibiotic therapy for refractory OAB and cannot be used to assess treatment response.

CHAPTER 8: CHANGES IN NGF LEVEL AND SYMPTOM SEVERITY FOLLOWING ANTIBIOTIC TREATMENT FOR REFRACTORY OAB

8.1 Introduction:

Animal studies have demonstrated a direct link between increased levels of NGF in bladder tissue and bladder dysfunction(Steers and Tuttle 2006) especially bladder overactivity(Chuang et al. 2001, Zvara and Vizzard 2007). These experimental data have been explored in human studies, with increased levels of urinary NGF reported in patients with overactive bladder (OAB) (Kim et al. 2006), detrusor overactivity (DO) and interstitial cystitis(Liu et al. 2010). NGF levels have also been found to decrease after successful antimuscarinic treatment for OAB(Cho et al. 2012, Liu et al. 2010). In the light of these data some authors have suggested that NGF levels could be considered as a biomarker for OAB, as well as an assessment of therapeutic outcome in patients with OAB or DO(Liu et al. 2010). Interestingly NGF levels are higher in OAB patients who do not respond to antimuscarinics but the reasons for this are unknown(Liu et al. 2011).

Patients with DO may present with symptoms of OAB such as urgency, urgency urinary incontinence, frequency and nocturia. Detrusor overactivity is a chronic condition which has a negative impact on life(Banakhar et al. 2012) and recent studies have shown that up to 88% of women with idiopathic DO have persistent

symptoms at long-term follow up(Garnett et al. 2009). However there is no consensus on the definition of refractory overactive bladder or refractory DO. In general patients who have not responded to first line management therapies such as behavioural modification, bladder retraining and anti-muscarinic therapy are considered to have refractory overactive bladder.

Overactive bladder and detrusor overactivity have a multifactorial aetiology(Diamond et al. 2012). An interesting hypothesis has recently emerged linking OAB symptoms and sub-clinical infection. A subset of women with OAB has persistent pyuria and low count bacteriuria(Khasriya et al. 2010, Walsh et al. 2011). Underlying untreated infection could therefore be one of the causes for increased NGF levels in women with refractory OAB symptoms.

Therefore, the objective of the present study was to assess change in urinary NGF levels following antibiotic treatment and measure concomitant changes in objective and subjective symptom severity in women with refractory OAB symptoms.

8.2 Materials and methods:

Women were recruited from a tertiary urogynaecology referral centre following ethical approval (REC reference number: 11/LO/1029) into this prospective six-week cohort study.

8.2.1 Inclusion and Exclusion criteria:

Adult women with overactive symptoms, and with an urodynamic diagnosis of detrusor overactivity, who had failed to respond to a combination of lifestyle adaptations, bladder retraining and antimuscarinics were included in the study. None of the women were on antimuscarinics during the study period but they had each previously had a minimum 12-week trial, including at least two antimuscarinics.

Women were excluded if they had a history of neurological dysfunction, bladder pain, bladder cancer, bladder or renal calculi, previous continence surgery, presence of voiding dysfunction, vaginal prolapse [≥ Stage II on POPQ], urinary tract infection by conventional microbiological criteria (colony count ≥10^5) or history of allergic reaction or side effects to more than one antibiotic of choice.

8.2.2 Study design:

Study participants were seen prior to and six weeks following antibiotic treatment. A clean catch midstream specimen of urine (MSU) was collected at full bladder at baseline and after six weeks of antibiotic treatment to measure the urinary NGF levels. Assessment of symptom improvement was done using validated questionnaires at baseline and six weeks after treatment. To establish normative ranges MSU samples were also obtained from asymptomatic subjects who did not suffer with overactive bladder symptoms. These patients were recruited from general gynaecology clinics.

8.2.3 Treatment:

Participants were treated with a six-week course of rotational antibiotics. Patient's previous sensitivities to proven urinary tract infection and drug allergies were taken into consideration prior to treatment. Three consecutive antibiotics were given for two weeks each; ciprofloxacin [1gm orally in two divided doses] to cover gram-positive and gram-negative organisms, doxycycline [200mg orally in two divided doses] to cover chlamydia trachomatis and atypical organisms like Mycoplasma hominis and ureaplasma urealyticum and cephalexin [1500mg orally in three divided doses] or Co-amoxiclav [1875 mg orally in three divided doses] to cover gram positive and gram-negative urinary pathogens.

8.2.4 Methods of assessment:

Objective and subjective assessment was performed at baseline and after six weeks of antibiotic therapy using a three-day bladder diary, Patients' Perception of Intensity of Urgency Scale (PPIUS)(Cartwright et al. 2011), King's Health Questionnaire and Patients' Perception of Bladder Condition questionnaire (PPBC)(Coyne et al. 2006).

The PPIUS is a tool designed to assess the intensity of urinary urge sensation. It has a 5-point scale ranging from 0 to 4, with grades 3 and 4 representing severe urgency and urgency incontinence respectively.

The PPBC is a reproducible validated single item questionnaire that assesses the patients' subjective impression of their urinary problems. The questionnaire has a six point scale with responses

ranging from ‘‘no problems at all’’ to ‘‘many severe problems”. The changes in PPBC score from baseline to week six of antibiotic treatment were stratified into four groups: deterioration (difference in scores is positive), no change (difference in scores is 0), minor improvement (difference in scores is negative in magnitude of 1), and major improvement (difference in scores is negative in magnitude of 2 or more) as previously described(Coyne et al. 2006).

8.2.5 NGF assay:

Collected urine samples were immediately refrigerated and centrifuged at 3000rpm at 4 ^{0}C for 10 minutes. Three millilitres of urine were also sent for measurement of urinary creatinine. The centrifuged supernatant urine was stored at -80^{0}C, until further processing. Urinary NGF levels were measured by enzyme linked immunosorbent assay (ELISA) using the NGF Emax Immunoassay System (Promega, Madison, WI, USA) as described in chapter 4.The measured urinary NGF levels were normalised against the concentration of the urinary creatinine to take into account the variation in urine concentration.

8.2.6 Statistical methods:

Based on previous studies(Liu et al. 2010) a sample size of 34 was estimated to provide 80% power for a half standard deviation change in NGF, and 90% power for a 60% of standard deviation change, with alpha set at 0.05. Spearman correlation test was used to assess the correlation between the baseline NGF values and the PPBC scores. Urinary NGF levels were compared before and after antibiotic

therapy using Wilcoxon signed rank test, and with levels from a parallel control group of asymptomatic women using Mann Whitney U test. The daytime frequency, nocturia, urgency scores and PPBC scores were compared before and after treatment using Student's t test or Wilson signed rank test as appropriate. Mann Whitney U test was used to compare the NGF levels after antibiotic treatment between the group of women who had no improvement in their PPBC scores and the group who did. (SPSS, Inc. version 14.0, Chicago, IL, USA)

8.3 Results

Thirty-nine symptomatic women with refractory detrusor overactivity were recruited who reported urinary urgency as well as urgency incontinence. In addition MSU samples were collected from 27 asymptomatic women who served as controls. Four patients (10.2%) were excluded from the final analysis due to discontinuation of antibiotics, commonly due to gastro intestinal side effects [n=3] and vulvovaginal candidiasis [n=1].

The median NGF/Cr level in the control group was 1.96 ng/g (25^{th}-75^{th} interquartile range, 0.1-11.47). The pre-treatment median NGF/Cr level in the symptomatic group was 21.09 ng/g (25^{th}-75^{th} interquartile range, 6.82 – 100.93). There was a significant difference in the median NGF/Cr levels between the control and the symptomatic group at baseline (Mann Whitney test, Z value -3.060; *p* value < 0. 005).

The baseline demographics and the clinical characteristics of the symptomatic group (n=35) are listed in Table 8.1. There was a statistical significant difference in the mean age between the symptomatic and asymptomatic group (Independent sample T test, p value =0.04). However there was no correlation between the NGF values and age (Spearman correlation test, rho 0.06; *p* value >0.05). There was a significant correlation between the baseline NGF/Cr values and the PPBC scores (Spearman's rho 0.34; *p* value< 0.05).

Mean Age	44.8(±14.9)*
Mean duration of symptoms (months)	22 (±5.6)*
Median Parity	2.0(1.0to 3.5)#
Median Number of antimuscarinics tried	3.0(2.0 to 4.0)#
Mean day time frequency	12.8(±3.5)*
Median Nocturia	2.0(1.0 to 3.0)#

Table: 8.1 Baseline demographics and clinical characteristics of patients.
***Values are expressed as: mean (standard deviation)**
 # Values are expressed as median (25^{th}-75^{th} interquartile ranges)

The NGF/Cr levels decreased significantly after six weeks of antibiotic therapy as shown in Table 8. 2 (Wilcoxon Signed rank test, Z value-2.424;*p*=0.015). OAB symptoms including daytime frequency, nocturia and urgency were also significantly improved by antibiotic therapy as shown in Table 8.3.

	Baseline		After 6 weeks of antibiotics		*p*
	Median	25th-75th IQR	Median	25th-75th IQR	
NGF/Cr levels ng/g	21.09	6.82-100.93	13.33	2.81-23.62	.015

Table 8.2. Urinary NGF/Cr levels at baseline and six weeks

	Pre-treatment	After 6 week course of antibiotics	*p*
Day time frequency	12.8(±3.5)*	8.7(±2.7)*	<0.005
Nocturia	2.0(1.0 to3.0)#	1.0(0 to 3.0)#	<0.050
PPBC scores	5.0 (4.0 to 6.0)#	2.0(1.0 to 4.0)#	<0.005
PPIUS scores	3.0(1.0 to 5.0)#	2.0(1.0 to 3.0)#	<0.005

Table 8.3. OAB symptoms at baseline and six weeks after antibiotic therapy
***Values are expressed as: mean (standard deviation)**
 # Values are expressed as median (25th-75th interquartile ranges)

The magnitude of improvement in PPBC after antibiotics therapy is as shown in Table 8.4. Overall 74% of women found a minor to major improvement in their symptoms after antibiotic treatment. The median NGF/Cr values after antibiotic treatment were lower in this group compared to that of women who had no improvement in their PPBC scores. However this difference was not statistically significant (17.41 vs. 10.61; Mann Whitney U test; p=0.52).

Magnitude of Improvement in PPBC	Proportion
No change = score of 0	26%
Minor improvement = score of -1	17%
Major improvement =score of -2 or more	57%

Table 8.4: Magnitude of improvement in PPBC scores

8.4 Discussion:

Urinary NGF/Cr levels were significantly higher in the symptomatic study group with refractory OAB symptoms when compared to the control subjects at baseline, which is in keeping with the results of other studies(Kim et al. 2006, Liu et al. 2010). Significant correlation existed between the baseline NGF/Cr values and the severity of urinary symptoms. Antibiotic treatment in women with refractory OAB symptoms was associated with a significant decrease in the NGF levels as well as improvement in patients' symptoms and PPBC (figure 8.1 &Table 8.3). Supporting this data, animal studies have shown a link between increased NGF levels and infection. Experimentally induced bacterial cystitis in animal bladders was found to result in increased NGF levels(Dupont et al. 2001). NGF is produced by the urothelium and detrusor in the bladder but other possible sources are eosinophils and macrophages (Aloe et al. 1997).Therefore women with refractory OAB symptoms may have inflammation secondary to infection, associated with increased NGF levels.

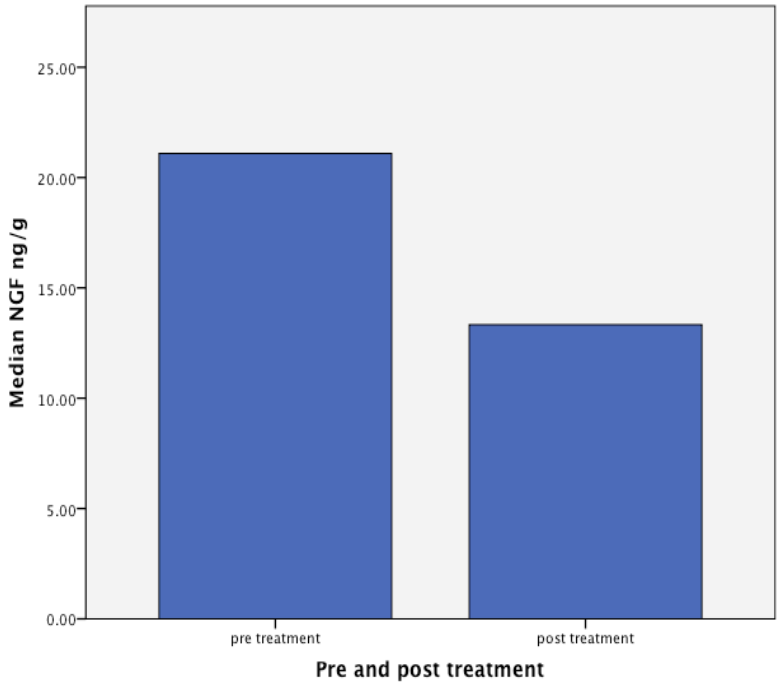


Figure 8.1:Median NGF/Cr levels before and after antibiotic treatment

The diagnosis of overactive bladder or detrusor overactivity is made only in the absence of urinary tract infection with a culture of ≥10^5 CFU/ml or other obvious pathology(Haylen et al. 2010). Interestingly it has been shown that the likelihood of having asymptomatic bacterial cystitis in women undergoing urodynamics is 6% in women with detrusor overactivity compared to 1% in women with urodynamic stress incontinence(Moore et al. 2000) and women who develop OAB in later life are more likely to suffer from urinary tract infections in their childhood(Salvatore et al. 2012)

In a study in women with lower urinary tract symptoms (LUTS) the incidence of bacteriuria was 29% when a threshold of 10^2 CFU/ml was chosen, compared to 15% at the routinely used threshold of 10^5 CFU/ml(Khasriya et al. 2010). Similarly, in a study using a threshold

of 10^3CFU/ml, 28% of women with OAB were found to have bacteriuria(Hessdoerfer et al. 2011). In women with acute exacerbation of OAB symptoms, the prevalence of bacteriuria on MSU samples at low count of $\geq 10^3$ was 39% compared to 6 % in controls(Walsh et al. 2011). Concerns have therefore been raised that urinary tract infections may be missed or under diagnosed in women with symptoms of overactive bladder if a threshold of 10^5 CFU/ml is used for diagnosis.

There is also evidence to suggest that infection caused by atypical organisms such as mycoplasma hominis; ureaplasma urealyticum and chlamydia not detected by routine urine culture methodology may be associated with overactive bladder symptoms. A retrospective observational study in patients with urinary symptoms showed a prevalence of 13.7% of atypical organisms(Latthe et al. 2008, Steers and Tuttle 2006, Zvara and Vizzard 2007)
Based on the available data, women with OAB seem to have an increased likelihood of having asymptomatic bacterial infection that may be missed due to usage of a higher threshold of 10^5 for diagnosis or lack of specific culture techniques.

Bacterial infection has been shown to trigger neutrophil infiltration into bladder mucosa and also production of cytokine IL-6. Therefore untreated infection could result in chronic cystitis and non-responsiveness to anti muscarinics.

Inflammation has been postulated to be one of the causative factors for the development of OAB(Hsiao et al. 2012, Saini et al. 2008). Bladder biopsies from patients with detrusor overactivity before intravesical botulinum toxin injection revealed presence of chronic inflammation in 59% of cases(Apostolidis et al. 2008). Animal and clinical studies on inflammatory biomarkers have provided evidence for the role of cytokines, prostaglandins and nerve growth factor in the development of lower urinary tract symptoms(Kim et al. 2006, Liu et al. 2010, Steers and Tuttle 2006, Tyagi et al. 2010, Zvara and Vizzard 2007).

We postulated that bladder inflammation might be the underlying pathogenesis for their refractory overactive bladder symptoms, which may occur as a result of chronic subclinical infection. Therefore we treated our patients with a combination of antibiotics to treat common urinary tract pathogens such as gram negative and gram-positive organisms as well as atypical organisms such as mycoplasma hominis, ureaplasma urealyticum and chlamydia. The main limitation of this study is the lack of evidence for infective aetiology in the treatment group. However treatment with antibiotics in women with refractory overactive bladder significantly decreased the urinary NGF levels (table 2). This study also shows a statistical significant improvement in the daytime frequency, nocturia and urgency and PPBC scores after antibiotic treatment. Twenty-six per cent of women who received antibiotic treatment did not find any improvement in their symptoms, whereas 74% of them found a minor to major improvement in their symptoms (Table 8.4). The

median NGF/Cr values after antibiotic treatment in women who had improvement in their PPBC scores were lower compared to those of women who had no improvement. However this difference did not reach statistical significance, which could be due to small numbers in the subgroups.

Antibiotics could have indirect immune modulatory effects in addition to antimicrobial effects(Tauber and Nau 2008). There is evidence that drugs such as Doxycycline, ciprofloxacin; cephalexin and Co-amoxiclav exert anti-inflammatory properties (D'Agostino et al 1998,Sun et al 2000,Miyachi et al 1986 & Casellas et al 1998). Therefore it is possible that the antibiotics that were used had an anti-inflammatory effect, which resulted in improvement of the overactive bladder symptoms rather than an anti-infective effect. It is also possible that the treatment with antibiotics had a placebo effect. However the improvement in the PPBC scores is far larger at 74% than most placebo studies where improvement levels of only 40-45% have been reported with placebo(Van Kerrebroeck et al. 2009). There is one study using sequential antibiotics compared with placebo but the study group were patients with interstitial cystitis, which showed no significant difference between the two treatment arms(Warren et al. 2000). This report is not applicable to the present study due to the differences in pathology as the patients had pain and no incontinence. Therefore we can be confident this is the first report of sequential antibiotic treatment of this group of patients. Although a small number of adverse events were found in this short-term study, it is important to keep in mind that microbial resistance

could occur with long-term of antibiotics particularly if the treatment is repeated for symptom recurrence.

The limitations of this study are inherent in the uncontrolled design, which could create a risk of biases and the use of patients who had failed first line therapies limits the generalizability. Although the NGF measurements were performed blind, limiting the risk of experimenter bias, without a group of untreated controls the changes observed may result from unmeasured factors. A placebo-controlled study is therefore required to quantify the therapeutic effect. These data provide a convincing rationale for the design of a controlled trial of this therapy, and it is important for such a study to assess the long term outcome of treatment.

Urinary NGF levels may be responsive to antibiotic treatment in women with refractory OAB. This key finding provides scope for the potential use of urinary NGF to assess treatment response in OAB patients. Furthermore targeted antibiotic treatment is associated with subjective and objective improvement in OAB symptoms. Antibiotics may be acting by an antibacterial or anti-inflammatory effect. Antibiotic treatment could be considered in women with refractory OAB symptoms before invasive treatment modalities such as botulinum toxin, neuromodulation and bladder augmentation. The use of NGF as a predictor of antibiotic treatment response merits testing in a blinded randomized trial.

CHAPTER 9

CONCLUSION

CHAPTER 9: CONCLUSION

An ideal biomarker should be easily measurable, reliable and valid .It should have either superior performance to existing tests or provide new information and improve existing tests. Most importantly it should help the clinician to manage the patients better.

There are few advantages of using urinary NGF as a biomarker in the assessment of patients with LUTD. Urine is easily accessible and obtaining a urine sample for NGF assay would be more acceptable from a patient's perspective since it is less invasive especially on comparison with urodynamics. Although cost analysis has not been done, NGF assays are not expensive since the cost of ELISA analysis is £4 per urine sample excluding lab consumables. Therefore urinary NGF assay can be less expensive and non-invasive on comparing to urodynamic assessment and more objective and less biased on comparing to patient reported outcomes.

However there are certain limitations with urinary NGF quantification. The assays are done in batches and there is no rapid turn over which affects its practicality in clinical practice.

Interindividual and intraindividual variability is a major concern with biomarkers and the latter is mainly due to lab errors or conditions specific to the individual (Mayeux 2004).The test-retest reliability of currently available urinary NGF assays by ELISA has

been reported for the first time in chapter 4. It is a reliable test with an ICC of 0.889 with almost perfect agreement. Test-retest assessments were done within a short time interval of four weeks and these measurements represent the reproducibility only within this particular time interval.

The criterion validity of measuring urinary NGF levels has been evaluated thoroughly with ROC analysis and sensitivity, specificity, PPV and NPV has been reported in chapter 5.The urinary NGF levels are significantly increased in patients with LUTS and different symptomatic groups such as OAB, SUI and BPS on comparing with controls. On ROC analysis, NGF levels demonstrate poor discriminant ability in differentiating different symptomatic and urodynamic groups [AUC <0.6] except for the OAB and non OAB group [AUC=0.66]. Using 13.0 ng/g as a cut off for urinary NGF/Cr concentration the test provides a sensitivity of 80.7% and specificity of only 39.4%, PPV of 53.33% and NPV of 70.42% in diagnosing OAB among patients with LUTS. NGF was also a good predictor of patients having OAB when multivariate logistic regression was performed to assess the effects of age and NGF on predicting participants having OAB with an adjusted odds ratio of 1.006; *p*=. 047.

Urodynamics was used as the reference standard to evaluate the diagnostic accuracy of NGF levels and there was no significant difference in the NGF levels between the different urodynamic groups such as DO, USI and MUI.

All the focus has been on OAB and IC/BPS but this study has demonstrated increased urinary NGF levels in stress urinary incontinence contrary to the results from Liu and co-workers. This finding supports the results from animal study with experimental urethrolysis and questions the specificity of urinary NGF levels since it is increased in various conditions of LUTD.

These results show that although Urinary NGF/Cr concentration is certainly increased in patients with LUTS especially in OAB, it cannot be used as a discriminatory tool. The limitations of urinary NGF assays seem to be low specificity, lack of discrimination power and wide standard deviations. It is difficult to establish normal ranges for NGF because NGF levels can be undetectable in OAB patients.

The link between NGF and inflammation was explored further in the study detailed on chapter 6.There was no relationship between NGF levels in OAB patients and the severity of trigonitis, erythema or trabeculation on cystoscopy or cystitis on histology. NGF cannot be the sole mediator of inflammation and there are several neurotransmitters and mediators of inflammation, which are involved in the pathology of bladder inflammation and OAB. Cystoscopy findings itself may be a poor indicator of bladder inflammation however the lack of association between NGF levels and severity of cystoscopic findings suggest either that NGF should not be used as surrogate markers of bladder inflammation or cystoscopic findings are not valuable in classifying degrees of inflammation.

We speculated that NGF levels should decrease after anterior repair in women who have had an improvement in their overactive bladder symptoms and this was explored further. [Chapter7]. However contrary to our speculation the urinary NGF levels were high after the anterior repair in women who had improvement of their overactive bladder symptoms. Although it is a pilot study the study has added some valuable information that measurement of urinary NGF levels after a surgical intervention or presence of prolapse may not be reliable and has to be interpreted with caution. A future study needs a larger study group and with a longer follow up of six months to ensure that surgical inflammation has settled.

To explore the role of NGF as a biomarker to assess therapeutic outcome, NGF levels were measured before and after antibiotic treatment for refractory OAB in the study described on chapter 8. About 74% of women found a minor to major improvement in their symptoms after antibiotic treatment and NGF levels were found to decrease significantly after six weeks of antibiotic therapy. Although the NGF measurements were performed blind, limiting the risk of experimenter bias, without a group of untreated controls the changes observed may result from unmeasured factors. The improvement after treatment could be due to variation in the measurement of biomarker.

However the findings of raised NGF levels in OAB and response to treatment provides scope for NGF as a potential inflammatory biomarker for OAB. Urinary NGF levels are increased in several conditions of LUTD, which reduces its specificity. Similar to cholesterol(Aronson 2005) or CRP, NGF may not be a good diagnostic biomarker but perhaps may have a role as a marker of treatment response.

Thus to conclude urinary NGF level measurement has limited role in assessment of women with LUTD and prolapse rejecting the null hypothesis.

The translation of biomarker from discovery to clinical practice is a difficult process and the research exploring the role of NGF as a potential biomarker is still in its infancy.

CHAPTER 10

RECOMMENDATIONS FOR FUTURE RESEARCH

CHAPTER 10: RECOMMENDATIONS FOR FUTURE RESEARCH

The results from this study have added new information with regards to the criterion validity of urinary NGF measurement, which helps in bridging the gaps in the evaluation of urinary NGF as a biomarker as well as corroborating and refuting some of the available evidence. However the role of urinary NGF as a diagnostic biomarker needs further evaluation and the following recommendations are put forward.

There are pitfalls with the NGF assay itself and this needs to be improved by standardizing the sample collection and the ELISA assays. All the reported studies in the literature have used Promega ELISA kits and the antibodies used are prepared from animal species but marketed for research studies to measure NGF levels only in tissue culture supernatants or tissue extracts. There are NGF ELISA kits prepared with human antibodies but these kits have not been employed to measure urinary NGF levels. The commercially available NGF ELISA kits need to be validated for measuring urinary NGF levels in human. The assays need to be developed to improve the detection of NGF in urine. Assays need to be quicker and simpler since the currently available assays are laborious and involve a 3 day process.

Measurements can be affected by prolonged intervals due to uncontrolled variables such as degeneration of protein. Further

studies with large numbers need to be done at various time intervals to account for variations in the assays. In this study only intra observer reliability after an interval of four weeks has been assessed and it is important to also test for interobserver reliability in future studies.

There are certain inherent problems with NGF quantification in urine sample. The urine NGF levels may not be exclusively derived from bladder. The only evidence is from a small experimental study by Steer et al. Tissue levels of NGF were assayed in organs other than the urinary bladder from normal and obstructed animals to ascertain whether the increases in bladder NGF were specific for the urinary tract. No significant increases were seen in NGF content in other tissue specimens(Steers and Tuttle 2006). More evidence is needed for the bladder specificity of urinary NGF levels .It is also important to assess the correlation between urinary and bladder NGF levels.

Urinary NGF levels can be influenced by various systemic disorders in which serum NGF levels are raised. This can result in false positives .In order to reduce the false positives the effect of raised serum NGF levels on urinary secretion need to be investigated and accounted for in future assays.

It is also possible that certain drugs can affect the levels of NGF such as progesterone(Choi et al. 2010) psychotropic drugs(Hassanzadeh and Rahimpour 2011), steroids (Schaper et al. 2009), anti-inflammatory drugs(Jang et al. 2006) (Hochstrasser et al.) ,

isoprenaline , phenylephrine(Persson et al. 1997)among many. These confounding factors need to taken into account and evaluated further in order to avoid bias.

Patient recruitment criteria need to be standardized in future studies that evaluate the role of urinary NGF as a diagnostic biomarker in LUTD. Larger studies are needed to assess the relationship between urinary NGF levels and anterior vaginal wall prolapse, BMI and ethnicity. There is a need for larger samples with age matched controls to evaluate and determine the accuracy, reliability, interpretability, and feasibility of NGF assays before we draw any conclusion. To implement urinary NGF as a biomarker in clinical practice the limitations in NGF quantification need to be addressed first.

Technical advances allow simultaneous measurement of biomarkers using multiplex technology. LUTD is multifactorial therefore NGF alone may be insufficient to act as a sole urinary marker for OAB. Measurement of polypeptides and other neurotrophic factors apart from NGF such as BDNF, which are implicated in the pathophysiology of bladder dysfunction, need to be evaluated. This may provide a scope for introducing a series of multiple biomarkers, which may serve as non-invasive diagnostic tools in assessment of patients with LUTD. Such biomarkers may be used alone or in combination to screen and diagnose various LUTD and may complement existing diagnostic tools such as frequency volume chart, bladder wall thickness, urodynamics etc. They may also be valuable in clinical

trials as well as monitoring therapeutic interventions in clinical practice.

Exploring and evaluating the role of NGF has helped us to understand the complex pathophysiological mechanisms involved in LUTD. This has also opened a new avenue of treatment for patients with LUTD where NGF can be an etiological factor. Recently antiNGF monoclonal antibody has been used to treat patients with interstitial cystitis in a phase 2 trial and has demonstrated promising results (Evans et al 2011). Treatment directed against NGF action is an exciting concept, which has scope for research in the management of patients with LUTD.

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