BIOMEDICAL MARKERS OF RESPONSE TO INTRAVESICAL BCG TREATMENT IN HIGH-GRADE NON-MUSCLE INVASIVE (PTA AND PT1) TRANSITIONAL CELL CARCINOMA OF THE BLADDER

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Abstract

Introduction: Intravesical Bacillus Calmette–Guérin (BCG) immunotherapy is the main treatment for bladder high-grade non-muscle invasive transitional cell carcinoma (HGNMITCC) following initial resection. Unfortunately, about 30% of patients will not respond to treatment and they carry a high risk of disease progression. The alternative, radical cystectomy, has major risks with high morbidity and mortality. The ability to predict the response to BCG treatment would be a useful tool in the selection of appropriate treatment modalities. This study investigated a variety of detectable immune responses in blood and urine to establish if there were differences between responders and non-responders to BCG treatment. We evaluated whether there were detectable immunological differences in blood or urine that could explain or predict outcome.

Material and Methods: Patients with HGNMITCC who were offered intravesical BCG treatment were included. Blood samples were obtained before induction of BCG treatment (6 instillations) and 8 weeks after induction. Urine samples were collected 4 hours after the final instillation.

In vitro stimulation of peripheral-blood mononuclear cells (PBMC) with tuberculin purified protein derivative (PPD) followed by antibody labelling of extra- and intracellular markers was used to assess the differentiation and activation status of T-lymphocytes and their subsets. Secreted cytokines were also measured after *in vitro* PBMC stimulation with PPD and lipopolysaccharide (LPS) stimulation of blood to explore correlations with the percentages of cytokine producing lymphocytes and secreted cytokines in urine.

Cell staining after *in vitro* Staphylococcus enterotoxin B (SEB), which was used as control, was also used to check for non-antigen specific differences.

Results: The clinical outcomes and the pre- and post-treatment immune response data were available for 43 cases. The median age was 71.5 year (range 49-90 years). There were 9 females and 34 male. Twenty-five patients reported being vaccinated against tuberculosis with BCG in the past or had evidence of a scar. After induction BCG immunotherapy treatment, 17 patients had tumour recurrences detected on cystoscopic surveillance, which were confirmed histologically.

Before induction of BCG treatment, the median percentage of PPD-inducible interferon-gamma (IFNγ)+ CD4 T lymphocytes was significantly lower in patients who had tumour recurrences in comparison to patients who had no recurrence; 0.26% (range 0-0.94%) and 0.43% (range 0–2.11%) respectively [*P* value = 0.0253]. Levels of secreted IFNγ measured after *in vitro* PPD stimulation were also significantly lower before treatment in patients who had recurrences after treatment; 1989pg/ml (range 38.47-38486pg/ml) and 8558pg/ml (range 581.9-91290pg/ml) respectively [*P* value = 0.0060].

The percentage of pre-treatment PPD-inducible interleukin-2 (IL2)+ CD4 T lymphocytes was also significantly lower in the recurrence group compared to the recurrence-free group; 0.15% (range 0-2.22%) and 0.61% (range 0.06-1.61%) respectively [*P* value = 0.0310]. Levels of IL2 measured post *in vitro* PPD stimulation were also significantly lower; median 187.1pg/ml (range 18.29-1155pg/ml) and 489.5pg/ml (range 113.8-1622pg/ml) respectively [*P* value = 0.0013].

The percentage of pre-treatment PPD-inducible tumour necrosis factor (TNF)+ CD4 T lymphocytes was found to be significantly lower in the recurrence group compared to the

recurrence-free; 0.45% (range 0 – 1.77%) and 1.03% (range 0-0-4.48%) respectively [*P* value = 0.0085].

Differences were also seen in the pre-treatment percentage of the polyfunctional (CD40L+ IFNγ+IL2+TNF+) T lymphocytes; 0.04% (range 0-0.26%)and 0.23% (range 0-1.3%) respectively [*P* value = 0.0013].

Similar significant differences were seen in the pre-treatment IL2+, TNF+ and CD40L+IFNγ+IL2+TNF+ after SEB stimulation, suggesting a general "non-PPD specific" immune differences between the groups.

Conclusion: Significant differences in immune responses were present between non-responders and responders to BCG immunotherapy treatment, before and after treatment. These differences may have clinical significance as predictors of outcome to intravesical BCG treatment. The presence of similar differences after SEB challenge, suggests a more generalised "non-antigen specific" immune variance that might influence response to such immunotherapy treatment. More research is recommended to confirm these findings as well as investigate the causes for such differences.

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1. Abbreviations

Ab	Antibody
AF647	Alexa Flour 647
Alexa700	Alexa Flour 700
APC	Antigen presenting cell
APC-Cy7	Allophycocyanin cyanin7
BCG	Bacillus Calmette-Guérin
BFA	Brefaldin A
BSA	Bovine serum albumin
BV421	Brilliant violet 421
BV571	Brilliant violet 571
CCR	Chemokine receptor
CD	Cluster of differentiation
CD40L	CD40-ligand
CI	Confidence interval
CIS	Carcinoma in situ
CUETO	Urological Club for Oncological Treatment
DMSO	Dimethyl sulfoxide
ECD	Electron coupled dye
EDTA	Ethylenediaminetetraacetic acid
EORTC	European Organisation for Research and Treatment of Cancer
FACS	Fluoresence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
g	Centrifugal force
h	Hour
HGNMITCC	High-grade non-muscle invasive bladder transitional cell carcinoma
IFNγ	Interferon gamma
IL1β	Interleukin-1 beta
IL2	Interleukin-2
IL4	Interleukin-4
IL6	Interleukin-6
IL8	Interleukin-8
IL10	Interleukin-10
IL17a	Interleukin-17a
IL12p70	Interleukin-12p70
IL13	Interleukin-13
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
min	Minute
mg	Milligram
ml	Millilitre
µg	Microgram
µl	Microliter
MMC	Mitomycin C
ng	Nanogram
NK cells	Natural killer cells

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-cy7	Phycoerythrin cyanine dye
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PPD	Purified protein derivative of *M. tuberculosis*
rs	Spearman's correlation coefficient
RPMI	Roswell Park Memorial Institute Medium
SEB	Staphylococcal enterotoxin B
SSC	Sideward scatter
TB	Tuberculosis
TCC	Transitional cell carcinoma
Th	T helper
TNF	Tumour necrosis factor
TNM	Tumour-node-metastasis
TURBT	Transurethral resection of bladder tumour
UICC	International Union Against Cancer

2. Introduction

2.1 Bladder Cancer

Bladder cancer is the 7th most common cancer in the UK with 10,000 new cases and 5,000 deaths each year. It is the fourth most common cancer in men and seventh in women. Over 90% of bladder cancer are transitional cell carcinoma (TCC) [1]. Marked differences have been reported in different ethnic groups, probably in part due to genetic variations [2], with a higher incidence in white than non-white populations.

Environmental exposure to tobacco and industry-related carcinogens are the primary risk factors for bladder cancer. The incidence of bladder cancer is two to three times higher among smokers compared to those who never smoked [3]. Alcohol consumption, dietary factors and the use of hair dyes have also been suggested as risk factors for bladder cancer [4-8].

At presentation, 70-80% of bladder TCC are non-muscle invasive disease. These are treated by transurethral resection of the bladder tumour (TURBT) followed by the use of intravesical chemotherapy drugs such as mitomycin C (MMC) and/or immunotherapy with the tuberculosis (TB) vaccine, Bacillus Calmette–Guérin (BCG). The remaining 20-30% of bladder tumours invade the muscle and are treated by cystectomy or palliation in advanced stages.

Bladder TCC has a high recurrence rate of 70% (similar stage) and progression rate of 35% (higher grade or stage) [9]. It is considered one of the most expensive malignancies to treat. The cost of diagnosis, treatment and 5-year follow up for patients diagnosed in the UK between 2001 and 2002 was £53.39 million with a mean cost per patient of £8349 [10]. In the United States the cost per case from diagnosis to death is $200,000 per patient [11].

BCG, an attenuated live strain of *Mycobacterium bovis*, is a well-known immunotherapeutic agent and the most common intravesical therapy for treating high-grade non-muscle invasive bladder transitional cell carcinoma (HGNMIBTCC). It was first used as a vaccine for TB in humans in 1921 [12]. Dr Alvaro Morales used intravesical BCG for the first time in 1976 to treat bladder cancer in 9 patients and discovered that it altered the recurrence pattern favourably [13]. Subsequently, intravesical BCG has been demonstrated to reduce the recurrence rate and the risk of progression to muscle invasive disease [14-16]. BCG is instilled into the bladder via a catheter with the recommendation to leave it for 2 hours to achieve maximum benefit. The induction course consists of 6 instillations once weekly. Subsequent use of BCG, so called maintenance BCG, is recommended but the optimum regime has not been determined and a minimum of 1 year is recommended [17]. BCG causes significant side effects in 90% of patients; the main symptoms experienced are dysuria, pyrexia, flu-like symptoms and haematuria and many patients do not complete the full BCG course [17].

The response rate to intravesical BCG ranges from 50% to 70% of those who complete the course. Cystectomy is offered to those who progress to muscle invasive disease or have recurrent HGNMITCC despite treatment. Between 30 and 50% of patients fail BCG treatment despite completing their treatment course [17]. Merz *et al.* reported a 30% risk of invasive disease and death from cancer if the disease is persistent at 9 months after treatment [18]. Solsona *et al.* found that 80% of patients who have persistent disease at 3 months progress to invasive disease [19]. In a series of 90 patients who were followed up over a 20 year period, Herr and Sogani reported improvement in survival following early cystectomy after BCG failure in superficial disease (92% vs. 56%) as well as invasive disease (54% vs. 36%)[20].

TNM Classification of bladder cancer

The tumour-node-metastasis (TNM) classification was first developed by a French surgeon, Pierre Denoix, between 1943 and 1952 to describe the extent of cancers in the body. It represents the different categories of cancer development; (T) refers to the tumour either by size or depth of invasion, (N) refers to lymph node involvement and (M) describes the distant spread or metastasis [21].

The objective of the TNM classification was to aid clinicians in categorising the extent of cancer, in order to help plan treatment and predict prognosis.

The International Union Against Cancer (UICC) published its first TNM recommendation in 1958, which was for breast and larynx cancers at that time. Over the years, the UICC published more TNM classification to involve 23 different body sites. The 7th edition was published in 2009 (Table 1) [22].

T Primary Tumour	
Tx	**Primary tumour cannot be assessed**
T0	**No evidence of primary tumour**
Ta	**Non-invasive papillary carcinoma**
Tis	**Carcinoma in situ: 'flat tumour'**
T1	**Tumour invades subepithelial connective tissue**
T2	**Tumour invades muscle**
	-T2a Tumour invades superficial muscle (inner half)
	-T2b Tumour invades deep muscle (outer half)
T3	**Tumour invades perivesical tissue:**
	-T3a Microscopically
	-T3b Macroscopically (extravesical mass)
T4	**Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall**
	-T4a Tumour invades prostate, uterus or vagina
	-T4b Tumour invades pelvic wall or abdominal wall
N Lymph Nodes	
Nx	**Regional lymph nodes cannot be assessed**
N0	**No regional lymph nodes metastasis**
N1	**Metastasis in a single lymph node in the true pelvis**
N2	**Metastasis in multiple lymph nodes in the true pelvis**
N3	**Metastasis in a common iliac lymph node(s)**
M Distant metastasis	
Mx	**Distant metastasis cannot be assessed**
M0	**No distant metastasis**
M1	**Distant metastasis**

Table 1: TNM classification of bladder tumours

Grading of bladder cancer

Another important factor in cancers is the grade of cellular aplasia. This varies from well differentiated to poorly differentiated tumour depending on the degree of resemblance to the original cells. In 1973 the World Health Organisation (WHO) published the grading for bladder cancers, which has been widely accepted and used. In 2004 the WHO published further updates on the grading of bladder cancer which was proposed by the International Society of Urological Pathology and the WHO (Table 2) [23].

Urothelial papilloma grading - WHO (1973)
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
Urothelial tumour grading – WHO (2004)
Flat lesions:
Hyperplasia (flat lesion without atypia or papillary)
Reactive atypia (flat lesion with atypia)
Atypia of unknown significance
Urothelial dysplasia
Urothelial Carcinoma in situ (CIS)
Papillary lesions:
Urothelial papilloma (which is a completely benign lesion)
Papillary urothelial neoplasm of low malignant potential
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

Table2: WHO grading of bladder tumours

In transitional cell carcinoma of the bladder the most important factors of prognosis are the grade and stage.

2.2 The immune system

2.2.1 T lymphocytes and cytokines

The immune system evolved to protect against infections by pathogenic microbes. It is subcategorised into the non-specific innate and the specific adaptive immune systems. The innate immune system includes phagocytes, granulocytes and mast cells, while the

adaptive immune system includes the humoral (B cells) arm and the cell mediated (T cells) arm that includes many phenotypes and functional subsets.

Although the innate and the adaptive systems recognise and react to pathogens in different ways, they interact and are dependent on each other in order to provide efficient immunity. When released from the thymus gland, CD4 and CD8 T cells are mature but naïve and typically carry the external markers CCR7, CD27 and CD28. Once they are exposed to antigens and start differentiating into functional cells, they change the expression of these external markers [24, 25]. Specific receptors expressed on cytotoxic CD8 cells recognise antigen presented in the context of MHC class I molecules and once stimulated, secrete cytokines and other inflammatory products in order to kill target cells. CD4 T cells interact with antigen-presenting cells (APC), and once stimulated, they can differentiate into several subtypes, including Th1, Th2, Th3 and Th17. Immune cells interact via direct cell-to-cell contact or via the secretion of cytokines, which are chemical messengers that help cells communicate, grow, differentiate or even die. Depending on their subtype, CD4 cells can secrete specific cytokines that can stimulate or suppress other immune cells to deal with pathogens [26].

Previous published reports revealed an increase in Th1 subsets and Th1-related cytokines in the bladder following BCG instillations [27, 28]. This would be expected as the Th1 cells operate against intracellular pathogens like *M. tuberculosis*, and in this case *M. bovis* BCG. The Th1 cells, once stimulated, secrete IFNγ, TNF and IL2 which recruit macrophages, natural killer (NK) cells, B-cells, and other Th1 cells into the area [29].

When BCG is administered in the bladder, macrophages phagocytose the bacteria and then migrate into the local lymphoid organs. Antigens are presented to the naïve helper T lymphocytes, which differentiate into Th1 cells that are better equipped to contain intracellular pathogen such as mycobacteria. Once differentiated into Th1 lymphocytes,

these cells migrate into the infected organ, in this case the bladder, to stimulate macrophages to kill ingested BCG cells [29].

In many immunology studies, functional T cells subsets capable of secreting cytokines are believed to have a key role in protection against pathogens. Cells that are capable of secreting multiple cytokines, termed "polyfunctional", and especially cells capable of secreting IFNγ, TNF and IL2 together, were reported to be important in protecting against TB and they were found to be a good screening tool for confirming diagnosis and evaluating TB vaccines [30-32]. In this study, functional subsets were explored for differences between the different patient response groups.

The main T helper 1 cytokines:

Interferon gamma (IFNγ): is secreted by activated T lymphocytes and NK cells and is a potent activator of macrophages, a key factor in the BCG immune reaction, and leads to up-regulation of surface major histocompatibility complex (MHC) class I and II molecules [33].

Interleukin-2 (IL2): IL2 is produced by activated T lymphocytes and it mainly induces T lymphocyte proliferation, regulates the immune activation and is also important for self-tolerance and haemostasis [34].

Tumour necrosis factor (TNF): Is secreted mainly by macrophages and also T lymphocytes and is believed to induce inflammation and stimulate T lymphocytes response. It can also induce tumour-cell apoptosis [35] and stimulate secretion of IL-12 in dendritic cells [36].

Other relevant cytokines:

CD40-Ligand (CD40L): mainly expressed on the surface of activated CD4 cells, but can also be found on B cells, granulocytes and dendritic cells. Th1 lymphocytes

can stimulate APC in two ways; mainly via the secretion of IFN-γ, but also by displaying CD40L protein which binds to CD40 on APC, such as macrophages, which triggers their activation [29].

Interleukin-17a (IL17a): once T lymphocytes are stimulated they can differentiate into either Th1 or Th2 cells. However more recently, another type of effector T lymphocyte has been identified. It secretes IL17a and is believed to act as an adjunct to Th1 or Th2 cells in their battle against pathogens [37].

Interleukin-1 beta (IL1β): IL1β is a pro-inflammatory cytokine that is mainly secreted by macrophages; it induces the production of prostaglandins and IL2. The IL1 family is believed to play an important role in the inflammation and the regulation of immune response [38].

Interleukin-4 (IL4): IL4 is mainly secreted by Th2 cells and believed to stimulate B-cells and proliferate naive T lymphocytes into the Th2 phenotype. It suppresses Th1 cells and macrophages to inhibit IFNγ and IL2 production [39].

Interleukin-6 (IL6): IL6 is secreted by T lymphocytes and macrophages and is involved in B-cell stimulation [40].

Interleukin-8 (IL8): IL8 is secreted by phagocytes and acts a chemokine attracting inflammatory cells like neutrophils, basophils and T lymphocytes [41].

Interleukin-10 (IL10): IL10 is secreted by many inflammatory cells including T lymphocytes and macrophages. It inhibits the secretion of several cytokines (INFγ, IL2, IL3, TNFα, TNF-β and GM-CSF) and hence it suppresses the proinflammatory response of Th1 cells and reduces unnecessary tissue damage caused by inflammation [42].

Interleukin-12p70 (IL12p70): IL12p70 is secreted mainly by macrophages and dendritic cells. The secretion of IL12p70 results in the activation and differentiation

of T lymphocytes. IL12 is required for INFγ production which is critical for Th1 function [43].

Interleukin 13 (IL13): IL13 is secreted by several immune cells and it is involved in the proliferation of B-cells, immunoglobulin production and macrophage activation [44].

2.2.2 Methods for detecting and measuring cytokines

There are several tools and techniques that have been described to measure cytokines; below are some of the commonly used methods.

Bioassays are based on observing the physiological response of a certain cytokine, for example by observing the effect of certain cytokine on a cell line. Because of the lack of accuracy they are rarely used given the presence of more advanced ways of measurement [45].

Immunoassays are based on using two types of antibodies, one that captures the cytokine and another that is linked to a signal molecule that can be measured. Enzyme linked immunosorbent assay (ELISA) is the most common form of immunoassay that uses the catalytic properties of enzymes to detect and measure immune reactions but can only detect one element at a time. The multiplex system allows the measurement of multiple cytokines at once reducing time and workload. The more advanced electrochemiluminescence technique measures cytokines using electrochemical stimulation on light emitting antibodies with high accuracy and with smaller sample size [45].

Flow cytometry streams cells tagged with fluorescent monoclonal antibodies (specific for surface or intracellular marker) through laser beams and counts them. This technique can measure the chemical and physical characteristic of cells, such as size, granularity and more detailed cell components such as DNA and gene expression. The advantages of flow

cytometry are the rapid ability to detect intracellular cytokines as well as cellular phenotypic identification. Modern variations of this technique now use multiple lasers and detectors that allow measuring of multiple markers at a time [46].

The use of flow cytometry to examine intracellular cytokines in T cells was first described by Waldrop *et al* [47]. This ability to stain surface and intracellular markers gives the opportunity to perform multiparametric analysis at a single cell level.

Nanoparticle-modified aptamers are short synthetic nucleotide sequences produced *in vitro* which can be either DNA or RNA that can bind to ligands (similar to antibodies) with high affinity and specificity. Ultrasensitive techniques that can measure cytokines using gold nanoparticle-modified aptamers have been described, but technical issues and availability of aptamers limit the use of this technique [48].

2.3 Bladder cancer and immunotherapy

2.3.1 The history of immunotherapy

The link between the immune system and cancer was first noticed in the 1880s when doctors observed remission of cancers after infections [49]. This led William Coley, a New York surgeon, to experiment on this phenomenon in 1883. He observed this when a cancer patient had two episodes of erysipelas, caused by *Streptococcus pyogenes,* followed by a complete remission of the cancer. He then started injecting cancer patients, who mostly had inoperable sarcomas, with streptococcal culture media later named Coley's toxin. A 10% response rate was observed [49]. Understandably, the use of this toxin did not last long given the low response rate and the morbidity associated with it. It is believed that Coley's experience was the birth of cancer immunotherapy [50, 51].

The TB vaccine BCG, derived from the bovine strain of tubercle bacilli *M. bovis*, was developed in the Pasteur Institute in Lille, France between 1903 and 1915 by the French bacteriologist Albert Calmette and his veterinarian assistant Camille Guerin. They demonstrated protection against TB when they tested a weaker strain on several cows. The weaker strain was developed from *M. bovis* by propagation on potato soaked in bile. It took 13 years of hard work and 230 successive cultivations to create a strain incapable of producing TB [52].

In 1929, Professor Raymond Pearl, a biostatistician at Johns Hopkins University, Baltimore, published an observational autopsy study on 816 cancer cases which were matched with 816 non-cancer cases; he found tuberculosis in 6.6% in the cancer group and 16.3% in the non-cancer group and concluded that TB had a protective effect against cancer. His findings, despite the weak association based on this data, gave birth to BCG immunotherapy [53].

Old and colleagues tested the anti-tumour effect of TB on guinea pigs; inoculating BCG with the tumour cells prevented the tumour cells from growing while in the absence of BCG the tumour grew, metastasised and killed the guinea pigs [54]. Zbar and colleagues reported similar findings [55]. Clinical use of BCG in 1969 [56] sparked interest in BCG immunotherapy for different cancers and better outcomes were reported for several cancers including breast, lung, melanoma, leukaemia, prostate, colon and kidneys [56-59], but they were soon replaced by more effective treatment.

Coe and Feldman experimented on guinea pigs and demonstrated strong delayed hypersensitivity reaction in their bladders [60]. With this finding and the criteria developed by Zbar [55], Dr Alvaro Morales, a urologist from Canada, tried topical BCG in the bladder. He published his data from 9 patients in 1976 [13] and since then, intravesical BCG has maintained its success story in the treatment of bladder cancer.

2.3.2 Mechanism of action

The mechanism of action of intravesical BCG immunotherapy is not fully understood. It has been suggested that when BCG is instilled into the bladder, it induces an influx of inflammatory cells including activated T lymphocytes, NK cells and macrophages [61]. This enhances the expression of MHC class II molecules on urothelial cells [62] resulting in the production of cytokines in the bladder mucosa which leads to a cellular immune response against tumour cells [63]. BCG is internalised by both urothelial and inflammatory cells such as neutrophils, and triggers an inflammatory cascade of cytokine release and immune cells recruitment (figure 1). Many studies have found elevated amounts of IL1, IL2, IL6, IL8 and IL12, TNF, IFNγ and granulocyte macrophage colony-stimulating factor (GM-CSF) in the urine of patients treated with BCG [28, 64].

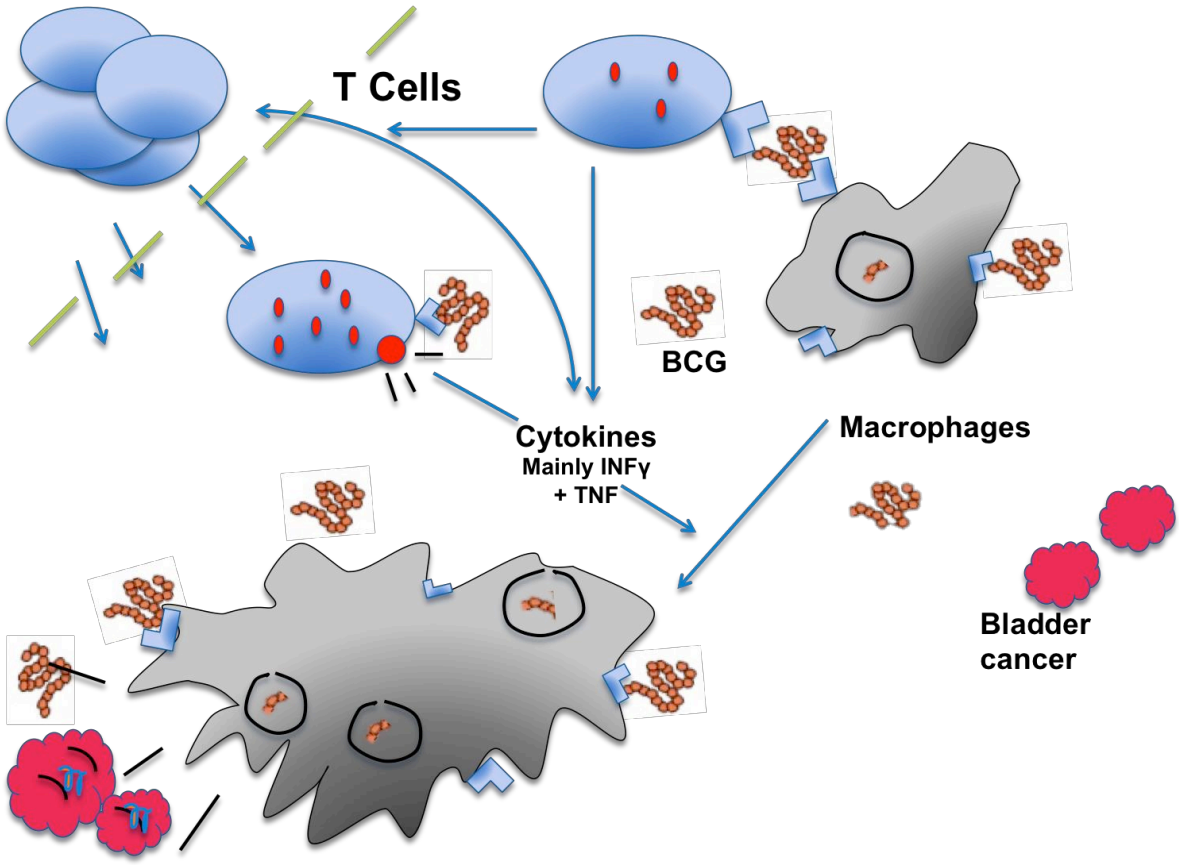

Figure 1: the local immune response after intravesical Bacillus Calmette-Guérin (BCG) immunotherapy. The bladder cancer cells escape the local immune surveillance. Once BCG is administered, antigen presenting cells (like macrophages) react with BCG initially and present it to the more specific T lymphocytes which in turn secretes inflammatory cytokines which will attract more immune cells to the area, directly influence the BCG-infected cells and help upgrade the macrophages and support it in its reaction to BCG. The cancer cells are believed to die as either collateral damage or directly targeted when immune cells are attracted to the bladder as a result to BCG immunotherapy.

2.3.3 Immunotherapy treatment failure

The use of BCG for HGNMITCC has been recommended by the European Association of Urology [17] and other networks [65]. This recommendation resulted from systematic reviews of randomised controlled trials; given the impact that intravesical BCG has on the recurrence and progression of those groups of patients.

In a systematic review of 25 trials, Han *et al* reported recurrence of up to 49.7% with patients who were not treated with BCG and this was reduced to 40.5% in patients who

received BCG (Odds Ratio 0.61, 95% CI 0.46 to 0.80, *P* value <0.0001) [66]. Shelley *et al* also reported similar findings and quoted a 56% reduction in the odds of recurrence (*P* value < 0.001) [67]. Better outcomes are reported when maintenance treatment is given [66, 68, 69].

2.3.3.1 Variation of immune response

Immune responses to BCG have been found to vary between individuals. In TB vaccination studies, variation in IFNγ response to mycobacterial antigens have been reported in BCG-vaccinated infants [70]. In another study involving 207 twin pairs, significant heritability for the immune responses to different vaccines was noted. Genetic factors, mainly outside the MHC class II locus, were found to play an important role in regulating immune responses to BCG [71].

In relation to bladder cancer, urine cytokines levels were evaluated after intravesical BCG treatment and found to vary between individuals but conclusions were inconsistent; some reported urinary IL8 acted as a marker of response [72-74], other reports suggested IL2 was a better marker of response [75, 76]. More recently Shintani *et al* suggested that urinary TNF might be essential for BCG anti-tumour activity and could act as an important response marker [28].

Schmidt *et al* [77] evaluated the peripheral blood lymphocyte response in 10 patients with superficial TCC treated with intravesical BCG. Blood samples were taken before the BCG course and before every further instillation and the authors then compared the *in vitro* proliferation responses of PBMC to BCG, which they presented as the stimulation index (SI). They reported variations in the SI between the 4 patients who had recurrences (SI < 5) and the 6 patients who did not (SI > 5).

Taniguchi *et al* evaluated the immediate immune response, locally and systemically, following each of the 6 BCG instillations in 21 patients. Urine (IL1β, IL2, IL6, TNF, IFNγ, Macrophage-CSF) and serum (IL2, IFNγ) cytokine levels increased after instillations. Peripheral blood NK cell activity also increased when measured three days after BCG instillation. The authors noted some variation in BCG-induced NK cell activity between the 16 patients who had no further recurrences and the 5 patients who had recurrences on follow up; tumour-free patients produced higher BCG-induced NK cell activity than tumour recurrence patients. The authors suggested the presence of a systematic cause of failure in response to BCG treatment [78].

More recently, Ardelt *et al* evaluated the IgA and IgM antibody responses to *M. bovis* BCG heat shock protein-65 as potential serum markers of response to BCG therapy in 16 patients who had intravesical BCG treatment for superficial bladder TCC. Variations in the antibody titres were noted between the 11 patients who responded and the 4 who had recurrences; non-responders had lower IgA and IgM levels to HSP65 before and after treatment in comparison to responders. Of note, one patient who initially failed BCG treatment and had a low antibody titre had a second BCG induction course, a strong antibody response was noted and the patient responded clinically [79].

The above examples show an expected local and systemic response to bladder BCG treatment that can be detected in urine and blood. Some show variations in local and systemic markers between responders and non-responders and based on that, some parameters have been proposed to act as treatment marker, but whilst presenting data suggestive of the role for underlying immune responses to BCG in determining the outcome of this therapy for TCC, the studies are small and larger definitive studies are required.

2.3.3.2 Definition of BCG failure

There is no specific consensus on the exact definition of BCG failure. Some consider recurrence after a single 6-week induction course as a failure of treatment but it has been shown that patients can respond after the 2nd cycle. Haaff *et al* reported a further 42% response in patients with carcinoma in situ and 46% in papillary recurrence following a second course of BCG [80]. Catalona *et al* reported that the risk of invasion and metastasis at entry into BCG therapy to be 7% and 5% respectively, and after one failed induction of intravesical BCG, this increased to 11 and 14% respectively. After 2 or more BCG cycles the rates of invasion and metastasis increased, if treatment failed, to 30% and 50% respectively [81]. Allen and Green defined BCG failure as recurrence (1) within 6 months of initial complete response, (2) following 2 courses of BCG, (3) while on maintenance or, (4) recurrent T1 tumour following one course of BCG [82]. The European Association of Urology considers BCG treatment to have failed (1) when muscle-invasive tumour is detected during follow-up, (2) when HGNMITCC is present at both 3 and 6 months; (3) when any deterioration of the disease under BCG treatment, such as a higher number of recurrences, higher T stage, higher grade, or the appearance of CIS, despite an initial response [17].

Due to the variation in the definition of BCG failure, and that presence of recurrence may not necessarily mean treatment failure, and to ensure objectivity, this dissertation will present the data based on presence or absence of recurrence after starting intravesical BCG treatment.

2.3.3.3 Markers of response:

Clinical markers

Many clinical factors have been reported to have an effect on success or failure of BCG treatment. Some reported on age [83, 84] and found that older patients are likely to fail treatment; Herr *et al* [83] reported reduced recurrence-free survival for patients older than 70 years. Other researchers found an association with gender [85-87]; Fernandez-Gomez *et al* [85] found that female gender (HR=1.71) was associated with increased risk of recurrence. Fernandez-Gomez *et al* [85] also found that multiple tumours at the time of resection (HR=1.54) to be associated with recurrence. The tumour size at the time of the initial resection was reported by Lopez-Beltran *et al* [88] to increase the risk of recurrence (*P* value=0.001). The tumour grade [89, 90] and the stage [87, 90] were also reported to increase the risk of recurrence and progression. The presence of carcinoma in-situ (CIS) was reported by many to increase the risk of recurrence and disease progression [85-87, 91]. Lin *et al* [92] found that lymphovascular invasion to be associated with reduced 5-year recurrence-free survival (*P* value <0.001) while Lotan *et al* [93] and Resnick *at al* [94] found lymphovascular invasion to be an independent predictor of nodal involvement while Saito *et al* [95] also found that recurrence-free and disease specific survivals to be reduced with lymphovascular invasion [92-95].

With regard to the BCG therapy, the number of BCG instillations was also found to effect outcome, and patients who don't complete the full course have a higher risk of recurrence [68, 96]. It has been also reported that patients with previous BCG immunisation have a better treatment outcome [97].

After induction BCG treatment, the presence of tumours at the first cystoscopic examination carries a high risk of further recurrence and disease progression [85, 91]. See Appendix 1 for summary table of published markers effecting response.

Biomarkers

In order for BCG to be internalised, it needs to bind to exposed fibronectin, which in turn becomes internalised by urothelial and tumour cells [98-101]. In animal models, adherence of BCG was inhibited by anti-fibronectin antibodies [99]. Boorjian *et al* reported higher recurrence rate in patients with anti-fibronectin clot inhibitor drugs [102].

When BCG is internalised by APC, a cascade of reactions happen that involve macrophages, NK cells and T lymphocytes resulting in more recruitment of these cells and release of inflammatory cytokines [103, 104]. An increased urinary white cell count (leucocyturia) has been reported as a marker of good response [165]. Interleukin-2 (which is secreted by activated T lymphocytes) in urine during and after induction BCG treatment has been reported by many as a potential marker [72, 105-107], while others suggested IL8 (which is secreted by activated macrophages) should be measured [73, 74, 108, 109] along with IL-18 [110].

Published reports [72-76] have shown an increase in urinary cytokine levels after intravesical BCG but they were inconsistent as markers of response. Also, previous studies [77-79] have shown that systematic immune responses occur in individuals receiving intravesical BCG for bladder cancer. As mentioned previously, some reports suggested that variation in the immune responses between different patients receiving intravesical BCG might explain or be associated with failure. This is likely to be related to natural variations in immune responsiveness. Schmidt *et al* [77] proposed using the peripheral blood lymphocyte stimulation index to predict response to treatment, though his finding was during and post intravesical treatment. Taniguchi *et al* [78] proposed using the post treatment peripheral blood NK cell activity against BCG as a marker to predict response, also a post treatment finding. While Ardelt *et al* [79] also recommended another treatment marker to predict response, the authors found that the post treatment IgA and IgM antibody

responses to *M. bovis* BCG heat shock protein-65 to differ in 16 patients studied and can differentiate responders from non-responders, though also proposing another example of a post treatment test.

Until the completion of this project, no reliable predictive test has been published and more importantly, no pre-treatment biomarkers has been identified that has promising potential to act as a predictive tool. See Appendix 1 for summary table of published markers effecting response.

2.4 Aims and hypotheses

2.4.1 Aims

The project aims to determine whether there is inter-individual variation in immune responses in patients undergoing intravesical BCG immunotherapy for HGNMITCC that is detectable in blood or urine samples and if these differences can be detected before treatment. If differences were found to be present, the project would aim to evaluate the significance of these differences and correlate them with outcome of treatment to determine whether these differences could act as predictors of outcome to treatment. The use of peripheral blood markers could help better understand the role of the systemic immune response in the success or failure of local immunotherapy treatment.

It is believed that Th1 cells migrate to the bladder after BGC instillation. As this could be the factor influencing the outcome to intravesical BCG treatment, T lymphocytes in the blood and their cytokine secretion capacity (especially IFNγ, TNF and IL2) were chosen as the primary focus for this PhD. The protocol originally described by Waldrop et al [47] and the use of multi-parametric flow cytometry were chosen, as this would allow us to study the T lymphocytes responsible for secreting the relevant inflammatory cytokines, to further identify T lymphocytes based on their phenotype, to explore the role polyfunctionality and determine whether any differences could be detected between patients receiving intravesical BCG treatment for bladder cancer.

As inflammatory cytokines can be secreted by different inflammatory cells and not purely by T lymphocytes, the PhD also examined secreted cytokines after challenge with various antigens including PPD. Electrochemiluminescence technology was chosen as it allowed the convenient and accurate measurement of multiple secreted cytokines at once including the Th1 cytokines and other exploratory cytokines.

The protocols for these techniques are described in the methods section below.

2.4.2 Hypotheses

1. There are immunological difference, in Th1 related cytokines, between responders and non-responders to intravesical BCG immunotherapy for HGNMITCC that can be detected before and after treatment in peripheral blood and urine samples.

2. Biomarkers can discriminate and predict the clinical outcome of BCG immunotherapy in patients receiving the treatment for HGNMITCC.

2.4.3 End points

The Th1 lymphocyte responses that were measured in this PhD in order to test the hypothesis include:

Primary end points

a. The percentages of CD4 T cells expressing IL2, TNF, or IFNγ in response to *in vitro* PPD stimulation.
b. The percentages of the most polyfunctional Th1 subset (CD40L+, IFNγ+, IL2+ and TNF+) in response to *in vitro* PPD stimulation.
c. The levels of Th1 related cytokines (IFNγ, IL2, TNF) were measured in the supernatant following over-night PPD stimulation.
d. The levels of cytokines (IFNγ, IL2, TNF) in urine following BCG treatment.

Other exploratory end points

Although our main aim was to investigate known Th1 responses this research presented us with an opportunity to investigate the role of other less established cytokines in the immune response to intravesical BCG – referred to as "exploratory" end points.

a. The percentages of CD4 T lymphocytes up-regulating CD40L and expressing IL17a in response to *in vitro* PPD stimulation were measured to detect differences between responders and non-responders to intravesical BCG treatment. The percentages of pure IL17a+ CD4 cells (IL17a+ but CD40L-, IFNγ-, IL2- and TNF-) were also measured and compared.
b. Explore whether these differences are antigen- specific or non-antigen specific by comparing the percentages of CD4 T lymphocytes (as above) in response to Staphylococcus enterotoxin B
c. The levels of other cytokines were measured in the supernatant following overnight PPD stimulation to explore differences (IL10, IL13, IL4, IL12p70, IL-1b, IL6, IL8).
d. The levels of other cytokines in urine following BCG treatment.
e. The levels of secreted cytokines following lipopolysaccharide (LPS) stimulation, a non-antigen specific test

3. Methods

3.1 Participants

Ethical Approval: This study was approved by the City Road and Hampstead Research Ethics Committee, reference number 11/LO/2039, as well as Brighton and Sussex University Hospitals' Research and Development Department and Brighton and Sussex Medical School's Research Governance and Ethics Committee. All patients gave written informed consent before enrolment in the study.

Patients and samples:

Between 01/07/2012 and 23/12/2013, all patients from a single centre (Brighton and Sussex University Hospitals) who underwent initial TURBT, had HGNMITCC and were offered intravesical BCG treatment were invited to participate in the study. Following written informed consent, relevant clinical information was collected from individuals and their hospital records. Patients followed a routine BCG (12.5mg intravesical OncoTICE® from Organon N.V) treatment schedule over 1 year; this started with a 6 weeks BCG induction course with weekly instillations, then further maintenance boosters of 3 instillations every 4 months.

15 ml of heparinised blood (Sodium Heparin, BD Vacutainer) was collected before treatment and 8 weeks after completion of induction treatment for patients who had intravesical BCG treatment for HGNMITCC.

Voided urine samples were collected in plain sterile universal containers (Sarstedt, Germany) before the start of BCG induction and 4 hours after the 6th and final BCG instillation. This schedule was based on a study by Shintani *et al* [28] that showed that urine samples 4 hours after the last instillation had significantly different cytokine profiles between responders and non-responders to treatment. See appendix 2.

3.2 Clinical examination and procedures

Cystoscopic surveillance: 6 weeks after completion of induction intravesical BCG treatment, patients were routinely scheduled for cystoscopic examination under general anaesthesia. Patients followed a standard cystoscopic surveillance programme which was performed following maintenance BCG treatment and every 4 months for 2 years after completing the BCG treatment, then 6 monthly for 2 years and then yearly thereafter.

Recurrence and bladder sampling: Recurrence was confirmed by the presence of TCC on histology. All patients had random bladder biopsies if no recurrence was identified visually. Patients who had recurrence at the first cystoscopy surveillance were offered to either undergo radical cystectomy, receive further intravesical BCG immunotherapy treatment or an alternative intravesical treatment modality (e.g Hyperthermic Mitomycin C treatment)

Due to the variation in the definition of BCG response and treatment failure as discussed earlier in section 2.3.3.3, this dissertation will present the data based on recurrence after starting BCG treatment into either:

A. **Recurrence-free**: patients who did not have any recurrence identified after starting BCG treatment and for the duration of follow-up [23 months, range 16-34 months].

B. **Recurrence:** patients who were confirmed to have recurrence at any stage after starting induction BCG treatment and within the study follow-up period.

3. 3 Laboratory protocols

3.3.1 Immunoflourescent T lymphocytes staining and flow cytometric assay:

This technique was used to evaluate and quantify the PPD-specific and non-PPD-specific (responses to SEB) T lymphocytes and their subsets in the peripheral blood and to measure their intracellular cytokine production. The technique used was similar to previous published reports [47, 111, 112].

Following a sequence of gating to identify the CD4 lymphocytes, by using the external markers (CCR7, CD27, CD28) CD4 cells could be identified in different maturation subsets and by using the intracellular markers (CD40L, IFNγ, IL2, IL-17a, and TNF). The percentage of CD4 T cells producing IL2, TNF or IFNγ in response to PPD stimulation, as well as CD40L and IL17a, were measured using intracellular staining methodology that is described in more detail below. Further exploratory analysis of gate combinations was performed for CD4 subsets (CCR7, CD27 and CD28 positive and negative combinations) as well as measuring the percentage of the functional CD4 subsets producing CD40L, IFN-γ, IL2, TNF and/or IL17a in several combinations.

3.3.1.1 Panel Development:

In order to characterise the T lymphocytes and determine their functional subsets a multicolour panel (11-colours) was developed. The multi-parametric flow cytometer LSR II was used with FACSdiva 6.1 software (BD Biosciences) with 633nm, 488nm and 405nm excitation lines. (See appendix 3 for flow cytometer filter layout).

The combination of fluorochromes was chosen based on 3 main factors:

1. Published recommendations [113, 114]; it is important to choose the brightest fluorochromes to minimise overlap between channels. As well, weak cellular

markers need brighter fluorochromes while unwanted or highly expressed markers need dim fluorochromes. For example, the Fixable Aqua (dim stain) was used to detect the unwanted dead cells, PerCP (dim stain) was used to detect CD4 (highly expressed marker) while BV421 (bright stain) was used to detect CD40L (weak cellular marker).

2. Recommended fluorochrome combinations from manufacturers and on-line multi-colour panel designer tool (see appendix 4).
3. Local knowledge and experience (Professor Florian Kern), as well as established running projects and local published work [112].

The selected panel combination was tested three times on stimulated blood samples from a healthy volunteer to ensure that the overlap between the fluorochromes was acceptable. Reagents were then titrated to determine the amount that achieved optimal staining allowing good separation between the positive and negative populations with low background staining levels. For each reagent, assay condition and at least 3 different concentrations of fluorochromes were tested. Non-stimulated samples were used for phenotypic markers while for activation markers both stimulated and non-stimulated samples were used in parallel. The optimal concentration was chosen based on the background staining and the stain index. For each marker, several fluorochromes were tested and compared with each other and then best combination was chosen for the panel.

The chosen Fluorochrome-conjugated antibodies used were as follows; anti-IL2-Fluorescein isothiocyanate (FITC), anti-IFNγ-Phycoerythrin cyanine dye (PE-Cy7) and anti-TNF-Alexa Flour 700 (Alexa700) were from BD Biosciences (San Jose, CA); anti-CD40L-Brilliant violet 421 (BV421), anti-CD3-Brilliant violet 571 (BV571), antiCD27-Phycoerythrin (PE), anti-CD4-Peridinin chlorophyll protein (PerCP), anti-IL17A-Alexa

Flour 647 (AF647) and CCR7-Allophycocyanin cyanin7 (APC-Cy7) were obtained from BioLegend (San Diego, CA); anti-CD28-Electron coupled dye (ECD) from Beckman Coulter (Fullerton, CA). As dead cells can cause high background non-specific staining, a viability stain was used to identify and exclude dead cells. Amine-reactive dyes penetrate the damaged cells as described by Perfetto *et al* [115], Fixable Aqua stain from Invitrogen (Paisley, UK) was used to discriminate between live and dead cells. The fluorochromes and the final concentrations are summarised in the table below (Table 3).

Laser	Excitation	Emission	Stain	Marker	Clone	Amount	Producer
Blue	494	520	FITC	IL2	5344.111	12µl	BD Biosciences
	480	575	PE	CD27	O323	3µl	BioLegend
	480	613	ECD	CD28	CD28.2	1µl	Beckman Coulter
	490	675	PerCP	CD4	OKT4	0.5µl	BioLegend
	480	767	PE-Cy7	IFNγ	B27	3µl	BD Biosciences
Red	635	668	AF647	IL17a	BL168	1µl	BioLegend
	650	719	Alexa700	TNF	MAb11	1.5µl	BD Biosciences
	650	774	APC-Cy7	CCR7	G043H7	5µl	BioLegend
Violet	405	421	BV421	CD40L	24-31	0.5µl	BioLegend
	367	526	Fixable Aqua	Live/Dead	-	0.5µl	Invitrogen
	405	570	BV571	CD3	UCHT1	0.5µl	BioLegend

Table 3: Summary of reagents, colour and concentration used.

PPD (Statens Serum Institute, Copenhagen, Denmark) dissolved in dimethyl-sulfoxide (DMSO, Fisher Scientific, Waltham, MA) with a final concentration of 10µg/ml. SEB (Sigma-Aldrich, St. Louis, MO) was chosen as it is a powerful mitogen that induces interferon synthesis in T lymphocytes [116], SEB was dissolved in DMSO with a final concentration of 5µg/ml. DMSO was stored at 1µg/ml concentration.

Fluorescence Minus One (FMO), which combines all the chosen reagents except for one, was done to properly interpret the flow-cytometry data by checking the sensitivity of the whole panel and overspill when all reagents are used (figure 2). This was performed for each of the fluorochromes chosen for the panel. Figure 2 show the FMO for all markers that are stained specifically with no overspill from other markers. For quality assurance and ensure the reproducibility of the data, the flow cytometer setting and voltages were automatically adjusted and validated by Cytometer setup and Tracking (or CST), from BD Biosciences (San Jose, CA), every time before acquisition. As well, Rainbow Beads from BD Biosciences (San Jose, CA) were used every time before acquisition to ensure standardisation of instrument and fluorescence linearity. As overlap is expected with this 11-panel design, compensation control was done for each antibody in the panel and automatic calculations were performed every time before acquisition of samples to adjust for the overlap.

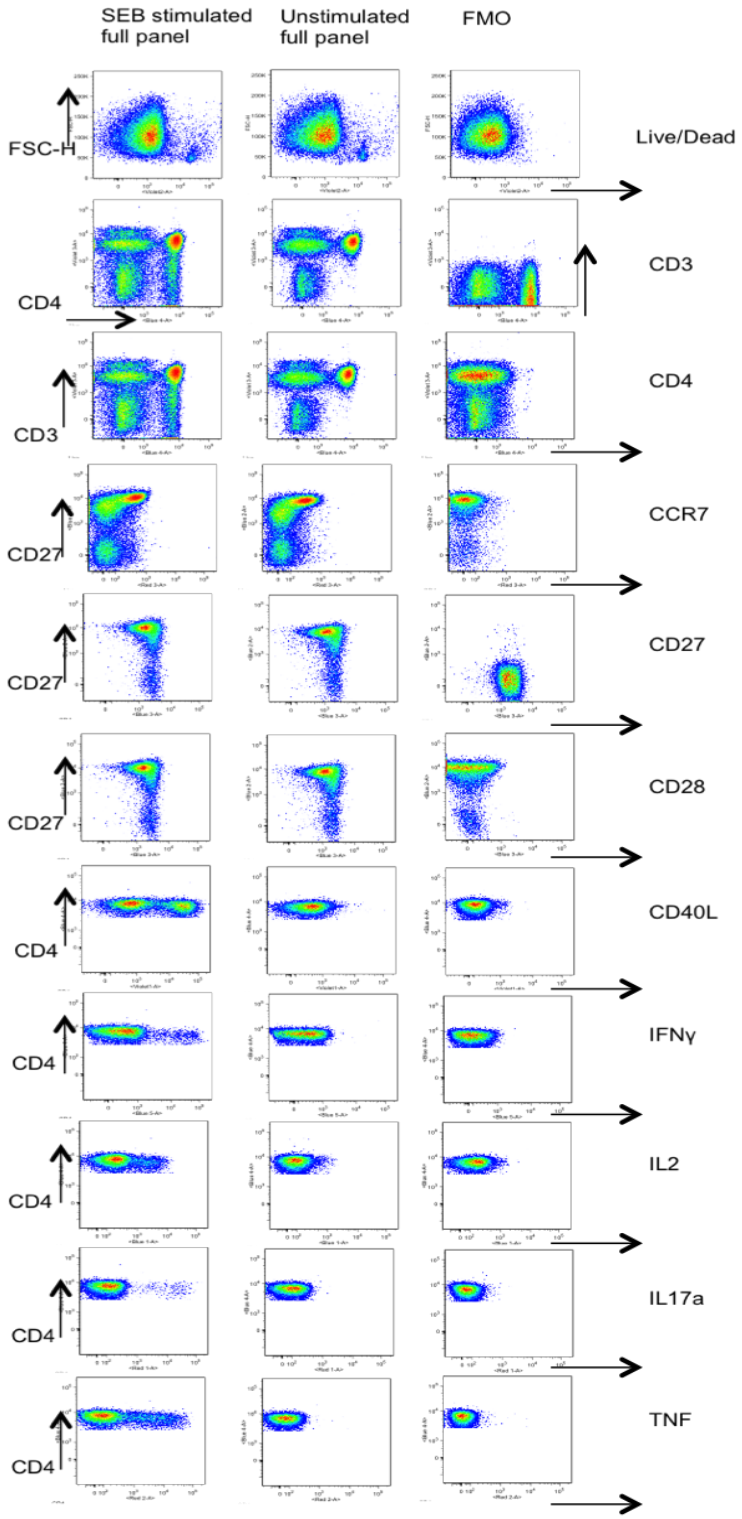

Figure 2: Full-panel Minus One (FMO) shown on pseudo-colour dot plot for reagents used. This combines all the chosen reagents except for one to properly interpret the data and check for overspill when all reagents are used and help in accurately gating for markers.

3.3.1.2 PBMC preparation and activation

Sample preparation and cell stimulation was similar to previous published reports [47, 111, 112]. Briefly, peripheral blood mononuclear cells (PBMC) were prepared from anticoagulated blood (sodium-heparin), diluted with sterile Phosphate-Buffered-Saline (PBS) (1:1) and layered onto Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) by density gradient centrifugation (20 minutes, room temperature, 1000 x g, no breaks; Centrifuge Multifuge 3S from Heraeus, Thermo Scientefic, UK) within four hours of blood collection. The PBMCs were then washed twice in sterile PBS (GIBCO BRL) (10 minutes, room temperature, 300 x g) and resuspended in RPMI 1640 medium (Biochrome) containing 10% heat-activated fetal-calf-serum (FCS) (Biochrome), 2 mM L-glutamine (Biochrome), and 100 IU of penicillin/streptomycin (Biochrome) to a volume of 5 x 10^6 cells per millilitre.

Three tubes containing 200µl (1 x 10^6 PBMC cells) cell suspension were mixed with either 5µg PPD, 2µg SEB as positive control or 2µl DMSO as negative control, bringing the total volume to 250µl. Cells were incubated for 2 hours and then 5µg Brefeldin A, which is a potent inhibitor of intracellular transport that prevents secretion of any produced cytokines and retains them inside the cell (20µg/ml; Sigma-Aldrich), was added bringing the total volume to 500µl. Cells were then washed (in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide and centrifuged for 8 minutes, 4°C, 400 x g; multifuge 3 S-R, Heraeus, Thermo Scientefic, UK) and then stained in pre-titrated surface antibodies for 30 minutes at 4°C. After a further wash (8 minutes, 4°C, 400 x g), lysis and permeabilisation reagents, from BD Biosciences (San Jose, CA), were used as per manufacturer's instructions. Cells were stained intracellularly for 30 minutes at 4°C. Cells were then washed (8 minutes, 4°C, 400 x g) and fixed in PBS containing 0.5% paraformaldehyde and then acquired on LSRII flow cytometer from BD Biosciences (San Jose, CA). (Figure 3)

Gating and data analysis: Data collected by multiparametric flow-cytometer were analysed using FlowJo software (Treestar, OR). The sequence of gating was as follows; lymphocyte gate (FSC-A versus SSC-A), doublets or clumps were gated out to enhance sorting purity by gating for singlets gate (FSC-A versus FSC-H), as functional cells were the target for this study, dead cells were gated out by gating for Live/Dead (Violet 2 versus FSC-H), CD3/CD4 gate (Blue 4 versus Violet 3), then subsets CCR7/CD27 (Red 3 versus Blue 2) and subsets CD28/CD27 (Blue 3 versus Blue 2). Intracellular markers were gated versus the selected CD4 as follows; CD4/CD40L (Blue4 versus Violet 1), CD4/IFNγ (Blue5 versus Blue4), CD4/IL2 (blue1 versus blue4), CD4/IL17A (Red1 versus Blue4) and CD4/TNF (Red2 versus Blue 4). (Figure 4)

Further analysis of gate combinations was performed for CD4 subsets (CCR7, CD27 and CD28 positive and negative combinations).

The negative control samples were subtracted from the PPD and SEB stimulated samples to standardise responses and remove spontaneous background activation.

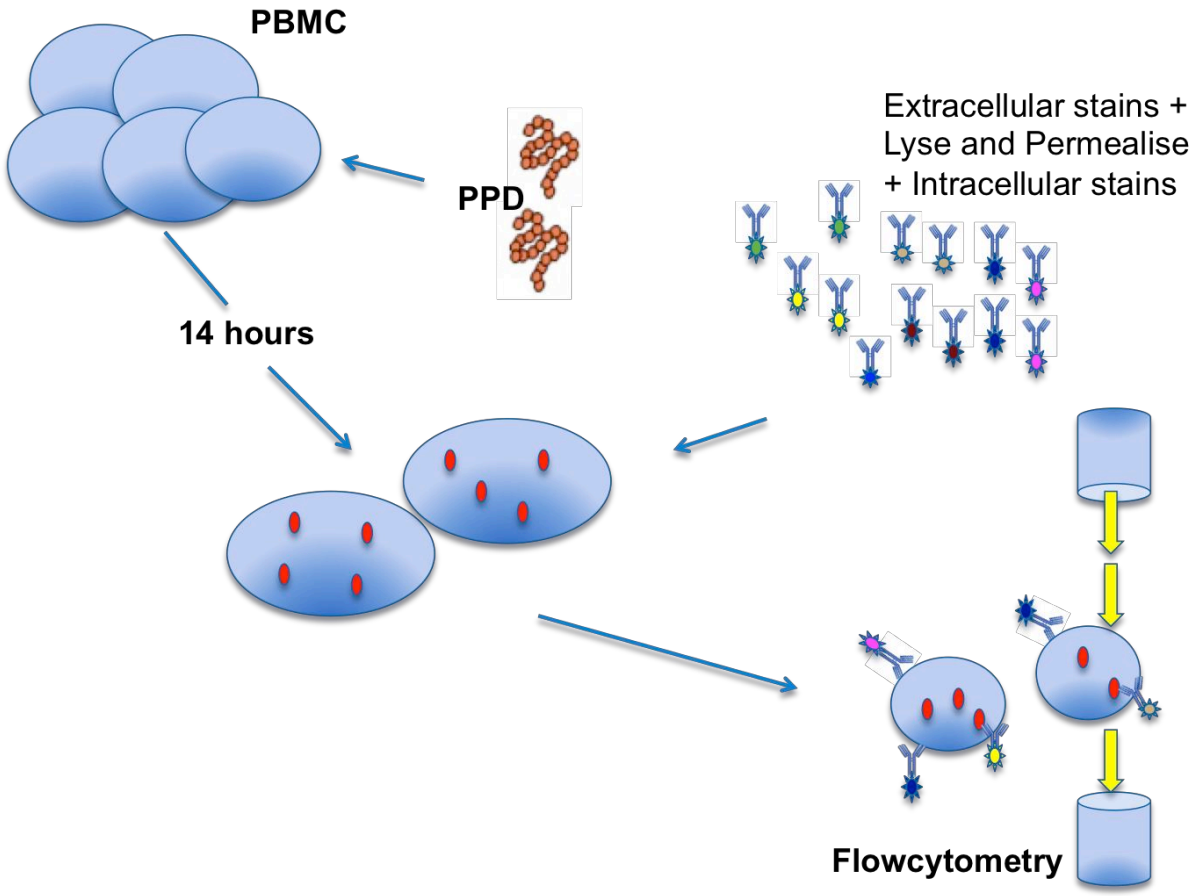

Figure 3: after peripheral blood mononuclear cells (PBMC) preparation, cells were stimulated overnight with Purified Protein Derivative (PPD), Staphylococcus enterotoxin B (SEB) as the positive control or Dimethyl sulfoxide (DMSO) as the negative control with Brefeldin A added to prevent the secretion of any produced cytokines. Samples were then stained with pre-titrated surface antibodies, lyse then permealise solutions added as per protocol, stained with pre-titrated intracellular antibodies and then washed. Cells were then fixed and acquired on LSRII flow cytometer.

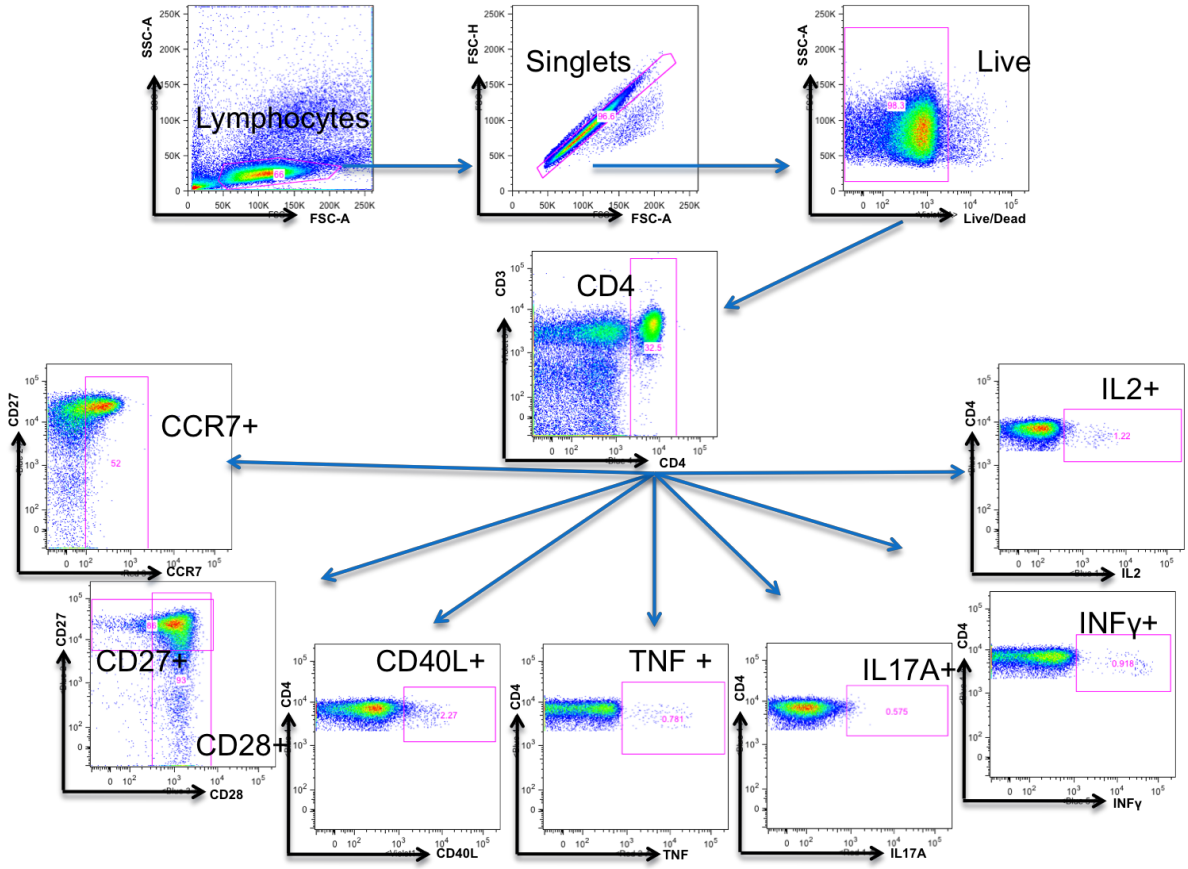

Figure 4: Flowjo gating sequence of stained peripheral blood mononuclear cells (PBMC) for the selected phenotypic markers. FSC-A versus SSC-A to gate for lymphocytes. FSC-A versus FSC-H to gate out clumped cells. Violet 2 versus FSC-H to gate for Live/Dead. Blue 4 versus Violet 3 for the CD3/CD4 gate. Red 3 versus Blue 2 for the CCR7/CD27 gates. Blue 3 versus Blue 2 for the CD28/CD27 gates. Intracellular markers were gated versus the selected CD4 as follows; Blue4 versus Violet 1 for CD40L, Blue5 versus Blue4 for IFNγ, blue1 versus blue4 for IL2, Red1 versus Blue4 for IL17A and Red2 versus Blue 4 for TNF.

Cell subsets: There is inconsistency in the classifications of T cells subsets based on external markers [24, 25, 117]. Okada *et al* considered CD4 lymphocytes with CCR7+CD27+CD28+ to be either "naïve" or "central memory" cells, CCR7-CD27+CD28+ may be considered "effector memory" cells, and CCR7-CD27-CD28+ or CCR7-CD27-CD28- may be considered "Th1" and "Th2" cells [24].

In constructing the antibody panel, CCR7, which is involved in directing lymphocytes into lymphoid tissues, was chosen as marker as it is thought to represent naïve or central memory cells and may also help in identifying effector subsets when excluded [118]. CD27, which is a TNF receptor that is up-regulated after stimulation of T lymphocytes, was used to identify effector memory subsets [119]. CD28 is involved in the signalling and proliferation of naïve cells and the production of IL2, which is vital for the survival of T lymphocytes and was also chosen for cell phenotyping [120].

For consistency and to avoid confusion, cells are presented using the surface markers CCR7, CD27 and CD28 to show differentiation subsets and the intracellular cytokine markers in different combinations to evaluate the different cell types within the cell differentiation subsets.

3.3.2 Secreted cytokines and Multiplex electrochemiluminescence detection

3.3.2.1 Secreted cytokines post PPD stimulation: For measuring secreted cytokines, PBMCs were prepared as described in section 3.3.1.2 above. The PBMCs (1 x 10^6 cells) were stimulated with 5µg PPD and incubated for 16 hours without adding brefeldin A. Tubes were then centrifuged briefly at 400g (no breaks) for 8 minutes and the supernatant collected and stored at -80°C until acquired in batches using electrochemiluminescence detection from Meso Scale Discovery (MSD) as explained below.

3.3.2.2 Secreted cytokines post LPS stimulation: Lipopolysaccharide was selected as an exploratory non-antigen specific test to evaluate differences between cases. The method used is similar to a previously published protocol [121]. Briefly, LPS (1mg/1ml, Sigma-Aldrich, St. Louis, MO) was diluted in double distilled water at a concentration of

100pg/µl. Two tubes were prepared with 100µl of fresh blood and a mixture of RPMI 1640 and 500pg of LPS to a total volume of 1ml. One tube was incubated for 4 hours (mainly for measurement IL1β) and the other for 24 hours (mainly for measurement IL10). Tubes were then centrifuged briefly for 5 minutes at 1800rpm (no breaks) and supernatant collected and stored at -80°C until acquired in batches using electrochemiluminescence detection from MSD as explained below.

3.3.3.3 Urine cytokine analysis: voided urine samples were collected in plain sterile universal containers (Sarstedt, Germany) before and 4 hours after completing the 6 week induction treatment. Samples were stored at -80°C prior to measuring the cytokines using electrochemiluminescence detection from MSD to as explained below

3.3.3.4 Multiplex electrochemiluminescence detection: The MSD kit was chosen as it offers a simple validated protocol, which can measure several variables at once using a small sample size with high sensitivity [122]. The MSD V-Plex pre-configured panel (10 human inflammatory cytokines) was chosen as it included the main targeted Th1 cytokines (IFNγ, IL2 and TNFα) as well as other cytokines (IL1β, IL4, IL6, IL8, IL10, IL12p70, IL13) that could be explored for immunologic differences between individuals

The main targeted cytokines were Th1 related (IFN-γ, IL2 and TNFα). To add to the value of this research and to explore whether there are immunologic differences between individuals that might involve cells other than Th1, the multiplex panel was expanded to involve 10 cytokines (T lymphocytes and non-T lymphocytes specific), which could also be detected using that protocol. The full cytokine panel used in this technique included (INFγ, IL1β, IL2, IL4, IL6, IL8, IL10, IL12p70, IL13 and TNFα).

The secreted cytokines were measured following overnight PPD stimulation using a sandwich immunoassay from MSD as per the protocol described below. Urine cytokines and post-LPS stimulation cytokines were also measured using this technique.

The detection range for each measured cytokines was as follows:

IFNγ: the lower detection limit was 0.245pg/ml and upper detection limit was 1410pg/ml. (see Figure 5).

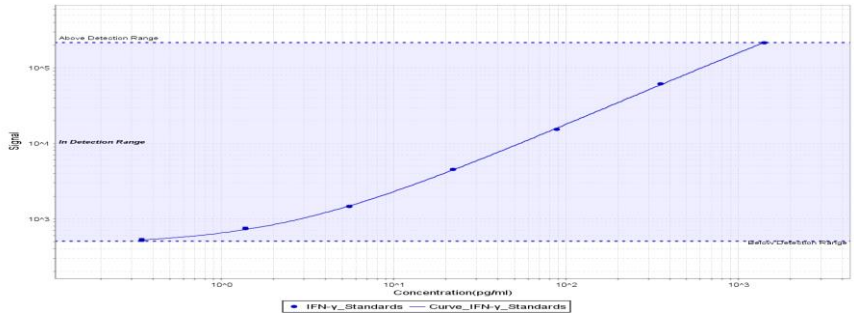

Figure 5: The IFNγ detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL1β: the lower detection limit was 0.0248pg/ml and the upper limit was 510pg/ml. (see figure 6).

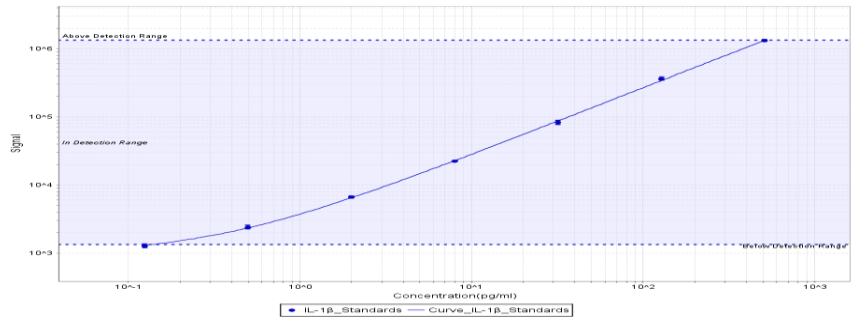

Figure 6: The IL1β detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL2: the lower detection limit was 0.0551pg/ml and the upper limit was 1470pg/ml. (see figure 7).

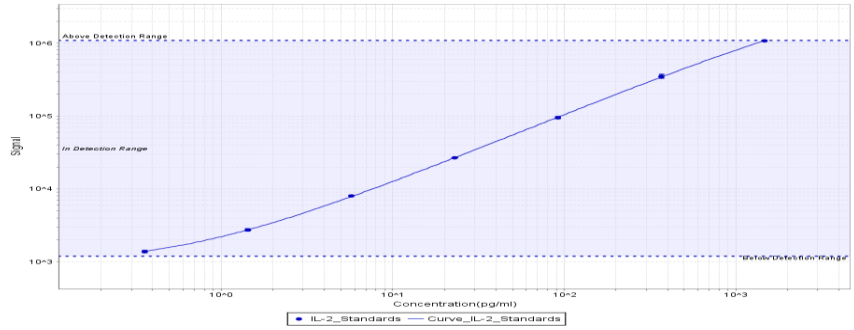

Figure 7: The IL2 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL4: the lower detection limit was 0.0184pg/ml and the upper limit was 216pg/ml. (see figure 8).

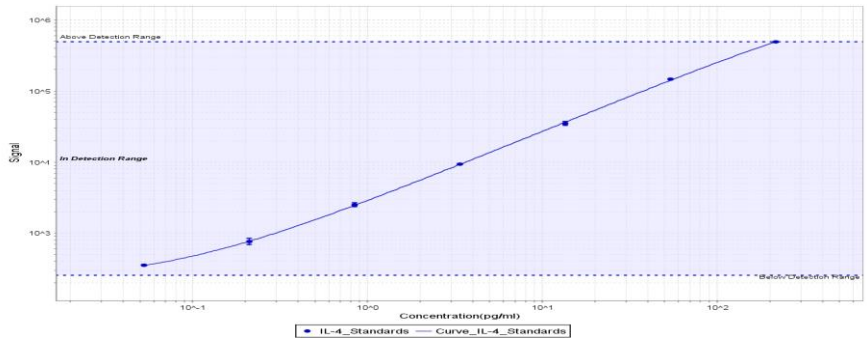

Figure 8: The IL4 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL6: the lower detection limit was 0.0615pg/ml and the upper limit was 769pg/ml. (see figure 9).

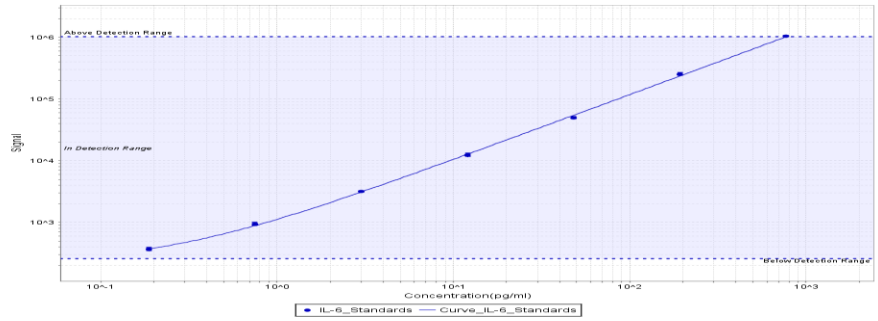

Figure 9: The IL6 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL8: the lower detection limit was 0.0482pg/ml and the upper limit was 507pg/ml. (see figure 10).

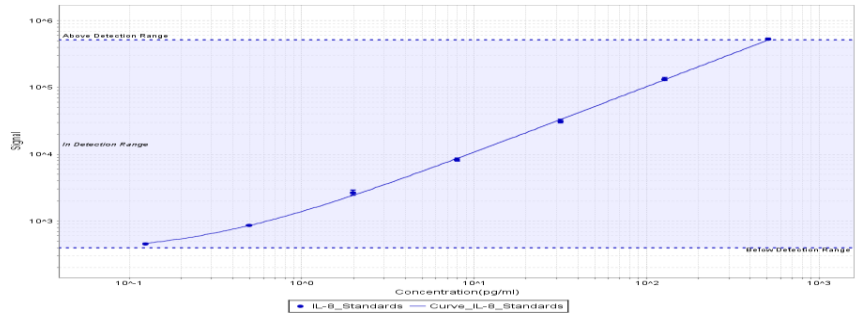

Figure 10: The IL8 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL10: the lower detection limit was 0.0269pg/ml and the higher limit was 324pg/ml. (see figure 11).

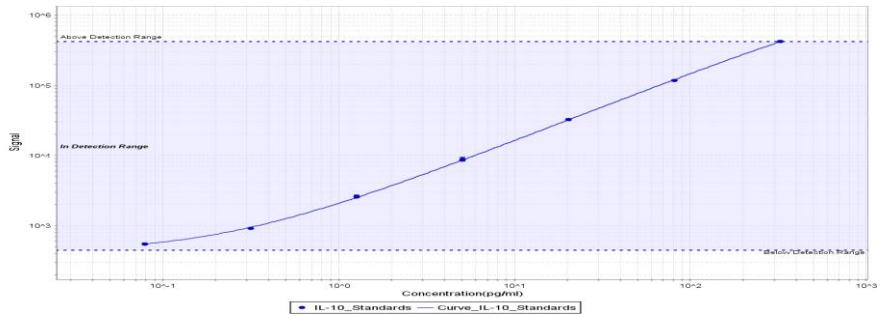

Figure 11: The IL10 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL12p70: the lower detection limit was 0.0752pg/ml and the upper limit was 413pg/ml. (see figure 12).

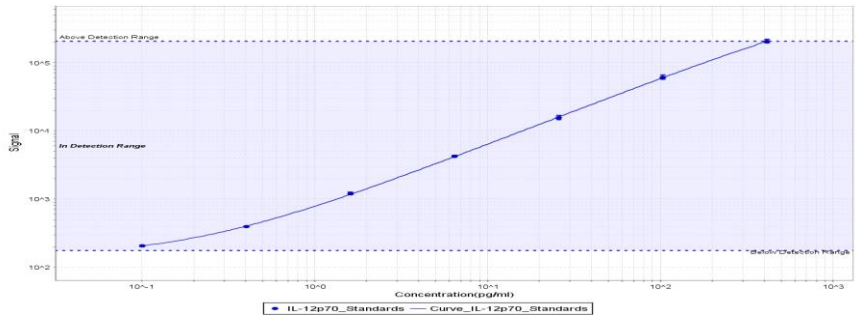

Figure 12: The IL12p70 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

IL13: the lower detection limit was 1.25pg/ml and the upper limit was 530pg/ml. (see figure 13).

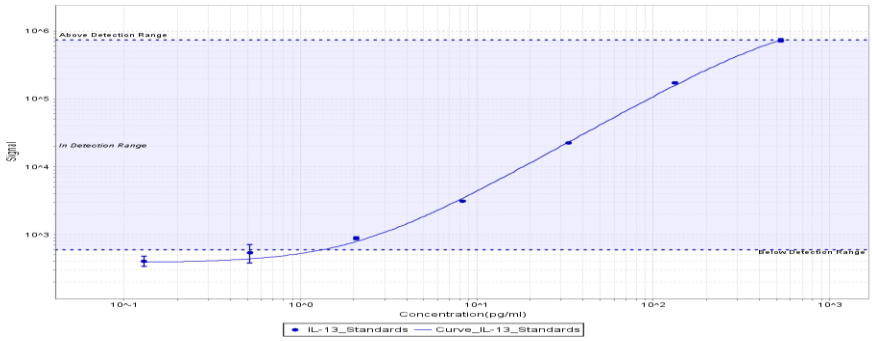

Figure 13: The IL13 detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

TNFα: the lower detection limit was 0.0520pg/ml and the upper limit was 316pg/ml. (see figure 14).

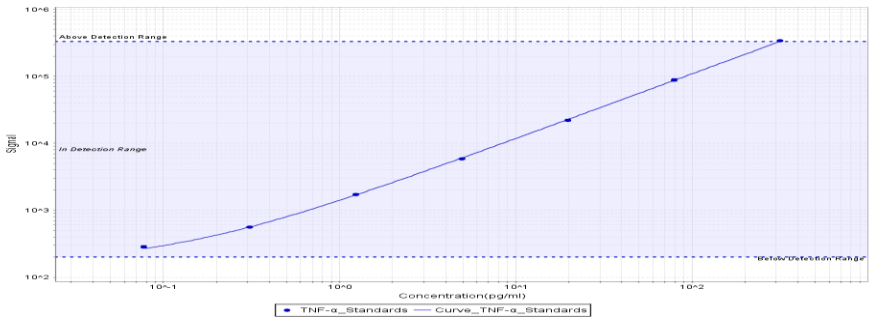

Figure 14: The TNFα detection curve for the multiplex electrochemiluminescence detection from Meso Scale Discovery.

Briefly; samples were defrosted and brought to room temperature followed by an 8 step calibration using a multi-analyte lyophilized calibrator according to the manufacturer's (MSD) protocol. Samples were diluted using diluent 2 as per the manufacture's protocol (MSD) (containing serum, blockers and preservatives) as follows;

- Secreted cytokines following over-night PPD stimulation: diluted 1:10 to measure all cytokines, and 1:1000 to re-measure IFNγ and IL8 when out of range.

- Supernatant following 4 hours LPS stimulation of fresh blood sample: undiluted to measure all cytokines, and diluted 1:100 to re-measure TNF and IL6 when out of range.
- Supernatant following 24 hours LPS stimulation of fresh blood sample: diluted 1:10 to measure all cytokines, and 1:100 to re-measure TNF and IL6 when out of range.
- Urine samples; undiluted samples to measure all cytokines, and 1:100 and 1:1000 dilutions to re-measure IFNγ and IL8 when out of range.

50µl of prepared samples were added to the designated wells on the multiplex plates. Plates were then sealed with an adhesive plate seal and incubated at room temperature while shaking for 2 hours. The plates were then washed 3 times with 150µl of wash buffer. 25µl of detection antibody solution was added to each well, which were then sealed and incubated with shaking for another 2 hours. The plates were then washed 3 times with 150µl of wash buffer. 150µl of 2X Read Buffer was added to each well and plates were then read using the MESO QUICKPLEX SQ 120 (MSD).

3.4 Statistical analysis

Excel was used for data tabling and initial analysis. GraphPad PRISM software (version 6) was used for graphs and statistical analysis. The data analysis was reviewed by Dr Rosemary Tate (School of Informatics, University of Sussex). The D'Agnostino-Pearson test was used to test the data for normality and as most of the data was not normally distributed, The Mann-Whitney test was used to compare values. As subgroups were relatively small, Fisher's exact test was used to compare the categorical variables (age, histology, multifocal disease and BCG immunisation). The Spearman's rank correlation coefficient (rs) was used to measure correlations between variables (e.g. association

between the percentages of CD4 lymphocytes); the cut-off value of rs= 0.3 was used for positive correlation and rs≥0.5 as evidence of strong correlation. *P* values of ≤0.05 were considered to be statistically significant. Where comparisons were made for 31 subsets, a Bonferroni correction with a factor of 31 was applied and *P* values ≤0.0016 were considered statistically significant.

To calculate predictive cut-off values I used Area under the ROC curve (receiver operator characteristic) with SPSS 23 from IBM with a random predictor threshold of ≥0.05.

Logistic regression was used to assess to relationship between variables and outcome SPSS 23.0 from IBM.

4. Results

Fifty patients were initially recruited who underwent transurethral resection of bladder tumour, histology revealed non-muscle invasive high risk (G3, T1 or CIS) bladder cancer and the patients opted to have intravesical BCG immunotherapy treatment. Four of these did not have the intended intravesical BCG immunotherapy due to a national shortage of BCG in the UK in 2012 and had intravesical Mitomycin C chemotherapy instead so were excluded. A further 3 patients were excluded because they did not complete the induction BCG treatment. Forty-three patients were eligible and the outcomes of their treatment were available for analysis. The median age for the study participants was 71.2 year (range 47-89 years). There were 9 females and 34 males. Twenty-five patients reported being vaccinated with BCG in the past or had evidence of a vaccination scar.

The patient's characteristics are presented in the table below (Table 4).

Seventeen cases were found to have recurrences at the first check cystoscopy (39.5%) and the median age of this group was 78 years (range 56-89 years). Twenty-six cases had no recurrence and the median age of this group was 69 years (range 47-87 years). Four cases had recurrences at the first surveillance cystoscopy (2 G2 recurrences, 1 G3 and 1 CIS recurrences). They were counselled on whether to have more intravesical BCG immunotherapy, have radical cystectomy or have an alternative treatment and all chose to continue with further intravesical BCG immunotherapy. They had no further recurrence during the follow up period and were therefore considered to have had a *successful* treatment outcome. The remaining 13 cases had either persistent recurrence at surveillance cystoscopy (4 cases), or had recurrence 6 months or after, i.e. following 2 courses of intravesical BCG treatment (2 cases) or had radical cystectomy due to recurrences after starting intravesical BCG treatment (7 cases). The characteristics of both groups are summarised in (Table 5).

Total number of patients	43
Median age (range)	71.2 years (47-89)
Male:Female	34:9
Original tumour Grade G3 CIS G3 + CIS CIS alone Stage Ta T1	 37 (86.1%) 19 (44.2%) 13 (30.2%) 6 (13.9%) 19 (44.2%) 24 (55.8%)
Lymphovascular invasion	0
Multifocal	12
Follow-up; median (range)	23 months (16-34 months)

Table 4: Patient's pre-treatment characteristics

	No Recurrence		Recurrence		*P* value
Number	26		17		
Age; median range	69 47-87		78 56-89		0.0721
Male : Female	21:5		13:4		0.5111
Original Histology Grade G3 CIS Stage Ta T1	 22 13 13 13	 84.6% 50% 50% 50%	 15 6 6 11	 88.2% 35.3% 35.3% 64.7%	 1.000 0.2635 0.3694 0.3694
Multifocal	5	19.2%	7	43.8%	0.2913
History of BCG immunisation	15	57.7%	10	62.5%	1.000

Table 5: Patient's post-treatment characteristics for recurrence and no recurrence groups.

4.1 Clinical Factors

Clinical factors, which may influence the response to intravesical BCG immunotherapy treatment, were evaluated and compared between groups. The age at start of BCG treatment, T stage of the initial resected bladder TCC according to the TNM classification, the presence or absence of CIS, single or multifocal disease at presentation and the BCG immunisation status prior to starting intravesical BCG immunotherapy treatment were compared between groups and also evaluated for association with recurrence. Other clinical information that were collected, like other medical conditions and the drug history were not analysed as no significant numbers or trends observed, as well it will not answer the primary question of this research which is to identify detectable biomarkers.

Comparing variables between groups:

Age:

The median age at the start of BCG treatment for the recurrence-free group was 69 years (range 47-87 years) while in the recurrence group the median was higher at 78 years (range 56-89 years). However, this did not reach the threshold for statistical significance [*P* value = 0.0721] (Table 6).

	Recurrence-free	**Recurrence**	***P* value**
Number	**26**	**17**	**0.0721**
Age; median range	69 47-87	78 56-89	

Table 6: Patient's age at start of BCG treatment: comparison between recurrence-free and recurrence groups- Mann Whitney test.

T stage

In the recurrence-free group, there were 13 cases (50%) that had Ta stage at initial resection, while there were 13 cases (50%) that had T1 stage. In the recurrence group, there were 11 cases (64.7%) that had T1 stage at initial resection and there were 6 cases

(35.3%) that had Ta stage. The difference in T stage between the two groups was not statistically significant [*P* value = 0.3694] (See table 7).

Data analysed	Recurrence-free	Recurrence	Total	*P* value
Ta	**13**	**6**	**19**	**0.3694**
T1	**13**	**11**	**24**	
Total	**26**	**17**	**43**	

Table 7: Comparison of T stage between recurrence-free and recurrence groups.

Carcinoma in situ

There were 13 cases (50%) that had CIS at initial resection before starting intravesical BCG treatment in the recurrence-free group and there were 13 cases (50%) that had no CIS at the initial resection. In the recurrence group, there were 6 (35.3%) that had CIS on the initial resection and there were 11 cases that had no CIS (64.7%) at initial resection. This was not found to be statistically significant [*P* value = 0.2635] (Table 8).

Data analysed	Recurrence-free	Recurrence	Total	*P* value
No CIS	**13**	**11**	**24**	**0.2635**
CIS	**13**	**6**	**19**	
Total	**26**	**17**	**43**	

Table 8: Comparison of patients CIS histology between recurrence-free and recurrence groups.

Focality

In the recurrence-free group, there were 5 cases (23.8%) that had multifocal disease at the initial resection while in the recurrence group there were 7 cases (43.8%) that had multifocal disease at presentation. Although the recurrence group had more multifocal disease at initial resection, this was not statistically significant on Fisher's exact test [*P* value = 0.2913] (Table 9).

Data analysed	Recurrence-free	Recurrence	Total	*P* value
Single	**16**	**9**	**25**	**0.2913**
Multifocal	**5**	**7**	**12**	
Total	**21**	**16**	**37**	

Table 9: Comparison of patient's focality histology between recurrence-free and recurrence groups.

BCG immunisation:

In total, there were 25 cases that reported to either being previously vaccinated against TB or had a BCG vaccination scar. Fifteen of these (57.7%) were in the recurrence-free group and there were 10 cases (58.8%) were in the recurrence group. In total, there were 18 cases that were not vaccinated and did not have a vaccination scar, of which 11 cases (42.3%) were in the recurrence-free group and 7 cases (41.2%) were in the recurrence group. There was no statistical difference between the groups [*P* value = 1.0000] (Table 10).

Data analysed	Recurrence-free	Recurrence	Total	*P* value
Not immunised	**11**	**7**	**18**	**1.000**
Immunised	**15**	**10**	**25**	
Total	**26**	**17**	**43**	

Table 10: Comparison of patient's previous immunisation between recurrence-free and recurrence groups.

Evaluating pre-treatment variables and association with recurrence

The relevant pre-treatment variables that were reported previously in published literature and that may influence the response to intravesical BCG immunotherapy treatment (age at start of treatment, T stage of the originally resected tumour, presence of CIS, multifocal disease at presentation and BCG immunisation status) were evaluated for association with recurrence after starting treatment.

Age

Age, as discussed previously, may influence response to intravesical BCG treatment. Herr *et al* [83] reported a 27% reduction in cancer-free survival for patients older than 70 years. We evaluated whether older age (> 70 years) was associated with higher risk of recurrence after starting intravesical BCG treatment.

There were 21 cases (48.8%) that were ≤70 years of age when they started intravesical BCG immunotherapy treatment, of which; there were 4 cases (19.1%) that had recurrences. There were 22 cases (51.2%) that were older than 70 years when they started the treatment

and of which; there were 13 (59.1%) that had recurrence after starting treatment. There were more recurrences in the group older than 70 years of age and this was found to be statistically significant [*P* value = 0.0122] (Table 11).

Data analysed	No Recurrence	Recurrence	Total
≤ 70 years	**17**	**4**	**21**
> 70 years	**9**	**13**	**22**
Total	**26**	**17**	**43**
Fisher's exact test; Odds ratio 6.139 (95% CI 1.542 – 24.45). *P* value = 0.0122			

Table 11: Association between patient's age and recurrence.

T stage

We evaluated if having T1 stage at initial resection is associated with higher risk of recurrence after starting intravesical BCG immunotherapy treatment. In total, there were 24 cases (44.2%) with T1 stage at initial resection and there were 19 cases (44.1%) with Ta stage. After intravesical BCG immunotherapy, there were 11 cases (45.8%) from the T1 stage that had recurrence while the remaining 13 cases (54.2%) had no recurrence. In the Ta stage, there were 6 cases (31.6%) that had recurrences and there were 13 cases (68.4%) that had no recurrences. On *Fisher's exact* test, there was no statistical difference between the two groups [*P* value = 0.3694].

Carcinoma in situ

We evaluated if having CIS at initial resection increases the risk of having recurrences after starting treatment. In total, there were 19 cases that had CIS in the histology prior to treatment, of which, there were 6 (31.5%) that had recurrence after treatment. While there were 24 that had no CIS prior to treatment and of which, there were 11 (45.8%) that had recurrences. On Fisher's exact test, CIS did not increase the risk of recurrence, [*P* value = 0.3694].

Focality:

We evaluated if having multifocal disease at initial resection increases the risk of having recurrences after starting intravesical BCG immunotherapy. There were 12 cases that had multifocal disease, of which; there were 7 cases (58.3%) that had recurrences. While 25 cases had a single tumour at the initial resection, of which; there were 9 (36%) that had recurrences after starting the treatment. The remaining patients had CIS with no papillary lesions.

Prior multi-focal disease at initial resection did not increase the risk of recurrence after treatment [*P* value = 0.2913].

Prior BCG immunisation:

We evaluated if prior BCG vaccination is associated with response to treatment. Before starting BCG immunotherapy, there were 25 cases that reported being vaccinated or had evidence of vaccination scar, of which, there were 10 cases (40.0%) that had recurrences. There were 18 cases that were not vaccinated prior to BCG treatment, of which; there were 10 cases (38.9%) that had recurrences. Prior vaccination did not influence the risk of recurrence [*P* value = 1.000].

4.2 Immunological Markers

In this section, immunological biomarkers measured in blood and urine samples, before and after intravesical BCG immunotherapy treatment, from our study population are presented.

4.2.1 Percentages of cytokine-producing CD4 T lymphocytes in response to PPD

4.2.1.1 Percentage of CD4 T lymphocytes producing IFNγ

When comparing the percentage of IFNγ+ CD4 T lymphocytes following *in vitro* PPD stimulation, there was a difference between the recurrence-free group and the recurrence group in the blood samples obtained before treatment. The median percentage of IFNγ+ producing CD4 T lymphocytes in the recurrence-free group was 0.43% (range 0 – 2.11%) while in the recurrence group, the median percentage was 0.26% (range 0 – 0.94%) and this was found to be statistically significant [*P* value = 0.0253] (Figure 15).

After induction intravesical BCG treatment, the median percentage of IFNγ+ CD4 T lymphocytes in the recurrence-free group was 0.39% (range 0.03 – 4.47%) while in the recurrence group, the median percentage was 0.12% (range 0 – 1.86%). This did not reach statistical significance [*P* value = 0.0744] (Figure 16).

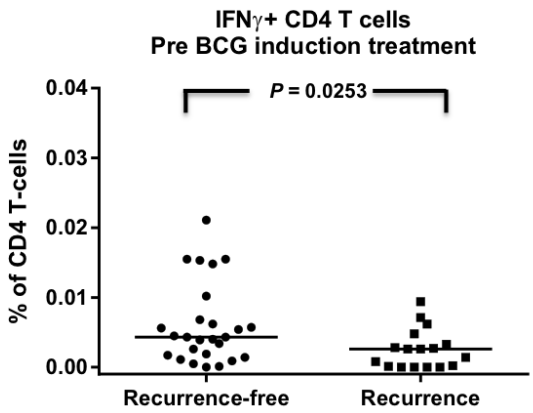

Figure 15

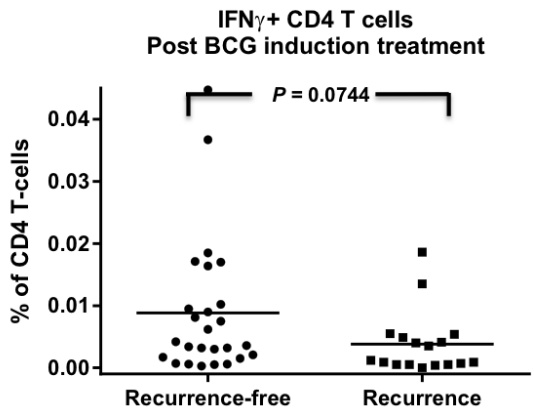

Figure 16

Figures 15 and 16: The percentage of IFNγ+ CD4 T lymphocytes post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (15) and after treatment (16).

4.2.1.2 Percentage of CD4 T lymphocytes producing IL2

The percentages of IL2+ CD4 T lymphocytes following PPD stimulation were also different between the recurrence-free group and the recurrence group in the blood samples obtained before treatment. The median percentage of IL2+ CD4 T lymphocytes in the recurrence-free group was 0.61% (range 0.06 – 1.61%) while in the recurrence group, the median percentage was 0.15% (range 0 – 2.22%). This was found to be statistically significant [*P* value = 0.0310] (Figure 17).

After induction intravesical BCG treatment, the median percentage of IL2+ CD4 T lymphocytes in the recurrence-free group was 0.55% (range 0 – 3.7%) while in the recurrence group; the median percentage was 0.39% (range 0.02 – 2.89%). This was not found to be statistically significant [*P* value = 0.6099] (Figure 18).

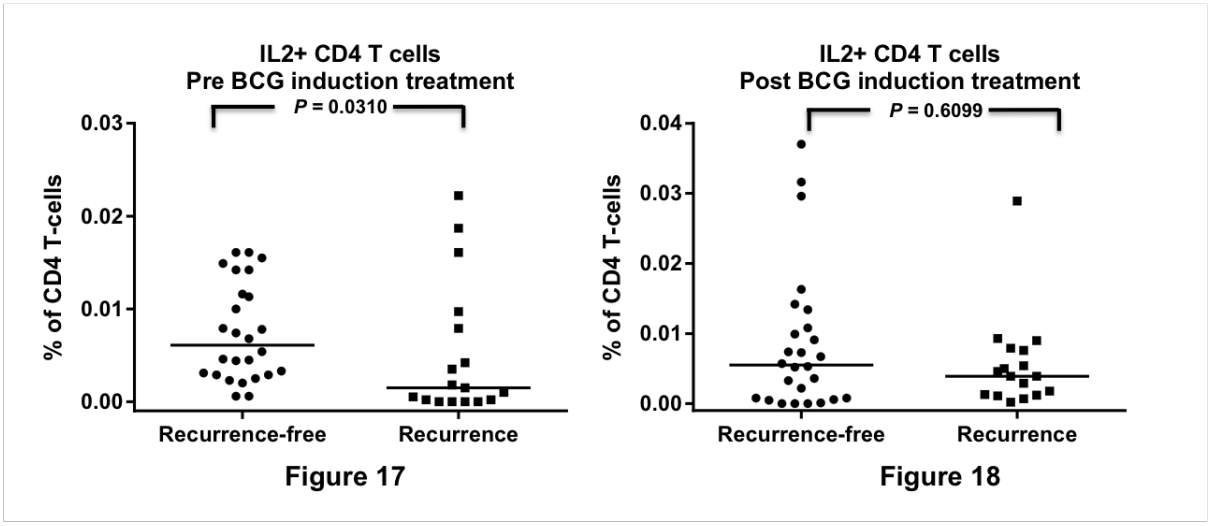

Figure 17 and 18: The percentage of IL2+ CD4 T lymphocytes post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (17) and after treatment (18).

4.2.1.3 Percentage of CD4 T lymphocytes producing TNF

The percentages of TNF+ CD4 T lymphocytes following PPD stimulation were also noticeably different between the recurrence-free group and the recurrence group in the blood samples obtained before treatment. The median percentage of TNF+ CD4 T lymphocytes in the recurrence-free group was 1.03% (range 0 – 4.48%) while in the recurrence group, the median percentage was 0.45% (range 0 – 1.77%). This was found to be statistically significant [*P* value = 0.0085] (Figure 19).

After induction intravesical with BCG treatment, the median percentage of TNF+ CD4 T lymphocytes in the recurrence-free group was 0.83% (range 0 – 4.77%) while in the recurrence group; the median percentage was 0.29% (range 0 – 4.25%). This was not found to be statistically significant [*P* value = 0.2747] (Figure 20).

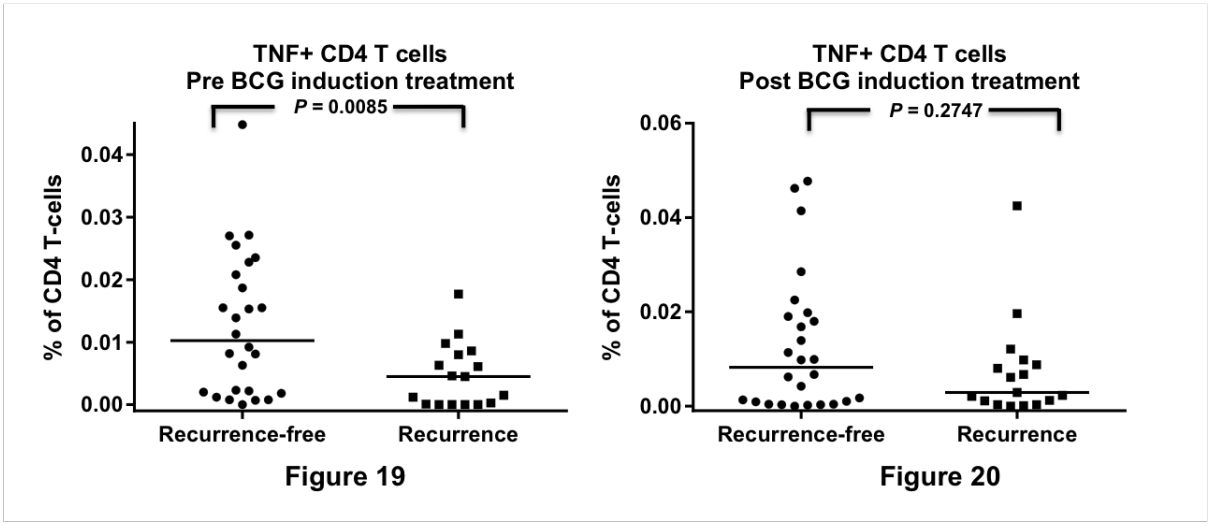

Figure 19 and 20: The percentage of TNF+ CD4 T lymphocytes post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (19) and after treatment (20).

4.2.1.4 Percentage of CD4 T lymphocytes producing CD40L

The percentages of CD40L+ CD4 T lymphocytes following PPD stimulation were not significantly different between the recurrence-free group and the recurrence group in the blood samples obtained before treatment. The median percentage of CD40L+ CD4 T lymphocytes in the recurrence-free group was 1.91% (range 0 – 7.49%) while in the recurrence group, the median percentage was 0.96% (range 0 – 3.84%). This was not found to be statistically significant [*P* value = 0.1452] (Figure 21).

After induction intravesical BCG treatment, the median percentage of CD40L+ CD4 T lymphocytes in the recurrence-free group was 1.29% (range 0 – 7.45%) while in the recurrence group; the median percentage was 1.66% (range 0 – 6.09%). This was not found to be statistically significant [*P* value = 0.8294] (Figure 22).

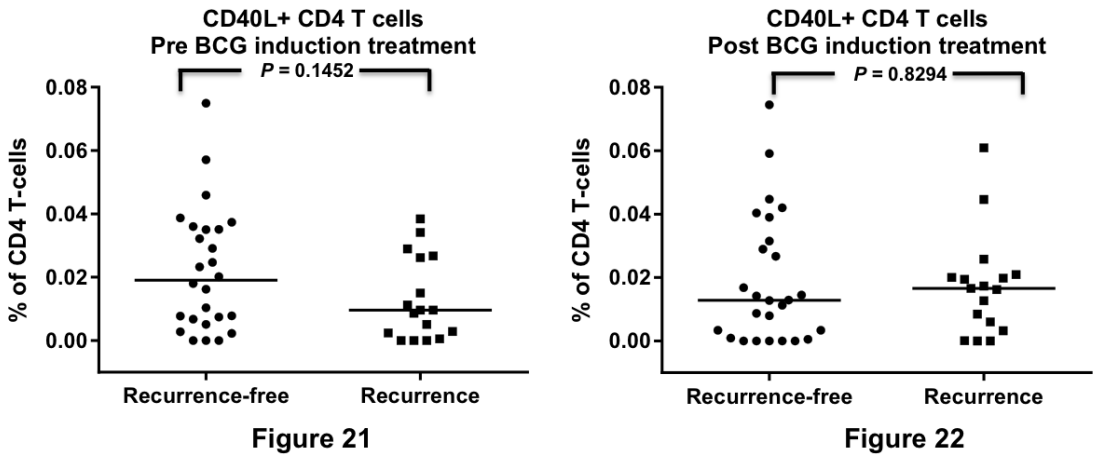

Figure 21 and 22: The percentage of CD40L+ CD4 T lymphocytes post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (21) and after treatment (22).

4.2.1.5 Percentage of CD4 T lymphocytes producing IL17a

The percentages of IL17a+ CD4 T lymphocytes following PPD stimulation were not significantly different between the recurrence-free group and the recurrence group in the blood samples obtained before treatment. The median percentage of IL17a+ CD4 T lymphocytes in the recurrence-free group was 0.07% (range 0 – 2.01%) while in the recurrence group, the median percentage was 0.08% (range 0 – 0.8%). This was not found to be statistically significant [*P* value = 0.6356] (Figure 23).

After induction intravesical BCG treatment, there was a noticeable difference between the two groups as the median percentage of IL17a+ CD4 T lymphocytes in the recurrence-free group was 0.14% (range 0 – 2.71%) while in the recurrence group; the median percentage was 0.04% (range 0 – 0.46%). This did not reach statistical significance [*P* value = 0.0928] (Figure 24).

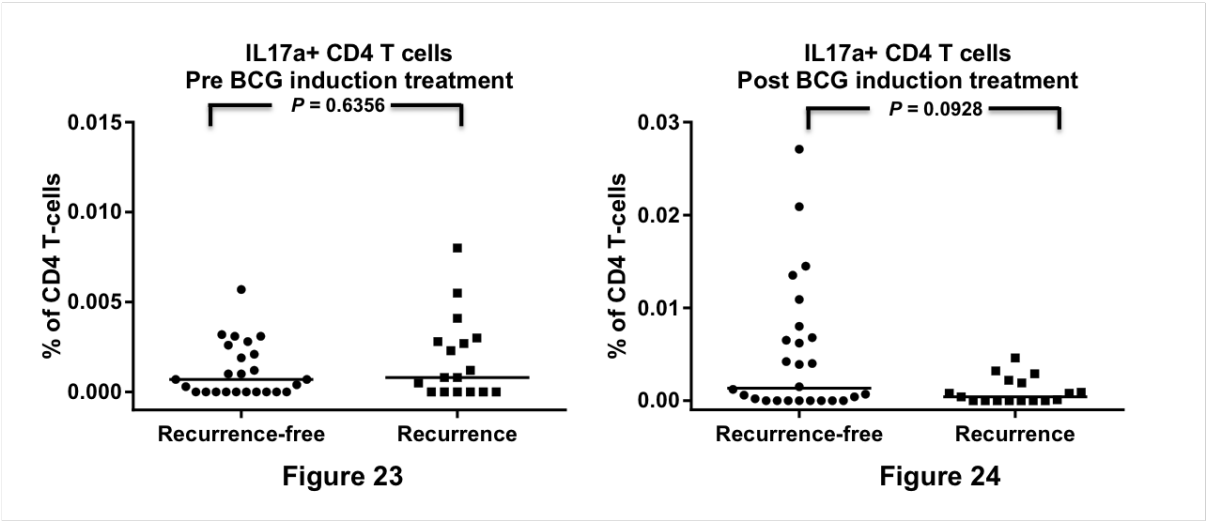

Figure 23 and 24: The percentage of IL17a+ CD4 T lymphocytes post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (23) and after treatment (24).

4.2.1.6 Percentage of functional T cell subsets

As previously discussed, some functional subsets have been reported in the literature to be vital for immunity against pathogens [30-32]. The percentages of PPD-induced functional subsets within the total CD4 lymphocytes population are summarised in table 12 below for the pre-treatment samples, and table 13 below for the post-treatment samples. The percentages of functional subsets for the CCR7+CD27+CD28+, CCR7-CD27+CD28+, CCR7-CD27-CD28+ and CCR7-CD27-CD28- are summarised in appendix 5.

Total CD4 lymphocytes								
CD40L	IFNγ	IL2	IL17a	TNF	Recurrence-free Median (range)	Recurrence Median (range)	*P* value
+	+	+	+	+	0% (0 – 0.04%)	0% (0 – 0.02%)	---
+	+	+	+		0% (0 – 0%)	0% (0 – 0%)	---
+	+	+		+	0.23% (0 – 1.3%)	0.04% (0 – 0.26%)	0.0013
+	+	+			0% (0 – 0.05%)	0% (0 – 0.05%)	---
+	+		+	+	0% (0 – 0%)	0% (0 – 0.78%)	---
+	+		+		0% (0 – 0%)	0% (0 – 0.03%)	---
+	+			+	0.1% (0 – 1.43%)	0.01% (0 – 0.32%)	0.0254
+	+				0.03% (0 – 0.15%)	0.01% (0 – 0.15%)	---
+		+	+	+	0.02% (0 – 0.11%)	0.02% (0 – 0.09%)	---
+		+	+		0% (0 – 0%)	0% (0 – 0.03%)	---
+		+		+	0.17% (0 – 0.95%)	0.05% (0 – 1.11%)	0.0258
+		+			0.05% (0 – 0.32%)	0.03% (0 – 0.47%)	---
+			+	+	0.01% (0 – 0.09%)	0% (0 – 0.14%)	---
+			+		0.01% (0 - 0.06%)	0% (0 – 0.05%)	---
+				+	0.23% (0 – 1.05%)	0.06% (0 – 0.41%)	0.0360
+					0.58% (0 – 6.01%)	0.51% (0 – 2.69%)	0.4114
	+	+	+	+	0% (0 – 0.01%)	0% (0 – 0%)	---
	+	+	+		0% (0 – 0%)	0% (0 – 0%)	---
	+	+		+	0% (0 – 0.14%)	0% (0 – 0.06%)	---
	+	+			0% (0 – 0.01%)	0% (0 – 0.02%)	---
	+		+	+	0% (0 – 0%)	0% (0 – 0%)	---
	+		+		0% (0 – 0.01%)	0% (0 – 0.04%)	---
	+			+	0.01% (0 – 0.51%)	0% (0 – 0.29%)	---
	+				0.04% (0 – 0.41%)	0% (0 – 0.47%)	0.0629
		+	+	+	0% (0 - 0.01%)	0% (0 – 0%)	---
		+	+		0% (0 – 0.02%)	0% (0 – 0.03%)	---
		+		+	0% (0 – 0.03%)	0% (0 – 0.02%)	---
		+			0.1% (0 – 0.38%)	0% (0 – 1.14%)	0.0636
			+	+	0% (0 – 0%)	0% (0 – 0.03%)	---
			+		0.04% (0 – 2%)	0.05% (0 – 0.55%)	0.5265
				+	0.05% (0 - 1.64%)	0% (0 – 0.14%)	0.0037

Table 12: the percentage of functional subsets within the total CD4 lymphocytes population in the recurrence-free and recurrence groups before starting intravesical BCG treatment for patients with high-grade non-muscle invasive bladder cancer. Bonferroni correction with a factor of 31 was applied and *P* values ≤0.0016 were considered statistically significant.

Total CD4 lymphocytes								
CD40L	IFNγ	IL2	IL17a	TNF	Recurrence-free median (range)	Recurrence median (range)	*P* value
+	+	+	+	+	0% (0 – 0.12%)	0% (0 – 0.02%)	---
+	+	+	+		0% (0 – 0%)	0% (0 – 0%)	---
+	+	+		+	0.23% (0 – 3.38%)	0.08% (0 – 0.88%)	0.1835
+	+	+			0% (0 – 0.12%)	0% (0 – 0.05%)	---
+	+		+	+	0% (0 – 0.01%)	0% (0 – 0%)	---
+	+		+		0% (0 – 0%)	0% (0 – 0%)	---
+	+			+	0.08% (0 – 2.09%)	0.03% (0 – 1.22%)	0.3236
+	+				0.02% (0 – 0.18%)	0.02% (0 – 0.09%)	---
+		+	+	+	0.03% (0 – 0.28%)	0.01% (0 – 0.09%)	---
+		+	+		0% (0 – 0.03%)	0% (0 – 0.01%)	---
+		+		+	0.05% (0 – 1.39%)	0.06% (0 – 1.63%)	0.6791
+		+			0.01% (0 – 0.67%)	0.07% (0 – 0.26%)	---
+			+	+	0% (0 – 0.14%)	0% (0 – 0.05%)	---
+			+		0% (0 - 0.09%)	0% (0 – 0.05%)	---
+				+	0.07% (0 – 0.9%)	0.05% (0 – 1.24%)	0.8472
+					0.57% (0 – 2.57%)	0.68% (0 – 2.95%)	0.4158
	+	+	+	+	0% (0 – 0.01%)	0% (0 – 0%)	---
	+	+	+		0% (0 – 0%)	0% (0 – 0%)	---
	+	+		+	0% (0 – 0.22%)	0% (0 – 0.03%)	---
	+	+			0% (0 – 0.01%)	0% (0 – 0.01%)	---
	+		+	+	0% (0 – 0%)	0% (0 – 0%)	---
	+		+		0% (0 –0.05%)	0% (0 – 0.04%)	---
	+			+	0.01% (0 – 0.42%)	0% (0 – 0.23%)	---
	+				0.08% (0 – 0.33%)	0% (0 – 0.15%)	0.0140
		+	+	+	0% (0 - 0.04%)	0% (0 – 0%)	---
		+	+		0% (0 – 0.04%)	0% (0 – 0.02%)	---
		+		+	0% (0 - 0.02%)	0% (0 – 0%)	---
		+			0.06% (0 – 0.17%)	0.02% (0 – 0.19%)	0.8159
			+	+	0% (0 –0.04%)	0% (0 – 0%)	---
			+		0.15% (0 - 2.57%)	0% (0 – 0.35%)	0.0618
				+	0.06% (0 - 0.81%)	0% (0 – 0.24%)	0.0029

Table 13: the percentage of functional subsets within the total CD4 lymphocytes population in the recurrence-free and recurrence groups after completing intravesical BCG treatment for patients with high-grade non-muscle invasive bladder cancer. Bonferroni correction with a factor of 31 was applied and *P* values ≤0.0016 were considered statistically significant. .

Percentage of the CD4 T lymphocytes subset producing CD40L+IFNγ+IL2+TNF+

As discussed earlier, polyfunctional T lymphocytes capable of secreting Th1 cytokines (IFNγ, IL2 and TNF) were reported to be important for immunity against TB. The frequency of polyfunctional subsets capable of secreting all the measured Th1 related

cytokines (CD40L, IFNγ, IL2 and TNF) were compared between the two groups and was found to be significantly different between the recurrence-free group and the recurrence group.

The percentages of the (CD40L+IFNγ+IL2+TNF+) polyfunctional CD4 T lymphocytes following PPD stimulation were significantly higher in the recurrence-free group; median percentage 0.23% (range 0 – 1.3%) while in the recurrence group, the median percentage was 0.04% (range 0 – 0.26%). This was found to be statistically significant [*P* value = 0.0013] (Figure 25).

After induction intravesical BCG treatment, although the median percentage of (CD40L+IFNγ+IL2+TNF+) CD4 T lymphocytes in the recurrence-free group was still higher than recurrence group; 0.23% (range 0 – 3.38%) and 0.08% (range 0 – 0.88%). This did not reach statistical significance [*P* value = 0.1835] (Figure 26).

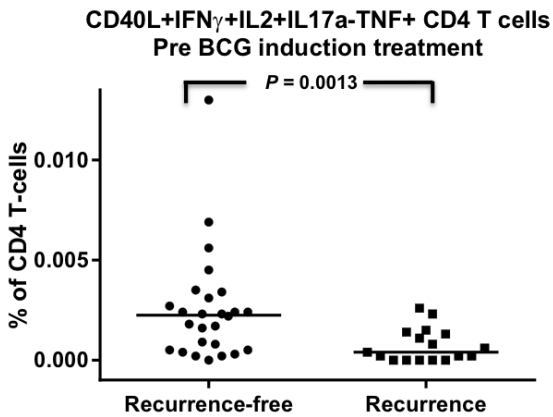

Figure 25

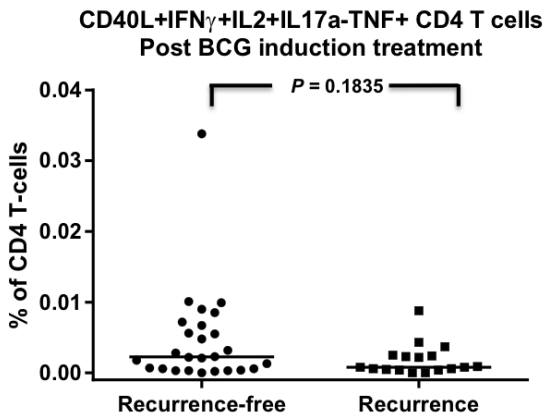

Figure 26

Figure 25 and 26: The percentage of CD40L+IL2+IFNγ+IL17a-TNF+ CD4 T lymphocytes post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (25) and after treatment (26).

4.2.2 Percentage of cytokine producing CD4 T lymphocytes in response to SEB

In order to clarify whether the differences seen in the percentage of cytokine producing CD4 T lymphocytes was PPD specific or not, responses to SEB stimulation, used as a positive control, were also evaluated for differences between the recurrence-free and recurrence groups.

4.2.2.1 Percentage of CD4 T lymphocytes producing IFNγ

The percentages of IFNγ+ CD4 T lymphocytes following SEB stimulation were not significantly different between the recurrence-free group and the recurrence group before treatment. The median percentage of IFNγ+ CD4 T lymphocytes in the recurrence-free group was 4.22% (range 0.68 – 10.86%) while in the recurrence group, the median percentage was 3.03% (range 0.05 – 10.97%). This was not found to be statistically significant [*P* value = 0.4326] (Figure 27).

After induction intravesical BCG treatment, the median percentage of IFNγ+ CD4 T lymphocytes in the recurrence-free group was 4.95% (range 0.14 – 15.22%) while in the recurrence group; the median percentage was 4.09% (range 0.87 – 10.33%). This was not found to be statistically significant [*P* value = 0.3260] (Figure 28).

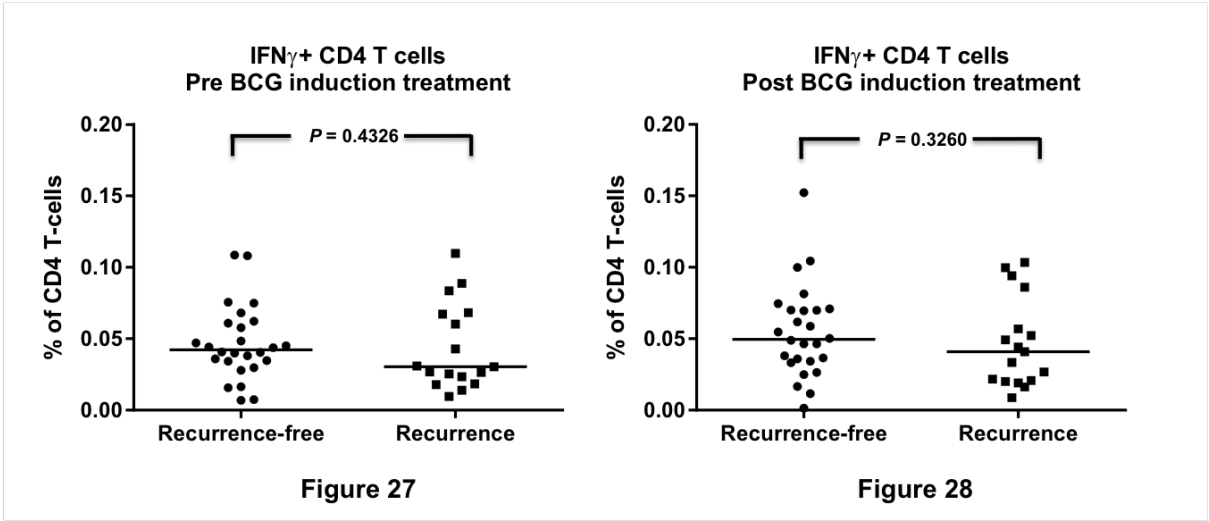

Figure 27 and 28: The percentage of IFNγ+ CD4 T lymphocytes post Staphylococcal enterotoxin B (SEB) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (27) and after treatment (28).

4.2.2.2 Percentage of CD4 T lymphocytes producing IL2

The percentages of IL2+ CD4 T lymphocytes following SEB stimulation were noticeably different between the recurrence-free group and the recurrence group before treatment. The median percentage of IL2+ CD4 T lymphocytes in the recurrence-free group was 10.48% (range 3.93 –23.28%) while in the recurrence group, the median percentage was 5.43% (range 0.97 –15.42%). This was found to be statistically significant [*P* value = 0.0014] (Figure 29).

After induction intravesical BCG treatment, the median percentage of IL2+ CD4 T lymphocytes in the recurrence-free group was 10.3% (range 0.66 – 27.11%) while in the recurrence group; the median percentage was 6.89% (range 2.73 – 22.39%). This difference did not reach statistical significance [*P* value = 0.0927] (Figure 30).

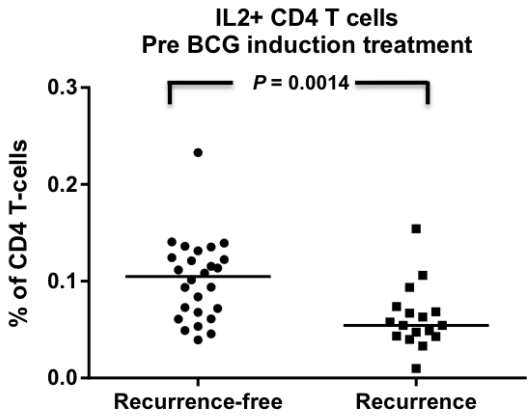

Figure 29

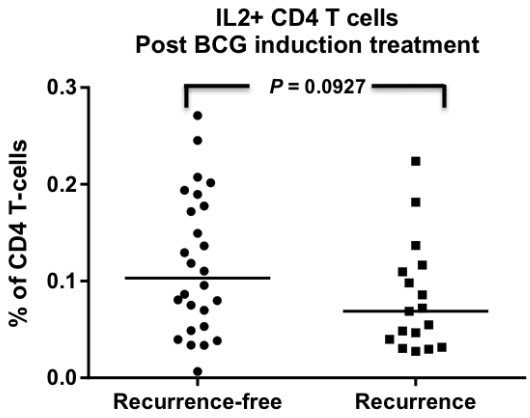

Figure 30

Figure 29 and 30: The percentage of IL2+ CD4 T lymphocytes post Staphylococcal enterotoxin B (SEB) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (29) and after treatment (30).

4.2.2.3 Percentage of CD4 T lymphocytes producing TNF

The percentages of TNF+ CD4 T lymphocytes following SEB stimulation were also noticeably different between the recurrence-free group and the recurrence group before treatment. The median percentage of TNF+ CD4 T lymphocytes in the recurrence-free group was 11.85% (range 4.95 – 24.12%) while in the recurrence group the median percentage was 8.31% (range 3.7 – 18.96%). This was found to be statistically significant [*P* value = 0.0323] (Figure 31).

After induction intravesical BCG treatment the median percentage of TNF+ CD4 T lymphocytes in the recurrence-free group was 12.87% (range 0 – 27.37%) while in the recurrence group; the median percentage was 8.14% (range 3.95 – 29.82%). This difference was also found to be statistically significant [*P* value = 0.0344] (Figure 32).

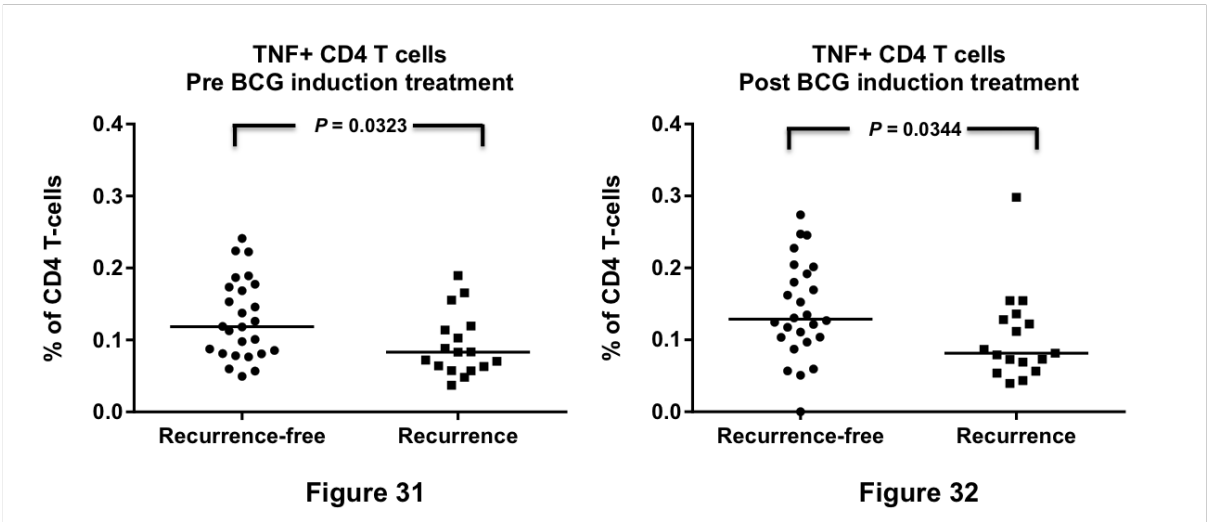

Figure 31 and 32: The percentage of TNF+ CD4 T lymphocytes post Staphylococcal enterotoxin B (SEB) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (31) and after treatment (32).

4.2.2.4 Percentage of CD4 T lymphocytes producing CD40L

The percentages of CD40L+ CD4 T lymphocytes following SEB stimulation were different between the recurrence-free group and the recurrence group before treatment. The median percentage of CD40L+ CD4 T lymphocytes in the recurrence-free group was 29.87% (range 3.97 – 47.46%) while in the recurrence group, the median percentage was 26.69% (range 17.07-63.74%). This was found to be statistically significant. [*P* value = 0.0232] (Figure 33).

After induction intravesical BCG treatment the median percentage of CD40L+ CD4 T lymphocytes in the recurrence-free group was 34.04% (range 3.29-52.06%) while in the recurrence group; the median percentage was 30.14% (range 18.60 - 47.18%). This was not found to be statistically significant [*P* value = 0.1168] (Figure 34).

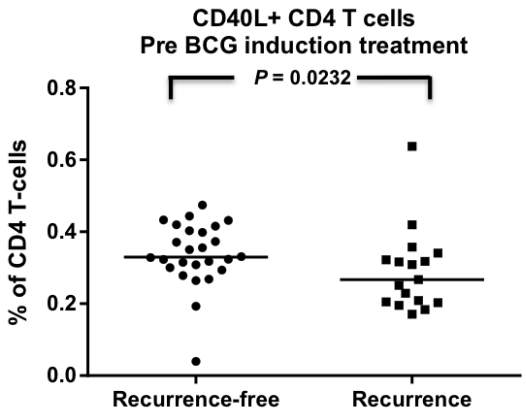

Figure 33

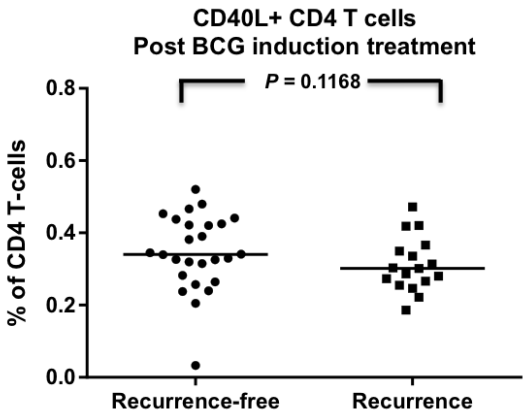

Figure 34

Figure 33 and 34: The percentage of CD40L+ CD4 T lymphocytes post Staphylococcal enterotoxin B (SEB) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (33) and after treatment (34).

4.2.2.5 Percentage of CD4 T lymphocytes producing IL17a

The percentages of IL17a+ CD4 T lymphocytes following SEB stimulation were different between the recurrence-free group and the recurrence group before treatment. The median percentage of IL17a+ CD4 T lymphocytes in the recurrence-free group was 0.63% (range 0 – 1.89%) while in the recurrence group, the median percentage was 0.35% (range 0 – 9.59%). This did not reach statistical significance [*P* value = 0.0831] (Figure 35).

After induction intravesical BCG treatment the median percentage of IL17a+ CD4 T lymphocytes in the recurrence-free group was 0.69% (range 0 – 1.72%) while in the recurrence group; the median percentage was 0.5% (range 0.02 – 12.7%). This was not found to be statistically significant [*P* value = 0.7077] (Figure 36).

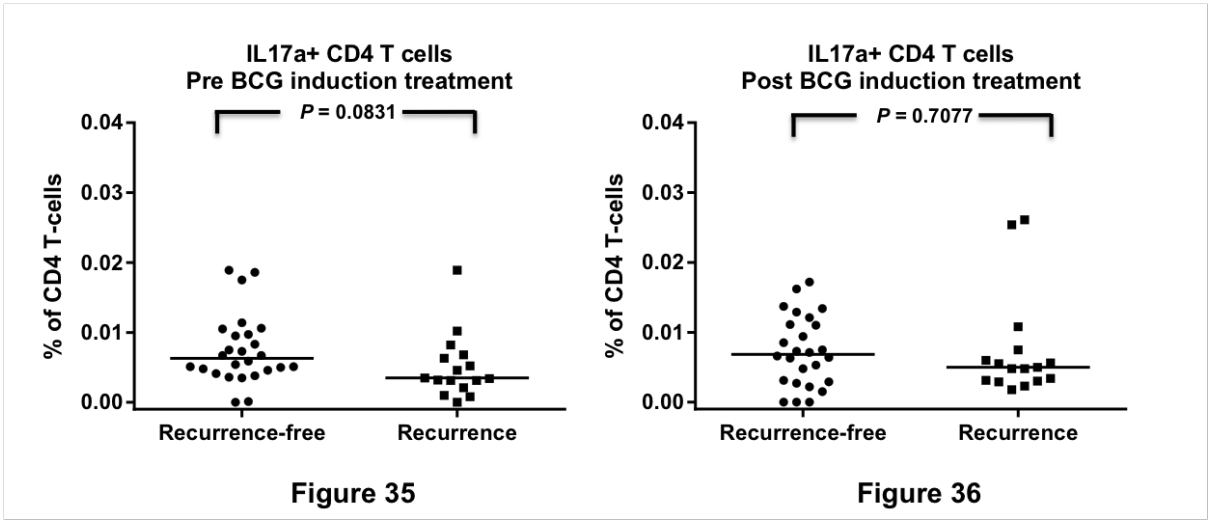

Figure 35 and 36: The percentage of IL17a+ CD4 T lymphocytes post Staphylococcal enterotoxin B (SEB) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (35) and after treatment (36).

4.2.2.6 Percentage of the CD4 T lymphocytes subset producing CD40L+IFNγ+IL2+TNF+

The percentages of CD40L+IFNγ+IL2+TNF+ CD4 T lymphocytes following SEB stimulation were different between the recurrence-free group and the recurrence group before treatment. The median percentage of CD40L+IFNγ+IL2+TNF+ CD4 T lymphocytes in the recurrence-free group was 1.83% (range 0 – 5.44%) while in the recurrence group, the median percentage was 1.14% (range 0.43 – 4.56%). This was found to be statistically significant [*P* value = 0.0264] (Figure 37).

After induction intravesical BCG treatment, the median percentage of CD40L+IFNγ+IL2+TNF+ CD4 T lymphocytes in the recurrence-free group was 2.29% (range 0 – 3.7%) while in the recurrence group; the median percentage was 1.26% (range 0.43 – 5.7%). This was not found to be statistically significant [*P* value = 0.1423] (Figure 38).

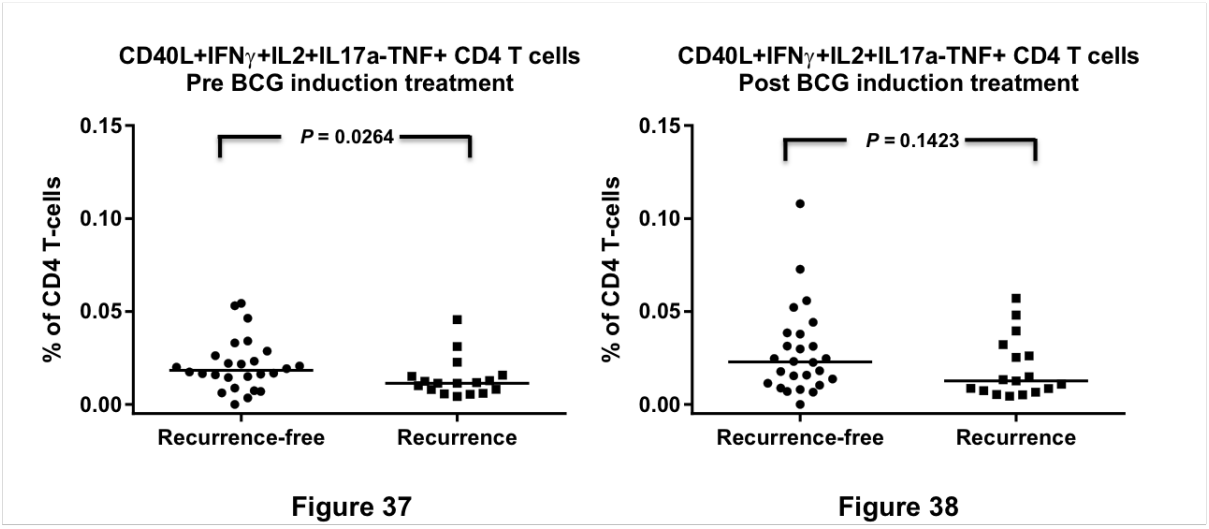

Figure 37 and 38: The percentage of CD40L+IFNγ+IL2+IL17a-TNF+ CD4 T lymphocytes post Staphylococcal enterotoxin B (SEB) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (37) and after treatment (38).

In summary, significant differences were found in the pre-treatment percentages of PPD-inducible IFNγ+, IL2+, TNF+ and the polyfunctional (CD40L+ IFNγ+IL2+TNF+) CD4 T lymphocytes, which were all significantly lower in the recurrence group. After induction BCG, these differences persist to after treatment but the gap between the two groups narrowed and these were not found to be statistically significant.

Similar significant differences were observed in the pre-treatment percentages of SEB-inducible IL2+, TNF+, CD40L and the polyfunctional (CD40L+ IFNγ+IL2+TNF+) CD4 T lymphocytes.

4.2.3 Secreted cytokines post PPD stimulation

Following overnight PPD stimulation of PBMC, the levels of secreted cytokines in supernatants were measured and evaluated for differences between the two groups. Levels of IFNγ, IL2 and TNF are described in detail below. Tables 18 and 19 summarise the results for the exploratory cytokines measured after *in vitro* PPD stimulation.

4.2.3.1 Secreted IFNγ

The level of IFNγ measured in supernatant following overnight PPD stimulation of PBMC were different between the recurrence-free group and the recurrence group before treatment. The median level of IFNγ in the recurrence-free group was 8558pg/ml (range 581.9 -91290pg/ml) while in the recurrence group the median level of secreted IFNγ was 1989pg/ml (range 38.5-38486pg/ml). This was found to be statistically significant [*P* value = 0.0060] (Figure 39).

After induction intravesical BCG treatment, the level of secreted of IFNγ in the recurrence-free group was 6217pg/ml (range 36.3-73940pg/ml) while in the recurrence group the median level of secreted IFNγ was 3982pg/ml (range 113.9-23655pg/ml). This did not reach statistical significance [*P* value = 0.3638] (Figure 40).

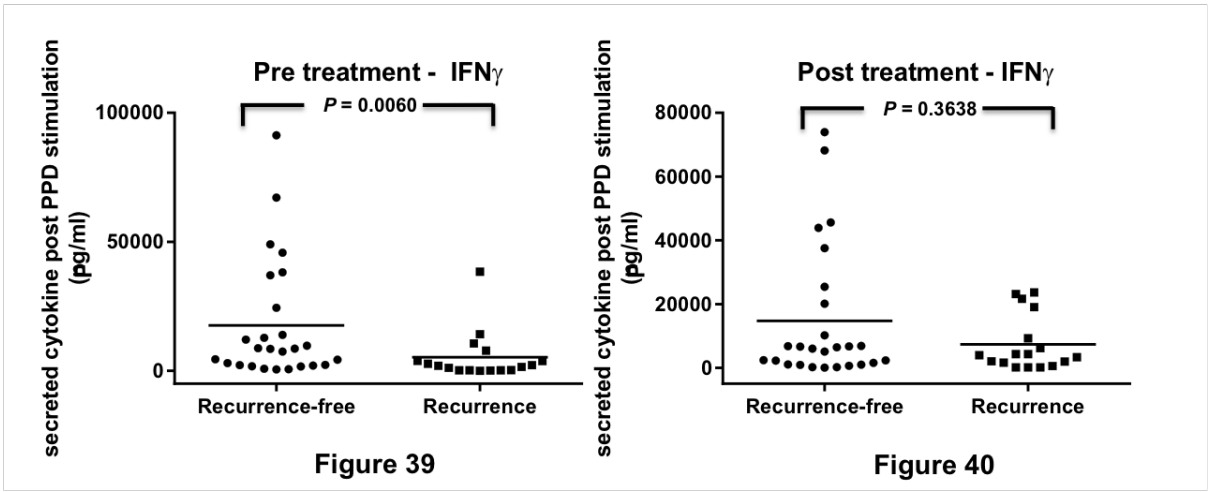

Figure 39 and 40: The levels of IFNγ measured in supernatant post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (39) and after treatment (40).

4.2.3.2 Secreted IL2

The level of IL2 measured in supernatant following overnight PPD stimulation of PBMC was also noticeably different between the recurrence-free group and the recurrence group before treatment. The median level of IL2 in the recurrence-free group was 489.5pg/ml (range 113.8-1622pg/ml) while in the recurrence group the median amount of secreted IL2 was 187.1pg/ml (range 18.3-1155pg/ml). This was found to be statistically significant [*P* value = 0.0013] (Figure 41).

After induction intravesical BCG treatment, the level of secreted of IL2 in the recurrence-free group was 342.9pg/ml (range 6-2732pg/ml) while in the recurrence group; the median level of secreted IL2 was 297.5pg/ml (range 39.9-1048pg/ml). This was not found to be statistically significant [*P* value = 0.3509] (Figure 42).

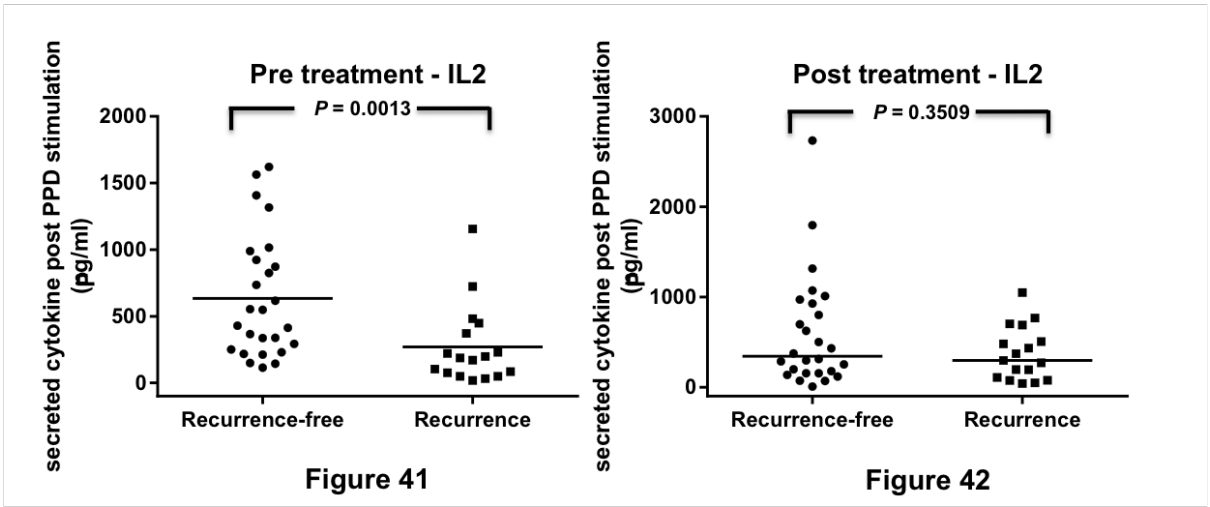

Figure 41 and 42: The levels of IL2 measured in supernatant post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (41) and after treatment (42).

4.2.3.3 Secreted TNF

The level of TNF measured in supernatant following overnight PPD stimulation of PBMC was not significantly different between the Recurrence-free group and the recurrence group before treatment. The median level of TNF in the recurrence-free group was 995.2pg/ml (range 412.6-3442pg/ml) while in the recurrence group the median level of secreted TNF was 778.1pg/ml (range 130.7 – 3089pg/ml). This was not found to be statistically significant, [*P* value = 0.3140], see figure 43.

After induction intravesical BCG treatment, the level of secreted of TNF in the recurrence-free group was 671.5pg/ml (range 176-5234pg/ml) while in the recurrence group the level amount of secreted TNF was 638.6pg/ml (range 106.7-2395pg/ml). This was not found to be statistically significant [*P* value = 0.3022] (Figure 44).

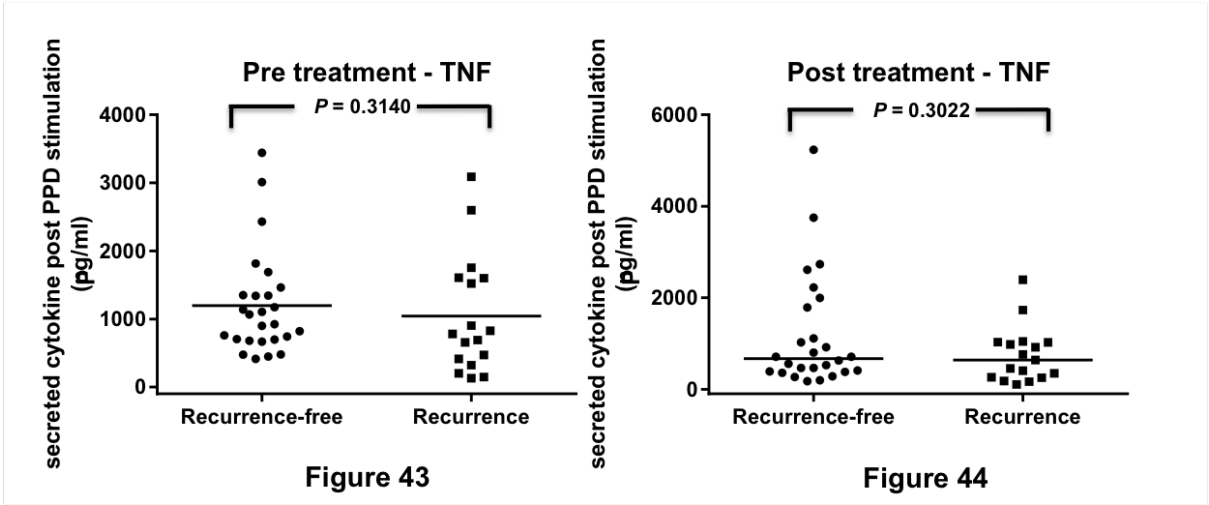

Figure 43 and 44: The levels of TNF measured in supernatant post tuberculin purified protein derivative (PPD) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (43) and after treatment (44).

4.2.3.4 Other Secreted Cytokines

The pre-treatment median levels of IL1β measured in supernatant post *in vitro* PPD stimulation was higher in the recurrence-free patients compared to the recurrence group (719.5pg/ml, range 147.6-3781pg/ml. and 387pg/ml respectively 32.8 -3259pg/ml, [*P* value 0.0163]). There was no significant difference after treatment.

There was no significant difference observed in the other exploratory cytokines summarised in tables 14 for pre-treatment samples and table 15 for the post-treatment samples.

Secreted cytokine	Recurrence-free Median (range)	Recurrence Median (range)	*P* value
IL1β	719.5pg /ml (147.6-3781pg /ml)	387pg/ml (32.8 – 3259pg /ml)	0.0163
IL4	31.7pg /ml (9.4 – 41.5pg /ml)	28.3pg /ml (5.5 – 32.94pg /ml)	0.8830
IL6	6601pg /ml (3602 – 17379pg /ml)	5953pg/ml (1465-14454pg/ml)	0.6242
IL8	53673pg/ml (5337-127666pg/ml)	55176pg/ml (13750-442048pg/ml)	0.8064
IL10	297pg/ml (131.9-1036pg/ml)	293pg/ml (71.6-583.7pg/ml)	0.1601
IL12p70	30.8pg/ml (8 -72.2pg/ml)	34.5pg/ml (11.5 – 58.11pg/ml)	0.4089
IL13	349.2pg/ml (140.6-1054pg/ml)	623.5pg/ml (62.2–1153pg/ml)	0.9412

Table 14: The summary of secreted exploratory cytokines measured by electrochemiluminescence after *in vitro* PPD stimulation from blood samples obtained before starting intravesical BCG treatment for bladder cancer.

Secreted cytokine	Recurrence-free Median (range)	Recurrence Median (range)	*P* value
IL1β	356.4pg /ml (64.6-2202pg/ml)	345.9pg /ml (58.5-1957pg/ml)	0.4471
IL4	31.20pg /ml (2.3-29.8pg/ml)	23.34pg /ml (5.6-29pg /ml)	0.0595
IL6	3992pg/ml (1364-13460pg/ml)	4451pg/ml (1482 – 15449pg/ml)	0.5565
IL8	51132pg/ml (7389-94443pg/ml)	46442pg/ml (18786-113825pg/ml)	0.3458
IL10	166.9pg/ml (9.9-748.6pg/ml)	182pg/ml (23.9-613.3pg/ml)	0.7928
IL12p70	28.9pg/ml (3.8- 75.4pg/ml)	24.12pg/ml (5.8 - 123.8pg/ml)	0.1558
IL13	367pg/ml (57.4-1391pg/ml)	412.1pg/ml (66.8 – 800.3pg/ml)	>0.9999

Table 15: The summary of secreted exploratory cytokines measured by electrochemiluminescence after *in vitro* PPD stimulation from blood samples obtained after completing induction intravesical BCG treatment for bladder cancer

In summary, significant differences were found in the pre-treatment levels IFNγ and IL2 measured in the supernatant following overnight PPD stimulation, which were significantly lower in the recurrence group.

4.2.4 Urine cytokines

As discussed earlier, several reports have found differences in post treatment urine cytokines levels between responders and non-responders to treatment [72, 73, 105-110]. The urine samples were collected before starting treatment and 4 hours after the 6th intravesical BCG instillation and differences were evaluated between the two groups. The pre-treatment urine samples had no measurable cytokines and hence no results can be presented and all urine results that are presented are for the post-treatment urine samples.

4.2.4.1 Urine IFNγ levels

The levels of IFNγ measured in urine following induction intravesical BCG treatment were different between the recurrence-free group and the recurrence group. The median level of IFNγ in the recurrence-free group was higher at 159pg/ml (range 1-39310pg/ml) while in the recurrence group the median level of IFNγ was 43pg/ml (range 0-7782pg/ml). This did not reach statistical significance [*P* value = 0.1103] (Figure 45).

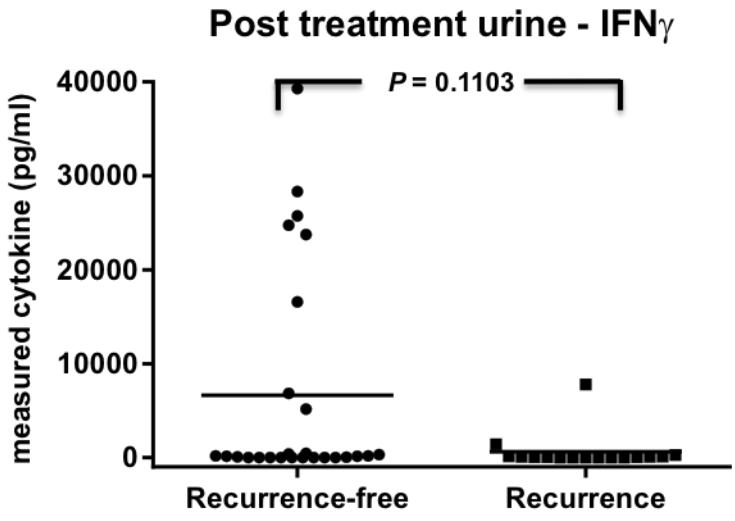

Figure 45

Figure 45: The levels of IFNγ measured in the urine in recurrence-free and recurrence groups after induction intravesical BCG treatment.

4.2.4.2 Urine IL2 levels

The level of IL2 measured in urine following induction intravesical BCG treatment was different between the recurrence-free group and the recurrence group. The median level of IL2 in the recurrence-free group was markedly higher; median 178.5pg/ml (range 4-1905pg/ml) while in the recurrence group, the median level of IL2 was 71pg/ml (range 1-1111pg/ml), this did not reach statistical significance [*P* value = 0.1198] (figure 46).

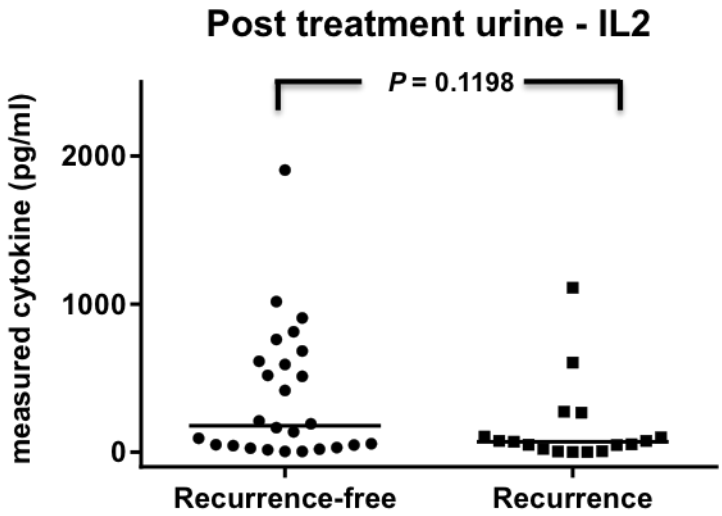

Figure 46

Figure (46); levels of IL2 measured in the urine of recurrence-free and recurrence groups after induction intravesical BCG treatment.

4.2.4.3 Urine TNF levels

The level of TNF measured in urine following induction intravesical BCG treatment were noticeably different between the recurrence-free group and the recurrence group. The median level of TNF in the recurrence-free group was higher at median 44.5pg/ml (range 0-654pg/ml) while in the recurrence group the median level of TNF was 14pg/ml (range 1-256pg/ml). This did not reach statistical significance [*P* value = 0.0764] (Figure 47).

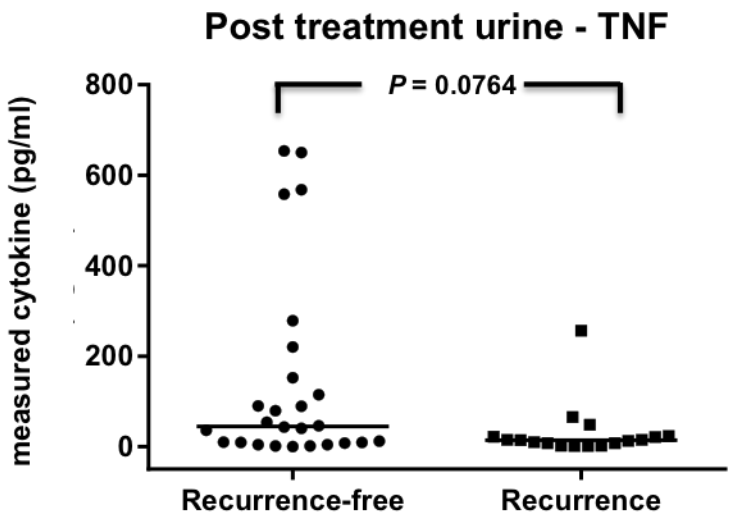

Figure 47

Figure 47: The levels of TNF measured in the urine of recurrence-free and recurrence groups after induction intravesical BCG treatment.

4.2.4.4 Other Urine Cytokines

The median level of IL4 measured in urine after the 6th BCG instillation was noticeably higher in the recurrence-free group compared to the recurrence group and this was statistically significant (3pg/ml; range 0-64pg/ml, and 1pg/ml; range 0-5pg/ml respectively. [*P* value = 0.0351]). The median levels of IL6 were also noticeably higher and this was found to be statistically significant (244.5pg/ml; range 2-1978pg/ml, and 126pg/ml; range 3-903pg/ml respectively *P* value = 0.0499). Median levels of IL8 and IL13 were also higher in the recurrence-free group, but this did not reach statistical significance.

The exploratory cytokines measured in urine after completing BCG induction treatment are summarised in table 16 below.

Urine cytokine	Recurrence-free Median (range)	Recurrence Median (range)	*P* value
IL1β	956.5pg/ml (14-17267pg/ml)	804pg/ml (4-3021pg/ml)	0.2144
IL4	3pg/ml (0-64pg/ml)	1pg/ml (0-5pg/ml)	0.0351
IL6	244.5pg/ml (2-1978pg/ml)	126pg/ml (3-903pg/ml)	0.0499
IL8	8247pg/ml (378-72713pg/ml)	3847pg/ml (530-19254pg/ml)	0.0977
IL10	6pg/ml (0-251pg/ml)	5pg/ml (0-79pg/ml)	0.1909
IL12p70	5pg/ml (0-29pg/ml)	2pg/ml (0-8pg/ml)	0.0671
IL13	40.5pg/ml (5-155pg/ml)	34pg/ml (6-78pg/ml)	0.2334

Table 16: The summary of urine exploratory cytokines measure after completing induction intravesical BCG treatment for bladder cancer

In summary, levels of urinary IFNγ, TNF and IL2 after treatment were not found to be significantly different between the recurrence and recurrence-free groups. In the exploratory cytokines measured, the post treatment levels of IL4 and IL6 were found to be significantly higher in the recurrence-free group.

4.2.5 Secreted cytokine levels post LPS stimulation

In order to evaluate the general immune responses to other antigens and evaluate if immunologic variations were similar to differences seen in the PPD stimulated samples, LPS was used to stimulate fresh blood samples following the previously mentioned protocol.

Fresh blood samples were stimulated in two batches,

- 4 hour LPS stimulation to measure IL1β
- 24 hour LPS stimulation to measure IFNγ, TNF, IL6, IL8, IL10 and IL13

4.2.5.1 IL1β levels 4 hours post LPS stimulation

The median level of IL1β measured in supernatant 4 hours following LPS stimulation of fresh blood was not significantly different between the recurrence-free group (median 33pg/ml; range 2-134pg/ml) and the recurrence group (49pg/ml; range 4-246np/ml) in the blood samples obtained before induction intravesical BCG treatment [*P* value = 0.2190] (figure 48). Similarly, after treatment the levels were not significantly different [median 47.5pg/ml (range 7-194pg/ml); median 42pg/ml (range 15-199pg/ml) respectively, *P* value = 0.6099] (figure 49).

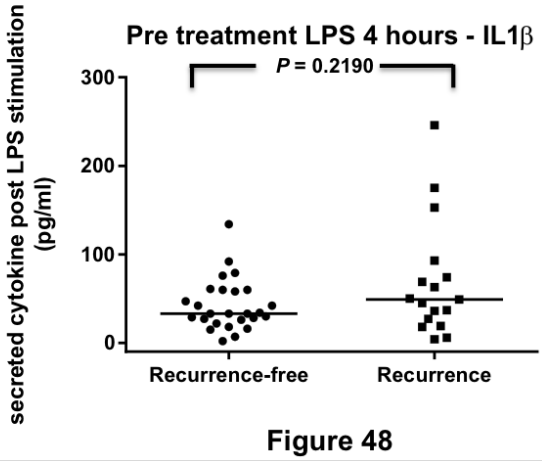

Figure 48

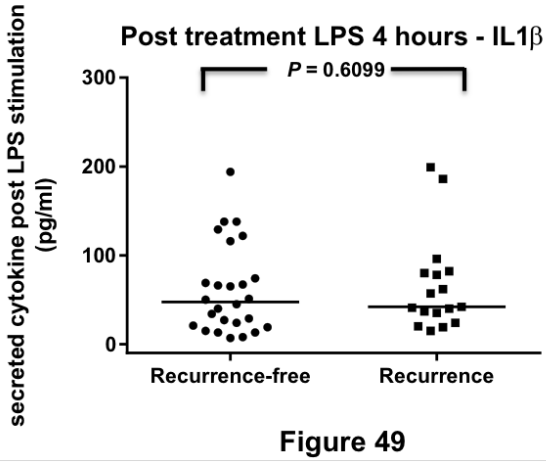

Figure 49

Figure 48 and 49: The levels of IL1β measured in supernatant post 4-hour Lipopolysaccharide (LPS) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (48) and after treatment (49).

4.2.5.2 IFNγ levels 24 hours post LPS stimulation

The level of IFNγ measured in supernatant 24 hours following LPS stimulation of fresh blood was different between the recurrence-free group and the recurrence group in the blood samples obtained before induction intravesical BCG treatment. The median level of IFNγ in the recurrence-free group was 449.5pg/ml (range 3.9 – 10068pg/ml) while in the recurrence group the median level of secreted IFNγ was 269.8pg/ml (range 5.1 - 6831pg/ml). However this difference was not found to be statistically significant [*P* value = 0.9468] (Figure 50).

After induction intravesical BCG treatment, the median level of secreted of IFNγ in the recurrence-free group was 590.5pg/ml (range 16.8-9862pg/ml) while in the recurrence group; the median level of secreted IFNγ was 273.5pg/ml (range 26.6- 5921pg/ml). This was not found to be statistically significant [*P* value = 0.4641] (Figure 51).

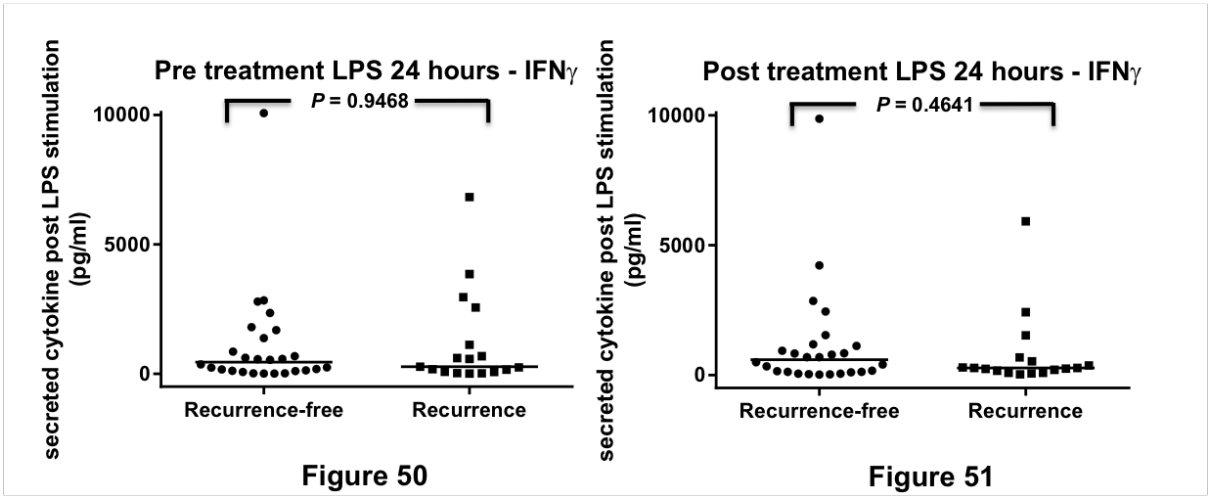

Figure 50 and 51: levels of IFNγ measured in supernatant post 24-hour Lipopolysaccharide (LPS) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (50) and after treatment (51).

4.2.5.3 TNF levels 24 hours post LPS stimulation

The level of TNF measured in supernatants 24 hours following LPS stimulation of fresh blood was no different between the recurrence-free group and the recurrence group in the blood samples obtained before induction intravesical BCG treatment. The median level of TNF in the recurrence-free group was 286.6pg/ml (range 17.9-1079pg/ml) while in the recurrence group the median level of secreted TNF was 338.8pg/ml (range 33.2-2245pg/ml) and this was not found to be statistically significant [*P* value = 0.3803] (Figure 52).

After induction intravesical BCG treatment, the level of secreted of TNF in the recurrence-free group was 335.4pg/ml (range 12 – 935.8pg/ml) while in the recurrence group; the median level of secreted TNF was 327.9pg/ml (range 89.4-649pg/ml). This was not found to be statistically significant [*P* value = 0.9663] (Figure 53).

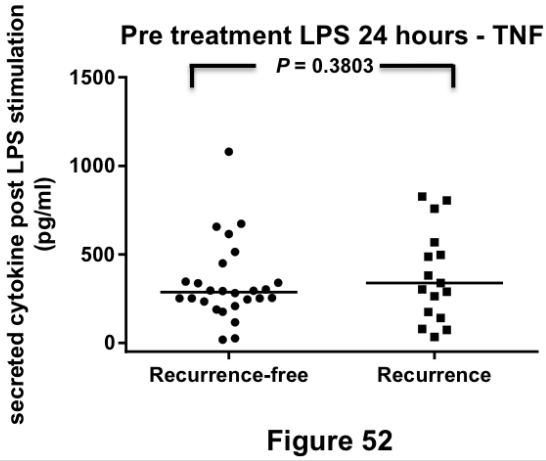

Figure 52

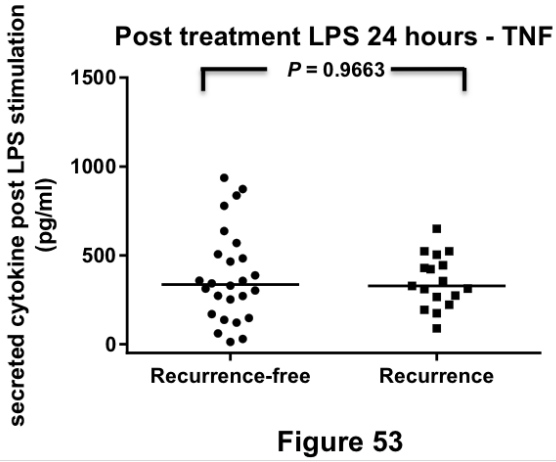

Figure 53

Figure 52 and 53: levels of TNF measured in supernatant post 24-hour Lipopolysaccharide (LPS) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment (52) and after treatment (53).

4.2.5.4 Levels of other cytokines 24 hours post LPS stimulation

No significant difference were observed (in the pre & post percentages) in the levels of other cytokines measured post 24 hours *in vitro* LPS stimulation of blood between the recurrence-free group and the recurrence group. Table 17 summarises the cytokine levels measured post LPS stimulation before treatment and table 18 summarises the cytokine levels measured post LPS stimulation after completing treatment.

Post-LPS cytokine	Recurrence-free Median (range)	Recurrence Median (range)	*P* value
4 hour IL1β	33pg/ml (2-134pg/ml)	49pg/ml (4-246np/ml)	0.2190
24 hour IFNγ	449.5pg/ml (3.9 – 10068pg/ml)	269.8pg/ml (5.1 -6831pg/ml)	0.9468
24 hour TNF	286.6pg/ml (17.9-1079pg/ml)	338.8pg/ml (33.2-2245pg/ml)	0.3803
24 hour IL2	2pg/ml (0.1-4.4pg/ml)	1.9pg/ml (0.1-5.7pg/ml)	0.9836
24 hour IL6	1003pg/ml (59.7 – 2866pg/ml)	1305pg/ml (136.9 – 4334pg/ml)	0.8308
24 hour IL8	1906pg/ml (558.6-5098pg/ml)	2445pg/ml (350.7 – 4313pg/ml)	0.4218
24 hour IL10	10.1pg/ml (0.9-40.2pg/ml)	8.7pg/ml (2.3-40.8pg/ml)	0.9607
24 hour IL13	36.5pg/ml (17.7-98pg/ml)	42.5pg/ml (15.7-84.2pg/ml)	> 0.9999

Table 17: Summary of cytokines measured post lipopolysaccharide (LPS) stimulation in the recurrence-free and recurrence groups before induction intravesical BCG treatment

Post-LPS cytokine	Recurrence-free Median (range)	Recurrence Median (range)	*P* value
4 hour IL1β	47.5pg/ml (7-194pg/ml)	42pg/ml (15-199pg/ml).	0.6099
24 hour IFNγ	590.5pg/ml (16.8-9862pg/ml)	273.5pg/ml (26.6- 5921pg/ml)	0.4641
24 hour TNF	335.4pg/ml (12 – 935.8pg/ml)	327.9pg/ml (89.4-649pg/ml)	0.9663
24 hour IL2	2pg/ml (0.1-8.281pg/ml)	1.7pg/ml (0.1-14.597pg/ml)	0.8894
24 hour IL6	1165pg/ml (44 – 3083pg/ml)	998.9pg/ml (282.6-2766pg/ml)	0.7501
24 hour IL8	2910pg/ml (307.5-12532pg/ml)	1958pg/ml (1027-10564pg/ml)	0.4183
24 hour IL10	12.6pg/ml (0.3-76.61pg/ml)	15pg/ml (2.5-34.8pg/ml)	0.9468
24 hour IL13	41.9pg/ml (23.2-115.9pg/ml)	37.3pg/ml (23.9 – 157.5pg/ml)	0.5675

Table 18: Summary of cytokines measured post lipopolysaccharide (LPS) stimulation in the recurrence-free and recurrence groups after induction intravesical BCG treatment

In summary, no significant difference was observed in the levels of cytokines measured 4 and 24 hours post LPS stimulation.

4.2.6 The change in immune response between the two time points

As discussed earlier, previous literature reported an increase in the immune response to BCG following each instillation [77, 78]. The changes in the immune responses I measured were evaluated between the two time points (before and after BCG induction) within the whole study population and within the two groups separately (recurrence-free versus recurrence)

4.2.6.1 The change in the percentages of CD4 T lymphocytes in response to PPD

4.2.6.1.1 Overall change between Pre and Post treatment levels

4.2.6.1.1.1 IFNγ+ CD4 T lymphocytes

The percentages of IFNγ+ CD4 T lymphocytes were not different between the two time points. The median percentage IFNγ+ CD4 T lymphocytes before treatment was 0.33% (range 0 - 2.11%), while after treatment the median percentage was 0.35% (range 0 – 4.47%). This was not found to be statistically significant [*P* value = 0.1249] (Figure 54).

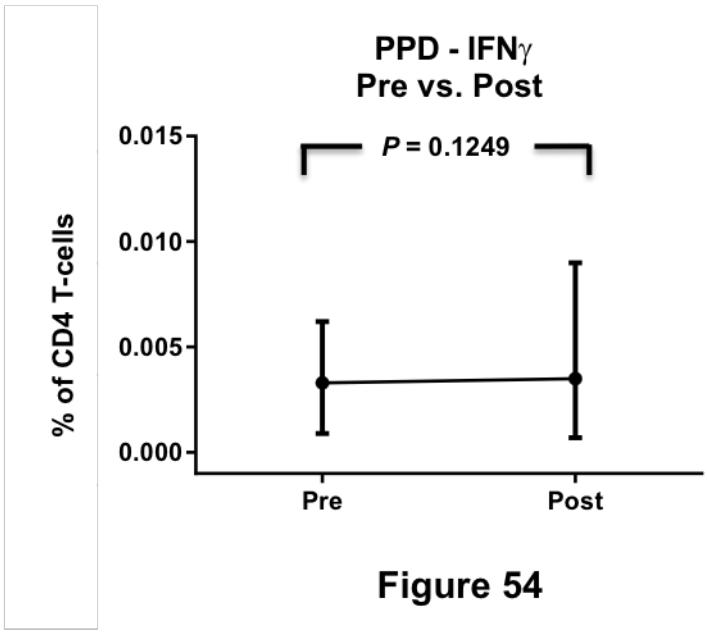

Figure 54

Figure 54: the change in percentages of IFNγ+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation from samples taken before and after induction intravesical BCG treatment.

4.2.6.1.1.2 IL2+ CD4 T lymphocytes

The percentages of IL2+ CD4 T lymphocytes are not different between the two time points. The median percentage IL2+ CD4 T lymphocytes before treatment was 0.44% (range 0 - 2.22%), while after treatment the median percentage was 0.5% (range 0 - 3.70%). This was not found to be statistically significant [*P* value = 0.8367] (Figure 55).

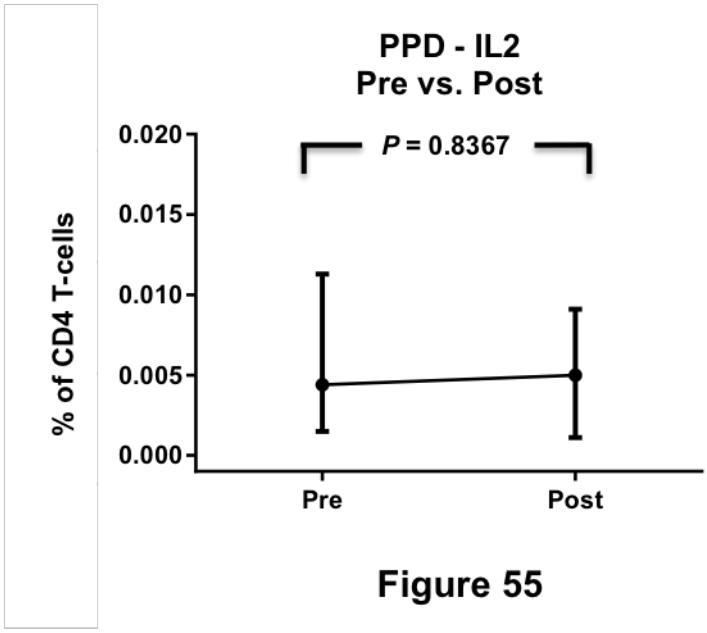

Figure 55

Figure (55): the change in percentages of IL2+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation from samples taken before and after induction intravesical BCG treatment.

4.2.6.1.1.3 TNF+ CD4 T lymphocytes

The percentages of TNF+ CD4 T lymphocytes are not different between the two time points. The median percentage TNF + CD4 T lymphocytes before treatment was 0.63% (range 0 - 4.48%), while after treatment the median percentage was 0.62% (range 0 - 4.77%). This was not found to be statistically significant [*P* value = 0.7831] (Figure 56).

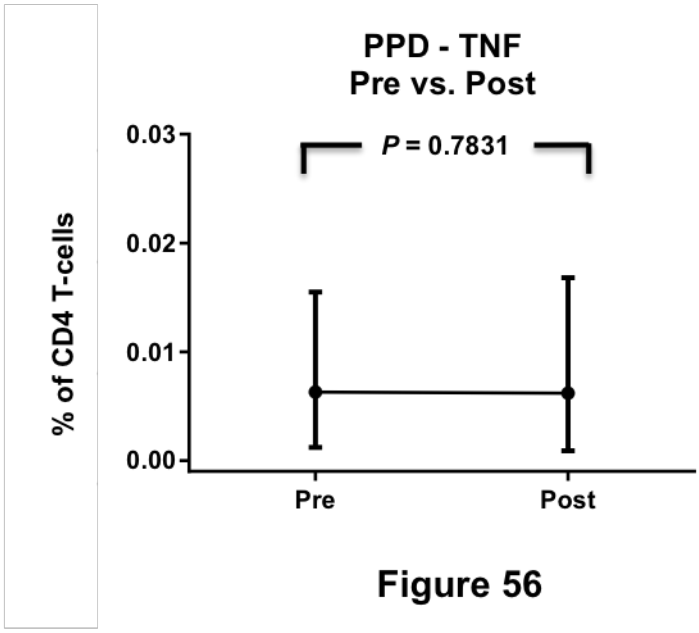

Figure 56

Figure 56: The change in percentages of TNF+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation from samples taken before and after induction intravesical BCG treatment.

4.2.6.1.1.4 CD40L+ CD4 T lymphocytes

The percentages of CD40L+ CD4 T lymphocytes are slightly different between the two time points. The median percentage CD40L+ CD4 T lymphocytes before treatment was 1.13% (range 0 - 7.49%), while after treatment the median percentage was 1.42% (range 0 - 7.45%). This was not found to be statistically significant [*P* value = 0.9408] (Figure 57).

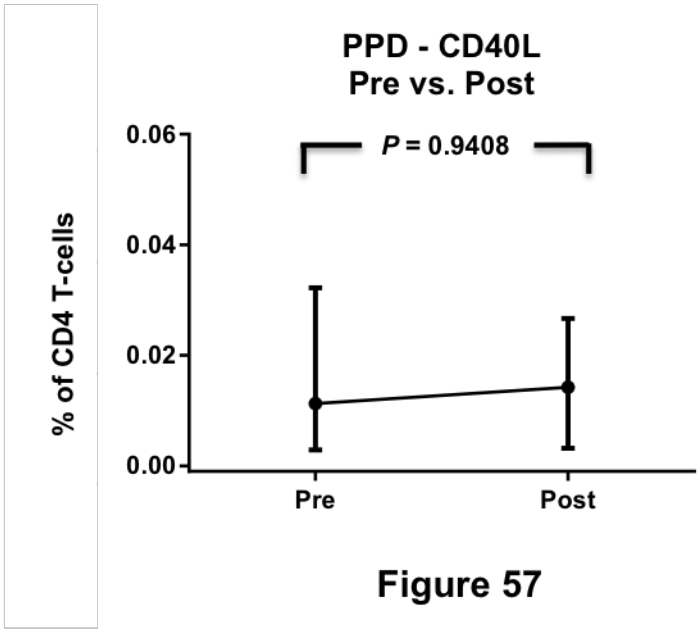

Figure 57

Figure 57: The change in percentages of CD40L+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation from samples taken before and after induction intravesical BCG treatment.

4.2.6.1.1.5 IL17a+ CD4 T lymphocytes

The percentages of IL17a + CD4 T lymphocytes are not different between the two time points. The median percentage IL17a + CD4 T lymphocytes before treatment was 0.08% (range 0 - 2.01%), while after treatment the median percentage was 0.08% (range 0 - 2.71%). This was not found to be statistically significant [*P* value = 0.2274] (Figure 58).

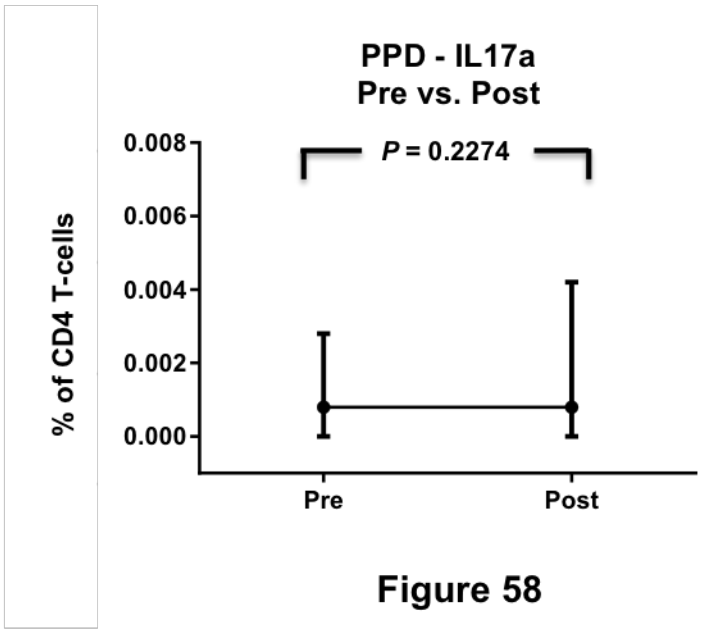

Figure 58

Figure 58: The change in percentages of IL17a+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation from samples taken before and after induction intravesical BCG treatment.

4.2.6.1.1.6 CD40L+IFNγ+IL2+TNF+ CD4 T lymphocytes

The percentages of CD40L+IFNγ+IL2+TNF+ CD4 T lymphocytes are noticeably different between the two time points. The median percentage CD40L+IFNγ+IL2+TNF+ CD4 T lymphocytes before treatment was 0.13% (range 0 - 1.30%), while after treatment, the median percentage increased in the whole study population and was 0.21% (range 0 - 3.38%). This was found to be statistically significant [*P* value = 0.0066] (Figure 59).

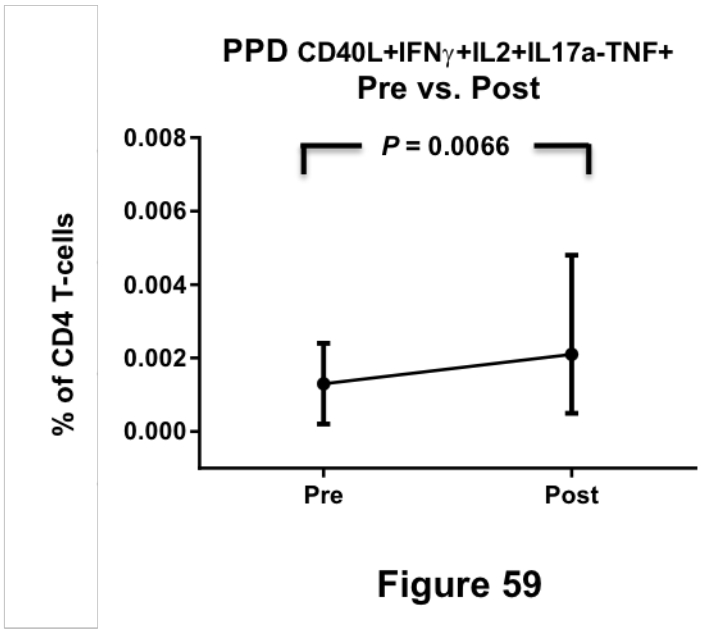

Figure 59

Figure 59: The change of percentages of CD40L+IFNγ+IL2+IL17a-TNF+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation from samples taken before and after induction intravesical BCG treatment.

4.2.6.1.2 The change between the two time points for the groups

No significant difference observed (in the pre & post percentages) between the recurrence-free group and the recurrence group. Percentages before and after treatment did not vary significantly with intravesical BCG therapy. Table 19 below summarises the change between the pre and post treatment percentages.

The difference in % of PPD induced T lymphocytes	Recurrence-free Median	Recurrence Median	*P* value
IFNγ+	**+0.12%**	**0%**	**0.6896**
IL2+	**-0.06%**	**+0.05%**	**0.6895**
TNF+	**-0.08%**	**+0.12%**	**0.5026**
CD40L+	**-0.16%**	**+0.6%**	**0.1797**
IL17a+	**+0.08%**	**-0.05%**	**0.0439**
CD40L+ IFNγ+IL2+IL17a-TNF+	**+0.02%**	**+0.08%**	**0.9853**

Table 19: Summary of difference in pre & post treatment percentages of CD4 T cells producing IFNγ, IL2, TNF, CD40L, IL17a or the functional subset CD40L+ IFNγ+IL2+IL17a-TNF+

4.2.6.2 The change in measured cytokine

4.2.6.2.1 Overall change between Post and Pre treatment levels

Similar to the percentage of CD4 lymphocytes, the levels of measured cytokines post *in vitro* PPD stimulation were also compared before and after treatment. Changes in IFNγ, IL2 and TNF are explained in detail below. Table 24 summarises the changes in the exploratory cytokines and table 25 summarises the changes observed between the recurrence-free and recurrence groups.

4.2.6.2.1.1 IFNγ

The level of IFNγ measured in supernatant post *in vitro* PPD stimulation is not significantly different between the two time points. The median level of secreted IFNγ before treatment was 3909pg/ml (range 38.5 – 91290pg/ml), while after treatment the median level was 4316pg/ml (range 36.3 -73940pg/ml). This was not found to be statistically significant [*P* value = 0.6197] (Figure 60).

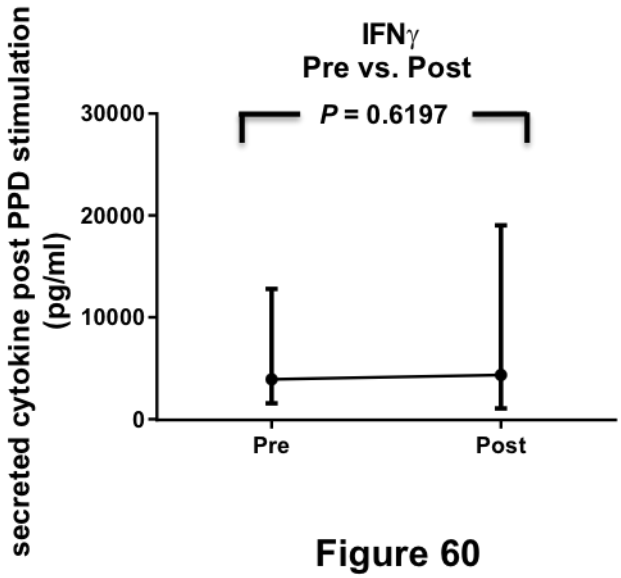

Figure 60: The level of measured IFNγ in supernatant between the pre and post treatment time points post *in vitro* tuberculin purified protein derivative (PPD) stimulation of PBMC.

4.2.6.2.1.2 IL2

The level of IL2 measured in supernatant post *in vitro* PPD stimulation is not significantly different between the two time points. The median level of secreted IL2 before treatment was 337.9pg/ml (range 18.3 – 1622pg/ml), while after treatment the median level was 311.5pg/ml (range 6 – 2732pg/ml). This was not found to be statistically significant [*P* value = 0.6976] (Figure 61).

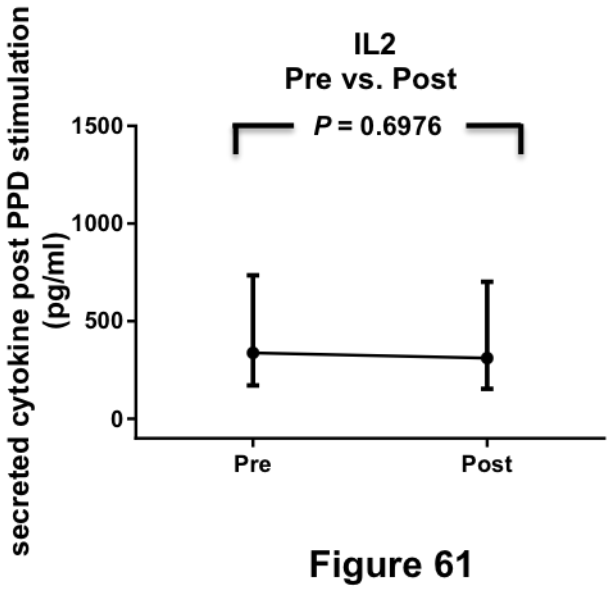

Figure 61

Figure 61: The level of measured IL2 in supernatant between the pre and post treatment time points post *in vitro* tuberculin purified protein derivative (PPD) stimulation of PBMC.

4.2.6.2.1.3 TNF

The level of TNF measured in supernatant post *in vitro* PPD stimulation is different between the two time points. The median level of secreted TNF before treatment was 900.9pg/ml (range 130.7- 3442pg/ml), while after treatment the median level was 638.6pg/ml (range 106.7 – 5234pg/ml). This was not found to be statistically significant [*P* value = 0.1964] (Figure 62).

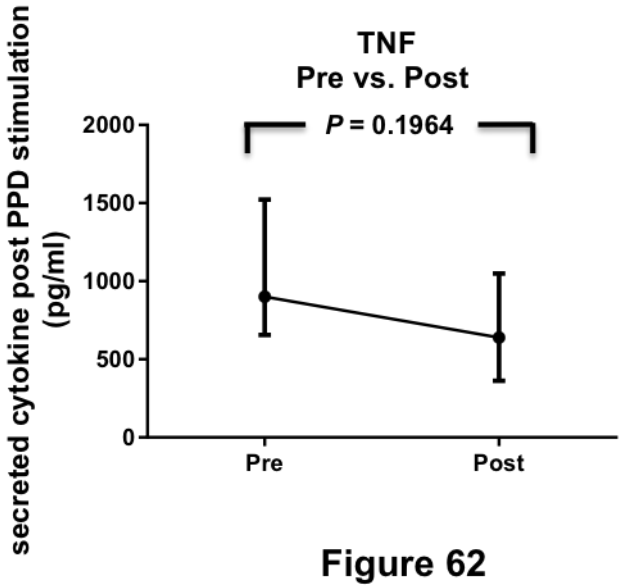

Figure 62

Figure 62: The level of measured TNF in supernatant between the pre and post treatment time points post *in vitro* tuberculin purified protein derivative (PPD) stimulation of PBMC.

4.2.6.2.1.4 The change in other measured cytokines

The levels of other cytokines measured in supernatant post *in vitro* PPD stimulation between the two time points are summarised in table 20 below.

Secreted cytokine	**Pre treatment level Median (range)**	**Post treatment level Median (range)**	***P* value**
IL1β	489.5pg/ml (32.8-3781pg/ml)	354.2pg/ml (58.5 -2202pg/ml)	0.0233
IL4	18.2pg/ml (5.5-41.5pg/ml)	13.7pg/ml (2.3 - 29.8pg/ml)	0.0007
IL6	6401pg/ml (1465–17379pg/ml)	4037pg/ml (1364 – 15449pg/ml)	0.0030
IL8	54373pg/ml (5337-442048pg/ml)	50121pg/ml (7389 - 113825pg/ml)	0.0583
IL10	293.7pg/ml (71.6-1036pg/ml)	173.4pg/ml (9.9 - 748.6pg/ml)	0.0074
IL12p70	31.1pg/ml (8-72.15pg/ml)	27.7pg/ml (3.8 -123.8pg/ml)	0.0810
IL13	527.9pg/ml (62.2–1153pg/ml)	412.1pg/ml (57.4 – 1391pg/ml)	0.6112

Table 20: The change in secreted cytokines post *in vitro* tuberculin purified protein derivative (PPD) stimulation of PBMC between the time points.

4.2.6.2.2 The change between the two time points for the groups

The levels of other cytokines measured in supernatant post *in vitro* PPD stimulation for the two groups between the two time points are summarised in table 21 below.

Secreted cytokine	Recurrence-free level Median difference	Recurrence post-pre level Median difference (range)	*P* value
IFNγ	-453.7pg/ml	+1594pg/ml	0.2024
IL2	-64.6pg/ml	+75.8pg/ml	0.2605
TNF	-221.2pg/ml	-320.8pg/ml	0.9858
IL1β	-255.6pg/ml	+35.1pg/ml	0.2302
IL4	-3.8pg/ml	-4.263pg/ml	0.8830
IL6	-1907pg/ml	-1192pg/ml	0.9273
IL8	-7454pg/ml	-7611pg/ml	0.8064
IL10	-141.2pg/ml	-36.2pg/ml	0.3168
IL12p70	-2.7pg/ml	-8.1pg/ml	0.4471
IL13	-1.3pg/ml	-17.3pg/ml	0.9217

Table 21: The change in the levels of secreted cytokines post *in vitro* tuberculin purified protein derivative (PPD) stimulation of PBMC between the two time points compared between the two groups

In summary, when comparing the overall change in the percentages of PPD-inducible CD4 T lymphocytes, there were no significant changes between the pre and post treatment levels of IFNγ, TNF, IL2, CD40L or IL17a+. There was a significant rise in the percentage of polyfunctional (CD40L+IFNγ+IL2+IL17a-TNF+) CD4 lymphocytes following BCG induction treatment.

When comparing between the recurrence-free and recurrence groups, there was a significant rise in the IL17+ CD4 lymphocytes after treatment in the recurrence-free groups.

There were no significant changes in pre and post treatment secreted cytokines following PPD stimulation.

4.2.7 Association between immunological markers

In order to evaluate the relationship between the measured Th1 inflammatory cytokines, the relevant markers were tested for associations using Spearman's rank correlation coefficient. The relationship between the different co-stimulatory cytokines were examined by first testing the correlation of the percentages of cytokine producing T cells (mainly IFNγ, TNF and IL2, as well as CD40L, IL17a and the polyfunctional T lymphocytes "CD40L+IFNγ+IL2+TNF+").

The relationship between the percentage of cytokine producing T lymphocytes and the measured levels of the cytokines was also examined, as well as the relationship between the cytokine producing cells in response to various antigens by comparing the percentages of IFNγ, IL2 and TNF following *in vitro* PPD and SEB stimulation.

4.2.7.1 Association between the percentages of CD4 T lymphocytes

The pre-treatment percentage of IFNγ+ T lymphocytes was found to correlate with the pre-treatment percentage of IL2+ T lymphocytes post *in vitro* PPD stimulation and this was found to be statistically significant [rs = 0.3569. *P* value = 0.0188] (figure 63).

The pre-treatment percentage of IFNγ+ T lymphocytes correlated strongly with the pre-treatment percentage of TNF+ T lymphocytes post *in vitro* PPD stimulation and this was found to be statistically significant [rs = 0.7577. *P* value < 0.0001] (figure 64).

The pre-treatment percentage of TNF+ T lymphocytes strongly correlated with the pre-treatment percentage of IL2+ T lymphocytes post *in vitro* PPD stimulation and this was found to be statistically significant [rs = 0.6255. *P* value < 0.0001] (figure 65).

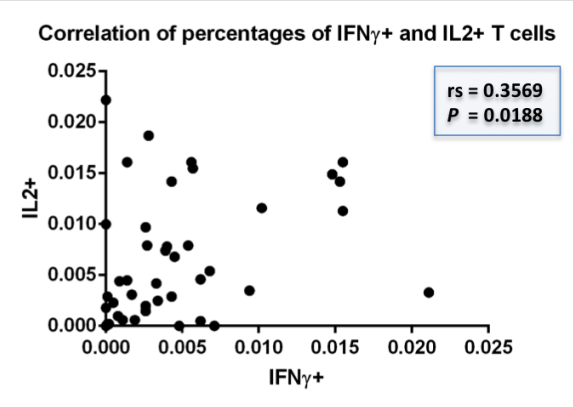

Figure 63

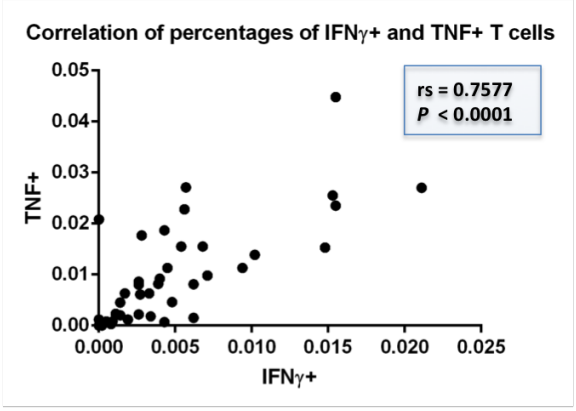

Figure 64

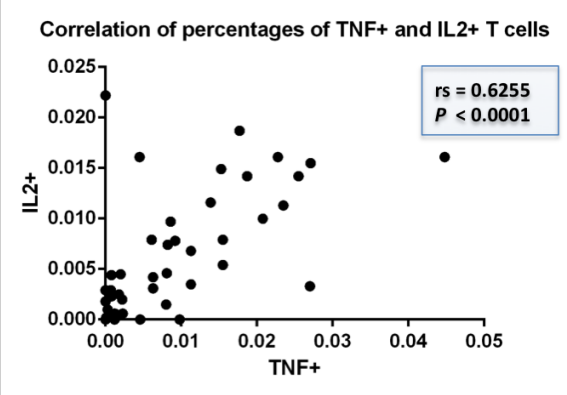

Figure 65

Figures 63, 64 and 65: The correlation between the percentages of IFNγ+ and IL2+ T lymphocytes (figure 63), IFNγ+ and TNF+ (figure 64), and TNF + and IL2+ (figure 65) after *in vitro* tuberculin purified protein derivative (PPD) stimulation from pre-BCG immunotherapy treatment samples.

The pre-treatment percentage of IFNγ+ T lymphocytes correlated strongly with the pre-treatment percentage of CD40L+ T lymphocytes post *in vitro* PPD stimulation and this was found to be statistically significant [rs = 0.5528. *P* value = 0.0001] (figure 66).

The pre-treatment percentage of IFNγ+ T lymphocytes did not correlate with the pre-treatment percentage of IL17a+ T lymphocytes post *in vitro* PPD stimulation and this was not found to be statistically significant [rs = 0.0894. *P* value = 0.5688] (figure 67).

The pre-treatment percentage of IFNγ+ T lymphocytes correlated strongly with the pre-treatment percentage of CD40L+IFNγ+IL2+TNF+ T lymphocytes post *in vitro* PPD stimulation. This was found to be statistically significant [rs = 0.6340. *P* value < 0.0001] (figure 68)

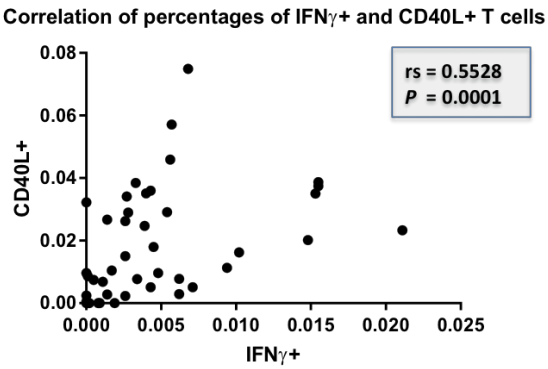

Figure 66

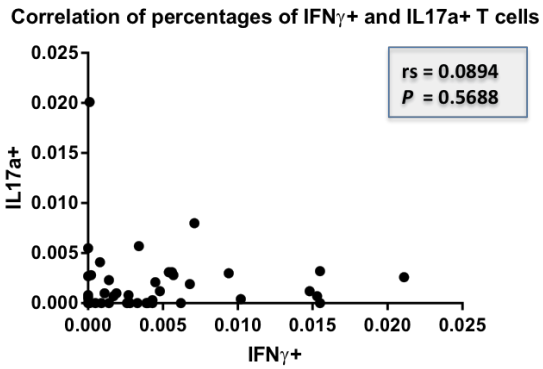

Figure 67

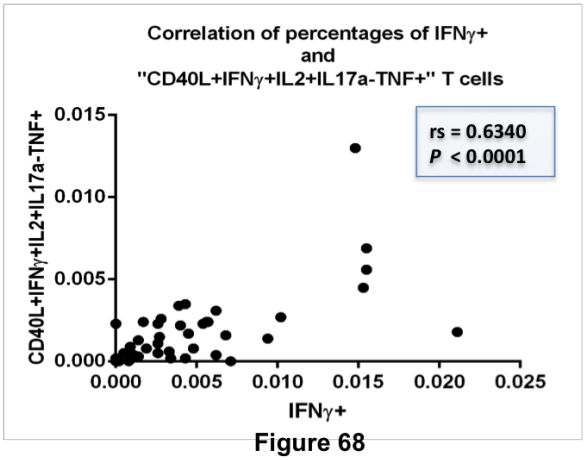

Figure 68

Figures 66, 67 and 68: The correlation between the percentages of IFNγ+ and CD40L+ T lymphocytes (figure 66), IFNγ+ and IL17a+ (figure 67), and IFNγ+ and CD40L+IFNγ+IL2+IL17a-TNF+ (figure 68) after *in vitro* tuberculin purified protein derivative (PPD) stimulation from pre-BCG immunotherapy treatment samples.

4.2.7.2 Association between the percentage of IFNγ+ T lymphocytes and measured secreted IFNγ levels

The pre-treatment percentage of IFNγ+ T lymphocytes was found to correlate with the pre-treatment levels of measured IFNγ in supernatant post *in vitro* PPD stimulation and this was found to be statistically significant [rs = 0.4366. *P* value = 0.0034] (figure 69). However, there was no correlation between the percentage of IFNγ+ T lymphocytes post *in vitro* PPD stimulation and the levels of measured IFNγ measured in urine after treatment [rs = 0.0713. *P* value = 0.6536], (figure 70).

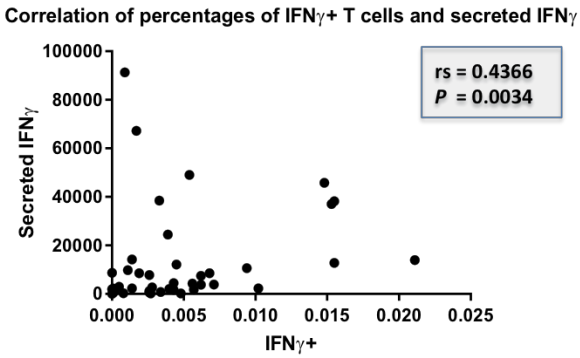

Figure 69

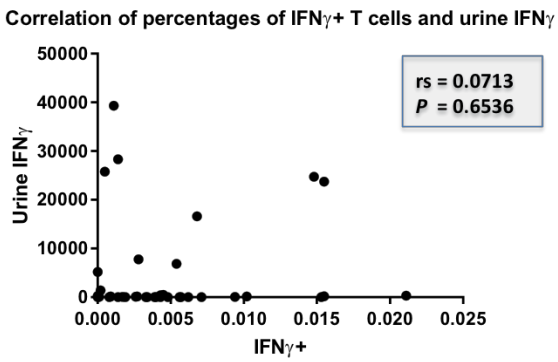

Figure 70

Figure 69 and 70: The correlation between the percentage of IFNγ+ T lymphocytes and the measured IFNγ (figure 69) in supernatant after *in vitro* tuberculin purified protein derivative (PPD) stimulation from pre-BCG immunotherapy treatment samples and correlation between percentage of IFNγ+ T lymphocytes and urine IFNγ after the 6th BCG immunotherapy instillation (figure 70).

4.2.7.3 Association between the percentage of IL2+ T lymphocytes and measured secreted IL2 levels

The pre-treatment percentage of IL2+ T lymphocytes strongly correlates with the pre-treatment levels of measured IL2 in supernatant post *in vitro* PPD stimulation and this was found to be statistically significant [rs = 0.5494. *P* value = 0.0001] (figure 71). However, there was no correlation between the percentage of IL2+ T lymphocytes post *in vitro* PPD stimulation and the levels of measured IL2 measured in urine after treatment [rs = 0.2460. *P* value = 0.1118] (figure 72).

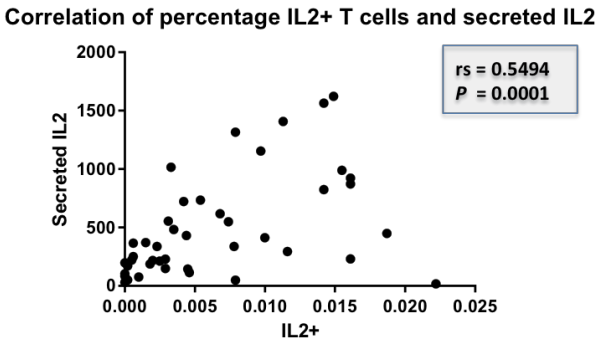

Figure 71

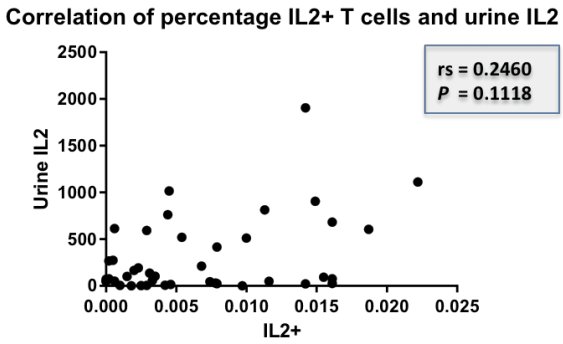

Figure 72

Figures 71 and 72: The correlation between the percentage of IL2+ T lymphocytes and the measured secreted IL2 (figure 71) after *in vitro* tuberculin purified protein derivative (PPD) stimulation from pre-BCG immunotherapy treatment samples and correlation between percentage of IL2+ T lymphocytes and urine IL2 after the 6th BCG immunotherapy instillation (figure 72).

4.2.7.4 The association between the percentage of TNF+ T lymphocytes and measured secreted TNF levels

The pre-treatment percentage of TNF+ T lymphocytes did not correlate with the pre-treatment levels of measured TNF in supernatant post *in vitro* PPD stimulation and this was not found to be statistically significant [rs = 0.1240. *P* value = 0.4281] (Figure 73). The pre-treatment percentage of TNF+ T lymphocytes post *in vitro* PPD stimulation did not correlates with the levels of measured TNF measured in urine after treatment and this was not found to be statistically significant [rs = 0.2477. *P* value = 0.1092] (Figure 74).

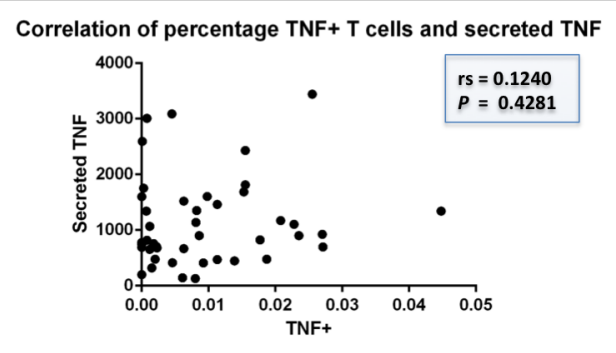

Figure 73

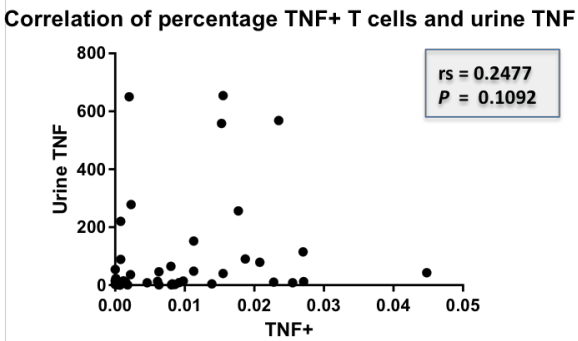

Figure 74

Figure 73 and 74: The correlation between the percentage of TNF+ T lymphocytes and the measured secreted TNF after *in vitro* tuberculin purified protein derivative (PPD) stimulation from pre-BCG immunotherapy treatment samples (figure 73) and correlation between percentage of TNF+ T lymphocytes and urine TNF after the 6th BCG immunotherapy instillation (figure 74).

4.2.7.5 The relationship between the percentages of T lymphocytes post PPD and SEB stimulations

The pre-treatment percentage of IFNγ+ T lymphocytes post *in vitro* PPD stimulation correlated with the percentage of IFNγ+ T lymphocytes post *in vitro* SEB stimulation and this was found to be statistically significant [rs = 0.4544. *P* value = 0.0022] (Figure 75).

Similarly, the pre-treatment percentage of IL2+ T lymphocytes post *in vitro* PPD stimulation correlates with the percentage of IL2+ T lymphocytes post *in vitro* SEB stimulation and this was found to be statistically significant [rs = 0.3966. *P* value = 0.0085] (Figure 76).

As well, the pre-treatment percentage of TNF+ T lymphocytes post *in vitro* PPD stimulation strongly correlates with the percentage of TNF+ T lymphocytes post *in vitro* SEB stimulation and this was found to be statistically significant. [rs = 0.6843. *P* value < 0.0001] (Figure 77).

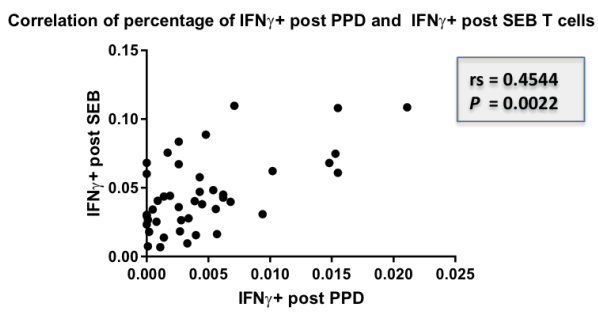

Figure 75

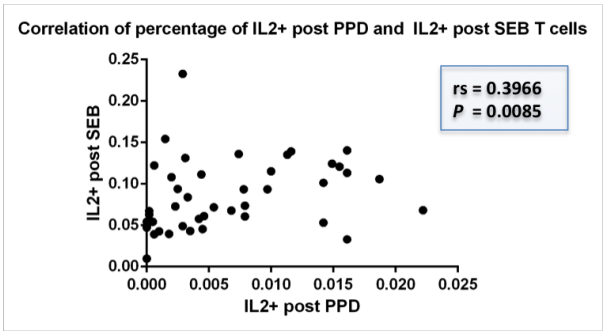

Figure 76

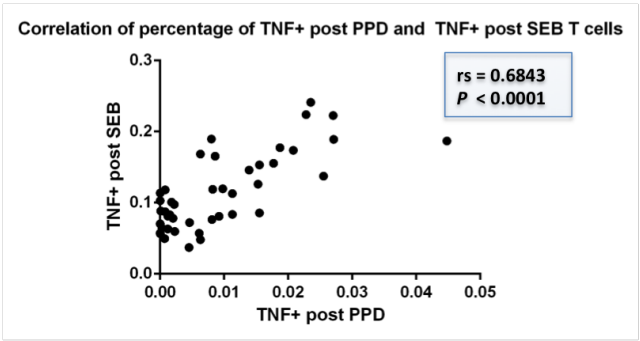

Figure 77

Figures 75, 76 and 77: The correlation between the percentages of IFNγ+ T lymphocytes after *in vitro* tuberculin purified protein derivative (PPD) stimulation and IFNγ+ T lymphocytes after *in vitro* Staphylococcal enterotoxin B (SEB) from pre-BCG immunotherapy treatment samples (figure 75). The correlation between the percentages of IL2+ T lymphocytes after *in vitro* PPD and SEB stimulations (figure 76). The correlation between the percentages of TNF+ T lymphocytes after *in vitro* PPD and SEB stimulations (figure 77)

4.2.7.6 The relationship between age and cytokines

In order to evaluate the relationship between age, which was found to be different between the two groups (recurrence-free and recurrence), and the measured inflammatory markers, the relevant markers and age were tested for associations using Spearman's rank correlation coefficient.

The relationship between age and the percentage IFNγ+ CD4 T lymphocytes, as well as secreted IFNγ are detailed below. The relationship between age and the other markers are summarised in table 22 below.

4.2.7.6.1 Age and IFNγ+ T lymphocytes

The pre-treatment percentage of IFNγ+ T lymphocytes post *in vitro* PPD stimulation correlated negatively with age and was found to be statistically significant [rs = -0.3561. *P* value = 0.0191] (Figure 78).

Similar relationship was observed between secreted IFNγ post *in vitro* PPD stimulation and age, this was found to be statistically significant [rs = -0.3302. *P* value = 0.0306] (Figure 79).

The percentage of IFNγ+ T lymphocytes post *in vitro* SEB stimulation also correlated negatively with age and this was found to be statistically significant [rs = -0.3097. *P* value = 0.0433] (Figure 80).

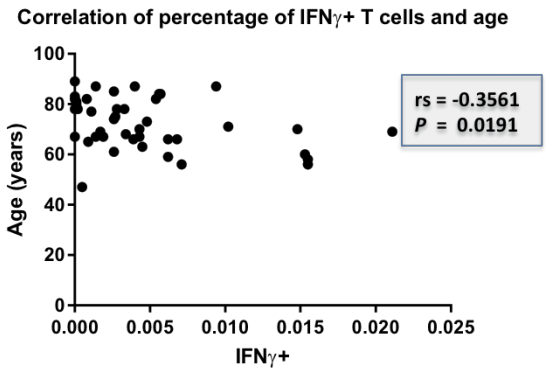

Figure 78

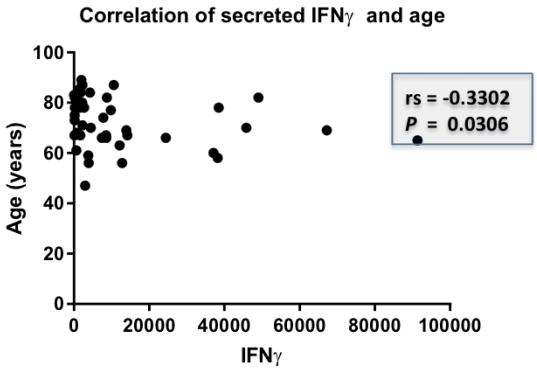

Figure 79

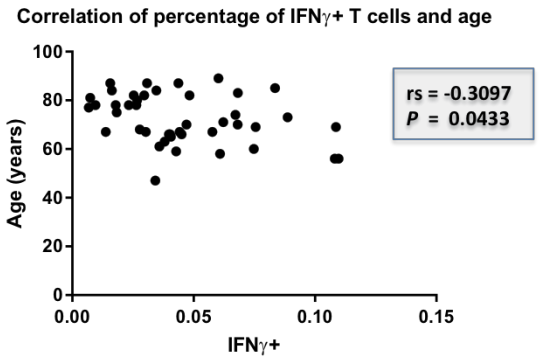

Figure 80

Figures 78, 79 and 80: The correlations between age and percentage of IFNγ+ CD4 T lymphocytes post *in vitro* tuberculin purified protein derivative (PPD) stimulation (figure 78), and levels of secreted IFNγ post *in vitro* PPD stimulation (figure 79) and percentage of IFNγ + CD4 T lymphocytes post *in vitro* Staphylococcal enterotoxin B (SEB) stimulation (figure 80).

4.2.7.6.2 Age and other immune responses measured

Correlation	Spearman rs	*P* value
Age and IL2+ CD4 lymphocytes	0.0645	0.6811
Age and TNF+ CD4 lymphocytes	-0.1136	0.4683
Age and CD40L+ CD4 lymphocytes	-0.0235	0.8810
Age and IL17a+ CD4 lymphocytes	0.0855	0.5885
Age and CD40L+ IFNγ+IL2+IL17a-TNF+ CD4 lymphocytes	-0.2111	0.1743

Table 22: The relationship between age and percentage of PPD-induced CD4 lymphocytes.

In summary, there were significant positive associations between the percentages of IFNγ+, TNF+, IL2+ and CD40L+ and the polyfunctional (CD40L+IFNγ+IL2+IL17a-TNF+) CD4 T lymphocytes following PPD stimulation. There were also significant positive associations observed between PPD and SEB inducible of IFNγ+, TNF+ and IL2+ CD4 lymphocytes. Age was found to be negatively associated with the levels of secreted IFNγ post PPD stimulation and the percentages of IFNγ+ CD4 lymphocytes following PPD and SEB stimulation.

4.2.8 Predictive value of pre-treatment markers

Because the overall population of 43 cases was not enough to allow for complex predictive and multivariate analysis, as this will dilute the sample even further and hence loose power. I performed predictive analysis for of the two most discriminatory markers, %IFNγ+ CD4 T cell and %CD40+IFNγ+IL2+IL17a-TNF+ CD4 T cells, as examples to highlight the impact of these pre-treatment markers on outcome and perhaps to be a base for future project.

4.2.8.1 Sensitivity and specificity of %IFNγ+ CD4 T cell

The computed AUROC model output (using SPSS 23.0) was converted (1= successful outcome and 0=unsuccessful outcome) and Figure (81) shows the ROC curve. Table 23 shows the different sensitivities and specificities for different cut-offs.

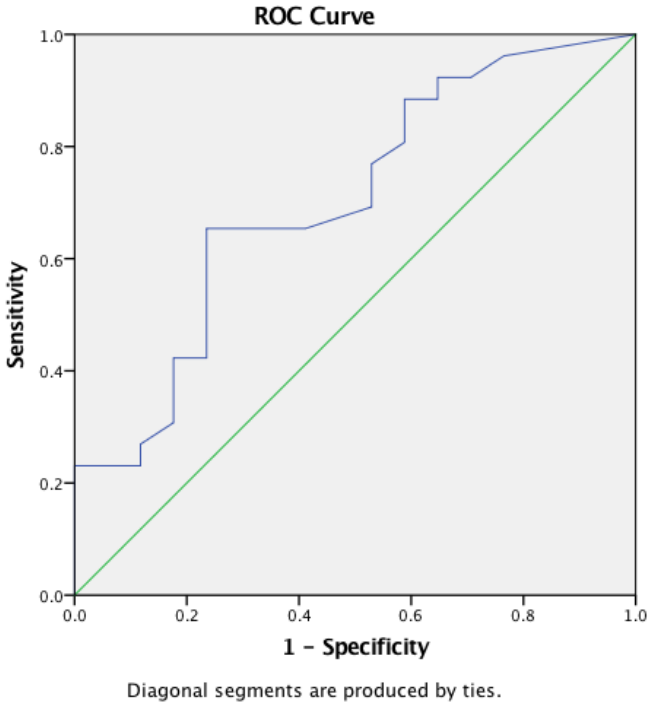

Figure 81: Area under the ROC curve (receiver operator characteristic) for the %IFNγ+ CD4 T cell and positive outcome from intravesical BCG treatment for the studied population. SPSS 23.0

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-1.000000	1.000	1.000
.000050	.962	.765
.000150	.923	.706
.000350	.923	.647
.000650	.885	.647
.000850	.885	.588
.001000	.846	.588
.001250	.808	.588
.001550	.769	.529
.001800	.731	.529
.002250	.692	.529
.002650	.654	.412
.002750	.654	.353
.003050	.654	.294
.003350	.654	.235
.003650	.615	.235
.003950	.577	.235
.004150	.538	.235
.004400	.462	.235
.004650	.423	.235
.005100	.423	.176
.005500	.385	.176
.005650	.346	.176
.005950	.308	.176
.006500	.269	.118
.006950	.231	.118
.008250	.231	.059
.009800	.231	.000
.012500	.192	.000
.015050	.154	.000
.015400	.115	.000
.018300	.038	.000
1.021100	.000	.000

Table 23: The sensitivities and specificities for the different cut-offs of the pre-treatment %IFNγ+ CD4 T cell and positive outcome from intravesical BCG treatment for the studied population based on the area under the ROC curve (receiver operator characteristic) SPSS 23.0.

No ideal cut-off can be obtained that can offer ≥80% sensitivity and ≥80% specificity. For example, a percentage of ≥0.00275 of IFNγ+CD4 T cells can predict a successful outcome with %65.4 sensitivity and %70.6 specificity.

4.2.8.2 Impact of pre-treatment markers on outcome

The impact of the %IFNγ+CD4 T cells on the outcome of treatment is perhaps better presented using a logistic regression model as described in the table 24 below. This shows that for every 1 x 10^{-4} increase in the %IFNγ+CD4 T cells, there is a %18.2 reduction in the odds of recurrence [β= 0.818. 95%CI 0.669-1.000. *P* value = 0.05].

	B	S.E	Wald	Df	Sig.	Exp(B)	95% CI for Exp (B)	
							Lower	Upper
%FNγ+ CD4 T cells	-.201	.103	3.846	1	.050	.818	.669	1.000
Constant	.363	.468	.601	1	.438	1.438		

Table 24: The relationship of %IFNγ CD4 T cells and recurrence after intravesical BCG treatment for high-risk non-muscle invasive bladder cancer based on logistic regression model SPSS 23.0.

Similarly for every 1x10^{-4} increase in the %CD40+IFNγ+IL2+IL17a-TNF+" CD4 T cells, there is %59.7 reduction in the odds of recurrence [β= 0.403. 95%CI 0.201 - 0.810. *P* value = 0.011] (see table 25).

	B	S.E	Wald	Df	Sig.	Exp(B)	95% CI for Exp

							(B)	
							Lower	Upper
%CD40+IFNγ+IL2+IL17a-TNF+ CD4 T cells	-.908	.355	6.523	1	.011	.403	.201	.810
Constant	.770	.514	2.240	1	.134	2.159		

Table 25: The relationship of %CD40+IFNγ+IL2+IL17a-TNF+ CD4 T cells CD4 T cells and recurrence after intravesical BCG treatment for high-risk non-muscle invasive bladder cancer based on logistic regression model SPSS 23.0.

5. Discussion

Intravesical BCG immunotherapy has now become the gold standard treatment for HGNMITCC following initial resection as it has been demonstrated to reduce the risk of recurrence [14-16]. However, about 30% of patients fail BCG treatment despite completing their treatment course putting them at significant risk of disease progression [17] and dying from their cancer [19]. Many clinical and pathological markers have been reported to increase the risk of BCG treatment failure [83-96, 123, 124]. But until now, none have been accurate enough to translate into a reliable prognostic test in clinical practice.

Several reports [28, 72-79, 105] demonstrated an increase in inflammatory markers in blood and urine samples from patients having intravesical BCG immunotherapy. Some reports found differences in the inflammatory marker levels between responders and non-responders after treatment, however, in all cases, the differences were reported either during or after completing intravesical BCG immunotherapy. Thus none of these studies was able to identify pre-treatment biomarkers that could usefully differentiate responders from non-responders prior to treatment or predict the outcome to this treatment.

This study is the first to prospectively measure pre-treatment and post-treatment immune responses and identify significant immunological pre-treatment differences that correlated with bladder tumour recurrence following intravesical BCG immunotherapy for HGNMITCC and this has significant potential to become a predictive tool to the outcome of treatment.

The percentages of PPD-inducible CD4 T lymphocytes producing the relevant Th1 cytokines; IFNγ, IL2 and TNF, were significantly lower in patients who developed tumour

recurrences following BCG immunotherapy compared to those who remained recurrence-free. The same was true for the polyfunctional T-cell subset producing/ up-regulating CD40L, IFNγ, IL2 and TNF at the same time.

These measured differences were in agreement with the secreted levels of IFNγ and IL2 after *in-vitro* PPD stimulation, but, interestingly, levels of secreted TNF did not vary between the two groups. The median levels of IFNγ, TNF and IL2 in the urine collected 4 hours after the 6^{th} instillation of intravesical BCG were higher in the recurrence-free group but this was not found to be statistically significant.

Very similar results were seen with SEB stimulation. Although SEB was used primarily as a positive stimulation control, it lends itself very well to the evaluation of non-antigen specific polyclonal responses. The percentages of IL2+ CD4 T lymphocytes and TNF+ CD4 T lymphocytes and the polyfunctional (CD40L+IFNγ+IL2+TNF+) T lymphocytes after SEB stimulation were significantly lower in patients who had recurrences, indicating a difference between the groups in regards to general immune responsiveness, i.e. not PPD-specific. However, why this did not extend to IFNγ+ CD4 T lymphocytes (as with PPD) remained unclear.

These novel findings seem to suggest that innate differences in the ability of T lymphocytes to produce Th1 cytokines prior to BCG therapy have significant effect on the outcome. Measuring Th1 cytokines before treatment has great potential to identify patients who will respond positively to treatment.

It is of note that a recurrence early after starting BGC treatment does not necessarily mean treatment failure, as some patients with early recurrences may still respond later after

further BCG treatment. From the studied population, four patients that had early recurrences responded positively to additional BCG instillations and had no further recurrence during the follow-up period. It is important to point out that patients in whom BCG treatment ultimately fails carry a significant risk of disease progression and death from their cancer. Since the definition of "BCG treatment failure" varies in the literature, my data analysis used recurrence during the follow-up period as the main end-point to ensure objectivity.

Clinical Factors

In a study with such a complex patient population, immunological factors cannot be considered in isolation. Other factors such as age and additional other clinical parameters also had to be evaluated with respect to therapy outcome. Indeed, previous published reports have demonstrated that older age comes with an increased risk of failing intravesical BCG treatment [83, 84], as well as tumour multiplicity [85], presence of CIS [89, 90], higher T stage [87, 90] and the absence of previous BCG immunisation [97].

Age

Joudi *et al* [84] reported reduced cancer-free survival in older age and especially older than 80 years. Also, Herr *et al* [83] reported a 27% reduction in cancer-free survival for patients older than 70 years of age (this is beyond 5 years of follow up). Both reports concluded that older patients carry a higher risk of treatment failure.

In my study, patients with recurrences were also older (median 78 years, range 56-89 years) than the recurrence-free patients (median 69 years, range 47-87 years), however the difference was not statistically significant [*P* value = 0.0721]. I also found that age above

70 years was associated with an increased risk of recurrence [Odds ratio 6.139; 95% CI 1.542 – 24.45. *P* value = 0.0122]. It is entirely conceivable that older age reduces the immune response to intravesical BCG treatment. When the relationship between age and *in-vitro* inducible IFNγ was examined, age showed a significant negative correlation with both the percentage of IFNγ+ T lymphocytes and secreted IFNγ following PPD stimulation. All these observations could be explained by immunosenescence, which is the age-dependent progressive decline in immunological responses, both innate and acquired [125]. Since clinical outcome to BCG treatment correlates with immune response, as shown in this thesis and in previous literature, immunosenescence could play an important role in influencing these responses and the outcome to treatment.

T stage

Several published reports found an increased risk of recurrence and progression with T1 stage compared to Ta stage [87, 90, 124, 126]. Sylvester *et al* [124] published a meta-analysis of 7 *European Organisation for Research and Treatment of Cancer* (EORTC) randomised controlled trials involving 2596 patients in order to calculate the short term and long term risks of recurrence and progression after transurethral resection of HGNMITCC. T stage was found to increase the risk of recurrence and more significantly it increases the risk of progression (appendix 5).

Aiming to develop a risk stratification model to provide estimates on recurrence and progression in patients who had BCG treatments, Fernandez-Gomez *et al* [85] reported on 4 Spanish Urological Club for Oncological Treatment (CUETO) in a meta-analysis of randomised controlled trials involving 1,062 patients who were treated with intravesical BCG. T stage was not found to increase recurrence but it was found to significantly increase the risk of progression (appendix 5).

In this study, the T stage was not found to differ significantly between the two groups. Among recurrence-free patients, 50% had T1 disease on initial resection compared with 64.7% in the recurrence group [*P* value = 0.3694]. The T stage was not found to be associated with increased risk of recurrence in this study group [*P* value = 0.3694]. This may be related to the relatively small number of patients in my study compared to the cited studies. As well, because of the shorter follow-up period, I was not able to correlate my results with disease progression.

Carcinoma in Situ **(CIS)**

Many reports have associated the presence of CIS with an increased risk of recurrence and progression [85-87, 91]. Losa *et al* [127] and Hurle *et al* [128] both reported increased risk of recurrence with CIS in univariate analysis [*P* value = 0.001]. Ovesen *et al* [129] found, in multivariate analysis, increased risk of progression with CIS [*P* value = 0.01]. Both, the EORTC [124] and CUETO [85] studies also associated the CIS histology as part of their risk stratification scores for recurrence and progression.

However, Pansadoro *et al* [130] in their multivariate analysis and 6 year follow-up found no association between CIS and BCG failure. Davis *et al* [131] also found no link in multivariate analysis and a longer follow-up of 10 years. Similarly in this analysis, I found that the presence or absence of CIS was not significantly different between the groups [*P* value = 0.3694], and in fact, the recurrence-free group had more CIS in their initial histology than the recurrence group, which was not statistically significant. CIS was not found to be associated with an increased risk of recurrence [*P* value = 0.7357].

Multifocal disease

The link between multifocal disease and recurrence is controversial. Smaller studies found no correlation between multifocal disease and recurrence [132, 133], while larger studies reported increased risk of recurrence and progression with multifocal disease [85, 134] suggesting the smaller cohorts may have been under-powered. The number of tumours was also reported to be associated with increased risk of recurrence [124].

In this study, the presence or absence of multifocal disease was clearly documented by the operating surgeon in the clinical notes, but the exact number of tumours was not always documented and so it was not possible to perform any analysis based on the exact number of tumours. The recurrence group had more multifocal disease at presentation (50%) compared to the recurrence-free group (23.8%). However this was not statistically significant [*P* value = 0.2913]. Multifocal disease at presentation was not associated with increased risk of recurrence [*P* value = 0.2913]. A larger sample size would be required to fully assess the link between multifocal disease and recurrence in this population.

BCG vaccination

Older literature reported an improved outcome when the PPD skin test converts from negative to positive following intravesical BCG therapy and also in patients who had previously been vaccinated with BCG [80, 135], i.e. had pre-existing immunity to BCG. However, randomised trials did not confirm this [136, 137]. In addition, Luftenegger *et al* [136] found no benefit from simultaneous intradermal and intravesical instillation, but reported a better response if patients develop a fever after treatment. Fevers were higher in patients who had a PPD positive skin test [136]. This suggests that previous exposure to BCG (or possibly other mycobacteria as the PPD skin response is not specific for BCG) leads to immunological memory that boosts the immune response to therapeutic BCG. In

contrast to this, Shinka *et al* [138] found no correlation between PPD skin test conversion and recurrence following intravesical BCG therapy. Moreover, Okamura *et al* [139] found no association between previous TB infection and response to intravesical BCG therapy. In my study, both the recurrence and the recurrence-free groups had similar number of BCG vaccinated patients [*P* value =1.000] and previous BCG vaccination was not found to be associated with reduced risk of recurrence [*P* value = 1.000]. However, many of the patients in my study would not have received routine vaccination due to their age in relation to the time BCG was introduced to the UK programme of immunisation. Furthermore, a larger sample size would be required to investigate this specifically.

Immunological Markers

A number of studies have evaluated a variety of immunological markers in the context of the response to intravesical BCG treatment for bladder cancer. Some studies simply confirmed an increase in immune responsiveness after BCG treatment. For example Zlotta *et al* [140] observed a significant increase in *in-vitro* PPD-induced PBMC proliferation after BCG treatment. Taniguchi *et al* [78] collected blood and urine samples from 21 bladder cancer patients before and on the day after each intravesical BCG instillation and observed an increase in both serum and urine cytokines. The authors reported an increase in the serum levels of IFNγ and IL2, as well as urine levels of IL1β, IL2, IL6, TNF and IFNγ. They concluded that the immune response to BCG involves systemic and local immunological factors. This prospective study provided insight into the body's response to BCG treatment and highlighted a bigger role for the immune system.

In my study, when looking at the overall studied-population, there was no significant increase in the immune response measured in blood after treatment (i.e. the percentage of PPD-induced CD4 lymphocytes or secreted cytokines after *in vitro* stimulation). Although

the post-treatment percentages of IFNγ+, IL2+ and TNF+ CD4 lymphocytes were significantly higher in patients who responded to treatment, these levels were similar to the pre-treatment levels. This implies that adequate levels of these functional lymphocytes are needed before treatment in order for BCG immunotherapy to generate its desired effect.

Other studies that compared immunological responses with respect to outcome also suggest that a higher degree of immune responsiveness correlates with successful treatment. For example, Schmidt *et al* [77] compared the BCG stimulation index (SI) of peripheral blood lymphocytes before and after BCG treatment and reported a significant difference between responders and non-responders. After 17 months follow-up, the authors reported that the 6 patients who had no recurrence had SI > 5 compared to the 4 patients who had recurrences and had SI < 5. The authors proposed using SI to monitor response to BCG treatment and predict recurrence after treatment. However, the SI was not different before starting treatment and would not help identifying patients or predicting outcome.

Antibody responses to BCG were also considered in the past. For example, Ardelt *et al* [79] evaluated peripheral blood IgA and IgM antibody responses to BCG's heat shock protein-65 (hsp65) in 16 patients who received intravesical BCG immunotherapy for bladder cancer. The authors reported no difference between patients before treatment when all patients had low titres. After the BCG induction course, titres rose significantly in the 12 patients who had no recurrences whereas they did not change in the 5 patients who had recurrences, but the authors did not specify when the recurrences happened. As a result, the authors proposed using anti-hsp65 IgA and IgM levels for monitoring treatment success, however, they were unable to correlate pre-treatment levels with clinical outcome.

IFNγ

IFNγ is known to play an important and critical role in promoting protective immune response against pathogens [141]. Humans with deficiencies in IFNγ production or signalling are highly susceptible to intracellular infections, which clearly emphasises the critical importance of IFNγ in preventing infections [142, 143].

In animal studies, mice with transplanted sarcomas rejected the tumours when given sublethal doses of LPS, while others could not fight the transplanted sarcoma when given IFNγ neutralising antibodies [144], highlighting the important role of IFNγ in protecting against tumours. It has also been reported that IFNγ prevents tumour proliferation [145] and its signalling pathway has been implicated in promoting tumour apoptosis [146, 147] and also in the inhibition of tumour angiogenesis [148, 149].

Due to the importance of IFNγ in generating immunity against pathogens and tumours, I examined IFNγ responses to evaluate the differences between responders and non-responders to BCG treatment. Unlike other studies, the percentages of IFNγ+ CD4 T lymphocytes after *in vitro* PPD stimulation of PBMC were found to vary significantly between individuals and were noticeably higher in patients who had no recurrences compared to patients who did. More importantly, the differences found in my study existed prior to starting treatment suggesting a possible role for innate or genetically determined immune responses in determining response to BCG therapy.

Elsasser *et al* [150] examined cytokine profiles in 18 patients receiving intravesical BCG for bladder cancer and observed treatment-induced changes in the PPD-inducible lymphocytes. The authors used a similar protocol to my protocol with PPD and SEB stimulation and extracellular and intracellular staining but used whole blood instead of PBMCs. The authors also reported similar baseline level for healthy controls and patients before treatment, but then observed an increase in PPD-inducible IFNγ+ CD4 T

lymphocytes from a baseline of 0.05% before treatment to 0.42% after treatment in the patients receiving BCG therapy. However, the authors did not find a correlation between pre-treatment levels and clinical outcome.

This stands in contrast to our own findings of a significant difference with respect to the same parameters between the recurrence-free patients and patients with recurrences and especially before starting BCG treatment [*P* value = 0.0253]. This may be explained by the larger sample size in my study and also the use of PBMC in my protocol, while Elsasser *et al* who studied only 18 bladder cancer patients and used whole blood stimulation. The pre-treatment %IFNγ+ CD4 T lymphocytes correlates significantly with outcome and for every 1 x 10^{-4} increase in the %IFNγ+CD4 T cells, there is a %18.2 reduction in the odds of recurrence [*P* value = 0.05].

The pre-treatment secreted IFNγ levels following overnight PPD stimulation of PBMC were noticeably higher in the recurrence-free group who secreted almost twice as much as the recurrence group. This is in agreement with the differences between the groups in regards to IFNγ+ CD4 T lymphocytes after PPD stimulation. This may be explained by the fact that following PPD stimulation *in vitro*, CD4 T cells are the main source of IFNγ production, while other subsets of immune cells that might, in principle, contribute to IFNγ production, such as NK cells, are unlikely to be directly activated by PPD and they were not formally analysed in this study.

Although the differences in secreted IFNγ persisted after the induction course of BCG treatment, the difference between the groups became smaller and was no longer statistically significant.

The presence of immune responses to PPD before BCG therapy suggests that, regardless of specific history of previous BCG vaccination or TB, these patients have had exposure to

mycobacterial antigens with memory responses that may boost the response to BCG therapy.

IL2

The effect of IL2 on cancer is best illustrated with malignant melanoma where T cells and NK cells infiltration is associated with tumour regression and this is enhanced by IL2 treatment [151]. Also, IL2 treatment has been found to shrink metastatic renal cell cancer in 15-20% of cases [152].

Interestingly, Taniguchi *et al* [78] reported an increase in IL2 levels in the serum and in the urine after starting intravesical BCG treatment. In my study, I observed marked differences in the percentages of IL2+ CD4 T lymphocytes after *in vitro* PPD stimulation of PBMC. The median percentage in the recurrence group was similar to that reported by Elsasser *et al* [150] across all patients (they did not evaluate differences between responders and non-responders). However, I found that the percentage of PPD-induced IL2+ CD4 lymphocytes was almost 4 times higher in the recurrence-free group, suggesting that higher pre-treatment percentages of IL2 producing lymphocytes contribute to generating a positive outcome.

I observed similar differences in percentages of IL2+ CD4 T lymphocytes after *in vitro* SEB stimulation of PBMC. The recurrence group had significantly lower percentages in the pre-treatment samples compared to the recurrence-free group; 5.43% and 10.48% respectively [*P* value = 0.0014]. The difference persisted after treatment but the gap between groups became smaller and was no longer statistically significant.

The level of secreted IL2 measured after *in vitro* PPD stimulation was also noticeably different between the groups before treatment and levels were almost 2.5 times higher in

the recurrence-free group compared to the recurrence group [*P* value = 0.0013]. There was no significant difference observed between the groups in samples taken after BCG induction treatment [*P* value = 0.3509]. The higher levels of secreted IL2 could reflect the higher numbers of IL2-producing cells.

The differences I observed in the percentages of IL2+ CD4 T cells and secreted IL2 after *in vitro* PPD stimulation suggest that adequate number of cells capable of secreting IL2, and in sufficient amount, are needed before starting treatment in order for BCG therapy to generate a positive outcome. This may be related to attracting T cells and NK to infiltrate the bladder tissue, however, this remains to be shown. A number of factors are likely to determine the levels of IL2 secreting cells in any individual including host genetic factors.

TNF

Although named for its anti-tumour properties, TNF is a multifunctional cytokine that plays a key role in inflammation as well as apoptosis and cell survival. TNF treatment has been found to enhance anti-tumour response in cancers like metastatic melanomas and sarcomas [153, 154].

Agarwal *et al* [155] evaluated the cytokine profile after *in vitro* phytohaemagglutinin (PHA) stimulation of PBMC from bladder cancer patients in comparison with healthy controls and found that bladder cancer cases had fewer TNF+ CD4 T lymphocytes than healthy controls, (12.8 +/- 4.49 and 17.6 +/- 5.96 respectively, [*P* value = 0.001]). Their findings suggest a difference in the general immune responsiveness in cancer patients that might also be a factor in cancer development. The authors did not stratify those bladder cancer patients by clinical parameters to identify possible differences between them. As a

result, no statement on the relationship between percentages of TNF+ CD4 lymphocytes and clinical outcome could be made.

In my study, I found significant differences in bladder cancer patients with regard to TNF+ CD4 T cells before intravesical BCG. Their percentage was significantly lower in patients who had recurrences compared to recurrence-free patients [*P* value = 0.0085] and the difference appeared to persist after treatment albeit not at a statistically significant level. However I saw no significant difference in the levels of PPD-inducible secreted TNF in samples taken before treatment. The levels of secreted TNF did not change significantly with treatment.

I also found that the percentage of SEB-inducible TNF+ CD4 T lymphocytes before treatment was significantly lower in the recurrence group compared with the recurrence-free group [*P* value = 0.0323] and this significant difference persisted after treatment [*P* value = 0.0344].

The disparity between intra-cellular TNF (percentage of positive CD4 T lymphocytes) and secreted TNF following *in-vitro* PPD stimulation may be explained by TNF secretion from other cellular sources (non T cells) in a non-antigen specific fashion (the main source of secreted TNF might be monocytes).

This is the first study to report a significant correlation between pre-treatment PPD-inducible TNF+ CD4 lymphocytes and absence of bladder cancer recurrence after BCG treatment. Like IFNγ and IL2, TNF is an effector cytokine and possibly involved in maintaining a strong local immune response after BCG therapy.

CD40L

T helper cells stimulate macrophages by secreting IFN-γ. However, the CD40L–CD40 pathway is an alternative, contact-dependent pathway, by which T helper cells can stimulate macrophages [29] which can also help in generating anti-cancer immune response. CD40L is not only present on the surface of activated CD4 T cells and other immune cells, but also present on tumour cells. The role of the CD40L-CD40 pathway in anti-cancer immunity is controversial; it can inhibit cancer growth via enhancing apoptosis of cancer cells via direct contact [156] but arguably, can also induce angiogenesis by releasing growth factors that can aid tumour progression [157].

In bladder cancer, Sandin *et al* [158] found the use of local CD40 agonist antibody in marine models mediated anti-tumour effects and suggested it could possibly become an alternative treatment for disseminated bladder cancer.

In my project, the percentages of CD40L+ CD4 T cells were measured after PPD stimulation to evaluate differences between the groups. There no significant difference observed before or after treatment between the two groups. Further research is needed to clarify the role of CD40L-CD40 pathway in bladder cancer and response to BCG immunotherapy treatment.

IL17a

It was reported that IL17a production is one of the early cellular responses after exposure to TB, which helps in recruiting T cells and neutrophils into the infected organ and aids in the granuloma formation and control of bacterial growth [159, 160]**.**

Takeuchi *et al* [161] evaluated the role of IL17a in BCG treatment for bladder cancer and found that IL17a production preceded neutrophil infiltration of bladder cancer in mice. In

IL17 deficient mice, neutrophil counts were reduced and BCG treatment lost its' antitumor effect.

In my project, no difference was found in the pre-treatment median percentages of the IL17a+ CD4 lymphocytes for the recurrence-free and recurrence groups and levels were generally much lower than those of other subsets of interest. However, after treatment, the median percentage of IL17a+ CD4 T lymphocytes doubled in the recurrence-free group to 0.14% while in the recurrence group the percentage remained low at 0.04%, though this difference was not found to be statistically significant. [*P* value = 0.0928].

The role of IL17a in the bladder is not fully understood, but an increase in the percentage of IL17a+ CD4 T lymphocytes following BCG treatment in the recurrence-free group may indicate the need for adequate levels of IL17a for BCG to induce its antitumor effect. Urine IL17a levels were unfortunately not measured, as this was an exploratory test when the protocol was developed. Testing IL17 in the urine after treatment or post-treatment bladder tissue samples might help understand the role of this cytokine in BCG immunotherapy.

Polyfunctional T lymphocytes

In some infectious models, polyfunctional T lymphocytes (producing IFNγ, IL2 and TNF at the same time) have a protective role [30]. Abel *et al* [31] found polyfunctional T lymphocytes to be important for protection against intracellular pathogens and hence polyfunctional T lymphocytes were used to evaluate vaccines in humans. Similarly, Sester *et al* [162] reported that cells secreting both IFN and IL2, which predominated in treated TB, latent TB and BCG vaccinated cases, helped discriminate active from latent TB.

Certain functional T lymphocyte subsets were reported to better distinguish patients with TB, for example Streitz *et al* [163], found that CD40L+IFNγ-IL2-TNF+ was the best in distinguishing cases with active TB from latent TB in highly exposed hospital staff.

In my study, the overall median percentage of the polyfunctional subset up regulating all the measured Th1 activation markers (CD40L, IFNγ, IL2 and TNF) increased in the whole study group from 0.13% before treatment to 0.21% after treatment [*P* value = 0.0066%]. Despite the small size of this subset, differences were noted between the treatment groups and were highly significant. Before treatment, the percentages of these polyfunctional T lymphocytes were significantly lower in patients who had recurrences (0.04% and 0.23% respectively [*P* value = 0.0013]), levels before treatment were found to correlate significantly with response and a $1x10^{-4}$ increase in the percentage of in this polyfunctional CD4 T cells is associated with %59.7 reduction in the odds of recurrence [*P* value = 0.011]. After treatment, the percentage was still noticeably lower in the recurrence group but was no longer statistically significant. These polyfunctional cells are included in the IFNγ producing, the IL2 producing and the TNF producing subsets discussed separately, and it was no surprise that they correlated with outcome to an extent. The overlap between subsets identified by different readouts and their combinations make the analysis extraordinarily complex and create multiple additional end-points.

It should be noted that the selection of cellular activation readouts is to an extent arbitrary and reflects current knowledge and trends. It is encouraging though that the majority of the end-points I selected were significantly different between the two groups and have substantial potential to act as markers to predict outcome to treatment. It will be the goal of future studies to identify cellular subsets whose presence/absence and frequencies will predict response to BCG treatment in bladder cancer with high certainty.

Urine markers

The exact mechanism of how BCG immunotherapy works is still unclear, but the immune reaction and granulomas generated locally have been found to correlate with recurrence-free and progression–free survival [164]. Urine, collected during or after intravesical BCG treatment, has been evaluated by many researchers in order to investigate the effects of immunotherapy and find markers that correlate with recurrence and progression. Saint *et al* [165] reported a better treatment response if patients suffered from treatment side effects and had higher urinary leukocytes measured 3 days after BCG instillation. The authors found that urine leukocytes higher than 1.65 x 10^6 /ml correlated with recurrence-free status [*P* value = 0.009]. This is not surprising given that it is the immune reaction that develops following BCG treatment is responsible for the desired therapeutic effect and death of the malignant cells

Taniguchi *et al* [78] reported that IFNγ becomes detectable in urine and that levels increase with the number of BCG instillations. However, Saint *et al* [105] and Watanabe *et al* [76] found no correlation between the urine levels of IFNγ and the response to BCG treatment.

In the population I studied, the levels of IFNγ varied between patients following treatment and although the median level was noticeably higher in the recurrence-free group compared to the recurrence group, this was not statistically significant [*P* value = 0.1103].

Haaf *et al* [166] was first to detect an increase in urine IL2 levels after BCG instillations, however, while some differences were observed between responders and non-responders, these were not statistically significant. On the other hand, Saint *et al* [105] found the levels of urine IL2 to be significantly different between responders and non-responders with levels less than 27pg/µmol being associated with an increased risk of recurrence [*P* value = 0.0009]. They proposed using IL2 as a promising biomarker for monitoring treatment progress. Similarly Watanabi *et al* [76] found urine IL2 to be significantly higher in

responders [*P* value = 0.003]. De Reijke *et al* [72] also reported that higher IL2 urine levels following the first BCG treatment were associated with increased recurrence-free survival [*P* value = 0.003].

In agreement with these observations, urine IL2 levels in my study were found to be higher in the recurrence-free group than in the recurrence group (178.5pg/ml versus 71pg/ml), however, it was not statistically significant [*P* value = 0.1198]. These results can be explained by the fact that the function of IL2 includes enhancing the ability to produce cytotoxic lymphocytes that can discriminately kill cancer cells without harming normal cells.

Of note, a mere difference between the two groups, even if significant, does not mean that a parameter can necessarily discriminate well when used as a diagnostic test. This will depend on the spread of values around the mean or median in each group.

Observations with urine TNF are similar to the ones with IL2. Watanabe *et al* [76] reported an increase in urine TNF after BCG instillations and Shintani *et al* [28] found a strong correlation between levels of TNF and absence of recurrence. I collected the urine in a similar fashion to Shintani *et al* and also found that in the recurrence-free group the TNF levels were noticeably higher but this did not reach the threshold for statistical significance [*P* value = 0.0764]. It is quite possible that many differences measured in relation to urine cytokines could become statistically significant if the study had been larger. A number of promising end-points should be studied in a future study with a larger cohort.

IL8 is secreted by activated macrophages and leads to the recruitment of inflammatory cells like neutrophils and T lymphocytes [41]. Rabinowitz *et al* [74] tested the levels of IL8 and found a significant difference between the levels of IL8 in the urine after BCG

treatment and proposed it as a treatment monitoring test. Thalmann *et al* [73] found levels of urinary IL8 to correlate with recurrence with levels below 4000ng/L bearing a 90% risk of recurrence. In the same way, Kumar *et al* [108] reported higher levels of urine IL8 in responders [*P* value = 0.001] with recurrence-free patients having more than 400pg/ml IL8 in urine. Sagnak *et al* [109] found levels of urine IL8 to be associated with recurrence-free survival [*P* value = 0.006]. In my study, the levels of urine IL8 in the recurrence group were less than half the levels measured in the recurrence-free group, and although the number of patients I had in my study was similar to the cited papers, the difference did not reach statistical significance [0.0977].

In this study, I also measured IL4, IL6 and IL12p70. The levels of these cytokines were all higher in the recurrence-free group than the recurrence group with differences reaching statistical significance for IL4 [*P* value = 0.0351] and IL6 [*P* value = 0.0499].

The function of IL4 is mainly to stimulate Th2 cells and B cells and to regulate or suppress Th1 cells [39], while IL6 is involved in the B cell activation [40]. Th1 cells and macrophages and cytokines (IFNγ, IL2, TNF and IL8) are believed to be the key players in a successful BCG immunotherapy reaction. The finding of significantly higher levels of IL4 and IL6 suggest the involvement of additional T lymphocytes subsets and potentially other immune and tissue-specific cell types. It is likely that the anti-tumour effect of BCG therapy depends on complex interactions between such cells on one hand and soluble mediators and receptors on the other.

Generally, the use of urine for immune screening is limited due to the technical problems of sampling, processing, storage and contamination. Also, urine testing can only be used following BCG treatment rather than a pre-treatment tool.

Non-antigen specific responses

Unlike the differences in secreted cytokines observed following *in vitro* PPD challenge, there was no noticeable cytokine difference between responders and non-responders in the supernatant following LPS challenge of fresh blood. This is likely because the LPS-induced immune response uses an alternative way to stimulate T lymphocytes which is mainly monocyte dependent and involves the release of type I interferon [167] rather than MHC-T cell receptor interactions that is required with PPD.

In summary, the pre-treatment percentages of INFγ+, TNF+, IL2+ T lymphocytes & "CD40L+IFNγ+IL2+TNF+" functional T lymphocyte subset as well as the levels of secreted INFγ and IL2 cytokines following *in vitro* PPD were found to be higher in the group who had no recurrence compared to the group which had recurrence of their cancer after BCG treatment. Possible explanations for this include previous mycobacterial antigen exposure, for example for PPD responses that are antigen specific. This previous exposure could result in immunological memory which in turn could boost the immune response to BCG treatment. In this study I did not find any correlation between previous BCG vaccination and whether or not cancer recurred, but the population number is small and the study may have been underpowered. Furthermore, PPD is a crude preparation and contains many antigens that are found in a wide range of non-tuberculous mycobacteria which are ubiquitous in the environment and to which we are widely exposed. Alternatively, the groups may have been equally exposed to mycobacterial antigens but other factors such as age or host genetics that regulates the absolute numbers of lymphocytes produced and influencing the body's response to many antigens. This could explain the similar findings observed with SEB stimulation where the patients who had recurrences after treatment had significantly lower TNF+, IL2+ T lymphocytes & "CD40L+IFNγ+IL2+TNF+" functional T lymphocyte subset.

Study limitations

One limitation of this study was the relatively small sample size, especially in regards to recurrences after the BCG immunotherapy treatment. One of the reasons for this is the prospective design as it is impossible to accurately predict the number of patients failing treatment. In the current cohort, around 40% had recurrences after starting BCG treatment giving a total of 17 patients in the recurrence group.

Also, during the recruitment process there was a national shortage of BCG immunotherapy treatment for 6 months, this meant no patients received intravesical BCG treatment for at least 6 months, which affected the overall number of patients eligible for recruitment for this study.

Although some predictive analysis was presented as examples, the small overall study population limited the ability to perform more in depth predictive analysis. Performing complex analysis would dilute the sample size even further and hence loose power and significance. This was in agreement with the opinion of the statistician and the supervising professor. A future study would need to recruit a larger population in order for such diagnostic or predictive tests to be performed.

Using the results obtained from this research and in order to confirm the differences observed in the pre-treatment %IFNγ CD4 T lymphocytes between the two groups (where the recurrence-free group had twice as much cells compared to the recurrence group) with a 95%CI and %80 statistical power, a future study will need at least 40 cases in each group. Appreciating the prospective design of any future study and knowing that the percentage of recurrence after BCG is about %40, then at least 100 cases will need to be prospectively recruited in order to obtain 40 cases in the recurrence group.

The other limitation was the relatively short follow up period (median 23 months, range 16 - 34 months), so that the results from this study represent a short-term clinical outcome

from intravesical BCG immunotherapy. Also, it was not possible to stratify patients based on disease progression as only 2 progressed during the follow up period. All outcomes from this study will be re-evaluated after 2 and 5 years to correlate immune responses with intermediate and long-term clinical outcome.

I also measured a large number of parameters in the study, so any positive findings will need to be replicated in much larger studies.

6. Outlook

The main aim of this study was to identify detectable immunological differences that correlate with clinical outcome. As some interesting differences have been detected in this study, a future aim would be to evaluate if these markers can act as a predictive tool, which can help in deciding on the best treatment modality offered to patients. The small study population and the short follow-up period were not enough to generate this. It is unlikely that any single parameter will be able to predict outcome, rather ultimately a predictive tool will be required that combines a number of clinical as well as immunological parameters.

The data generated by this research, not only leads to the possibility of predicting outcome, but also suggests that immunological responsiveness prior to treatment is central to the outcome to BCG immunotherapy in bladder cancer. The is the most useful stage at which to be able to predict response as patients who are unlikely to respond can be identified and directed early to an alternative therapy.

This raises many questions, for example, what are the reasons for these immune differences? What is the role of host factors in determining the response to BCG, including age and host genetic make-up? Will it be possible in future to manipulate the factors associated with a good response to improve the prognosis for patients with bladder cancer?

To answer these questions, a future study will have to include more prospective patients (see study limitation) and provide for a longer follow-up. Such a study might be able to establish a predictive test for the outcome of BCG immunotherapy. Understandably, a reliable, robust and validated predictive test will need years to develop.

Also, by increasing the population size, the correlation between the immune differences and age will be investigated more thoroughly. A future study will also look at genetic factors, and to what extent host genetic variation can explain the findings observed in this

project. Ultimately I am hoping that this work will form the basis for future investigations to establish a test that can reliably predict failure of BCG immunotherapy and provide information as to how the treatment could be modified to increase its effect, for example by adjuvants.

7. Conclusions:

This ethically-approved case controlled study is the first study to prospectively evaluate immunological differences pre- and post-BCG immunotherapy treatment for high-grade non-muscle invasive bladder cancer and report significant pre-treatment differences between responders and non-responders to the treatment. I anticipate, with further research, these markers could be developed into a useful clinical tool that will discriminate between those who are likely to respond to BCG and those who are unlikely to respond, leading in the long term to better outcomes for patients with bladder cancer.

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9. References:

1. Cancer Research UK. 2013 [cited 2013 02/08]; Available from: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/bladder/?script=true.
2. Kim, E.J., et al., *Genotypes of TNF-alpha, VEGF, hOGG1, GSTM1, and GSTT1: useful determinants for clinical outcome of bladder cancer.* Urology, 2005. **65**(1): p. 70-5.
3. IARC Tobacco Smoking, *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.* IARC, 1985. **38**: p. 244-268.
4. Brownson, R.C., J.C. Chang, and J.R. Davis, *Occupation, smoking, and alcohol in the epidemiology of bladder cancer.* Am J Public Health, 1987. **77**(10): p. 1298-300.
5. Zeegers, M.P., et al., *Elevated risk of cancer of the urinary tract for alcohol drinkers: a meta-analysis.* Cancer Causes Control, 1999. **10**(5): p. 445-51.
6. Sun, C.L., et al., *Dietary soy and increased risk of bladder cancer: the Singapore Chinese Health Study.* Cancer Epidemiol Biomarkers Prev, 2002. **11**(12): p. 1674-7.
7. Bruemmer, B., et al., *Nutrient intake in relation to bladder cancer among middle-aged men and women.* Am J Epidemiol, 1996. **144**(5): p. 485-95.
8. Gago-Dominguez, M., et al., *Use of permanent hair dyes and bladder-cancer risk.* Int J Cancer, 2001. **91**(4): p. 575-9.
9. Alexandroff, A.B., et al., *BCG immunotherapy of bladder cancer: 20 years on.* Lancet, 1999. **353**(9165): p. 1689-94.
10. Sangar, V.K., et al., *The economic consequences of prostate and bladder cancer in the UK.* BJU Int, 2005. **95**(1): p. 59-63.
11. Konety, B.R., G.F. Joyce, and M. Wise, *Bladder and upper tract urothelial cancer.* J Urol, 2007. **177**(5): p. 1636-45.
12. Fine, P., et al., *Issues relating to the use of BCG in immunization programs*, W.H.O.D.o.V.a. Biologicals, Editor. 1999, WHO: Geneva.
13. Morales, A., D. Eidinger, and A.W. Bruce, *Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors.* J Urol, 1976. **116**(2): p. 180-3.
14. Herr, H.W., et al., *Bacillus Calmette-Guerin therapy alters the progression of superficial bladder cancer.* J Clin Oncol, 1988. **6**(9): p. 1450-5.
15. Pawinski, A., et al., *A combined analysis of European Organization for Research and Treatment of Cancer, and Medical Research Council randomized clinical trials for the prophylactic treatment of stage TaT1 bladder cancer. European Organization for Research and Treatment of Cancer Genitourinary Tract Cancer Cooperative Group and the Medical Research Council Working Party on Superficial Bladder Cancer.* J Urol, 1996. **156**(6): p. 1934-40, discussion 1940-1.
16. Sylvester, R.J., et al., *Bacillus calmette-guerin versus chemotherapy for the intravesical treatment of patients with carcinoma in situ of the bladder: a meta-analysis of the published results of randomized clinical trials.* J Urol, 2005. **174**(1): p. 86-91; discussion 91-2.
17. Babjuk, M., et al., *EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2013.* Eur Urol, 2013.
18. Merz, V.W., et al., *Analysis of early failures after intravesical instillation therapy with bacille Calmette-Guerin for carcinoma in situ of the bladder.* Br J Urol, 1995. **75**(2): p. 180-4.
19. Solsona, E., et al., *The optimum timing of radical cystectomy for patients with recurrent high-risk superficial bladder tumour.* BJU Int, 2004. **94**(9): p. 1258-62.
20. Herr, H.W. and P.C. Sogani, *Does early cystectomy improve the survival of patients with high risk superficial bladder tumors?* J Urol, 2001. **166**(4): p. 1296-9.
21. Denoix, P., *Enquête permanente dans les centres anti-cancéreux*. Vol. 1. 1946: Bulletin.Instut Nationald'hygiene.

22. Sobin, L., M. Gospodarowicz, and C. Wittekind, *TNM classification of malignant tumours (UICC International Union Against Cancer)*. 7 ed. 2009, New York: Wiley-Blackwell.
23. Sauter, V., et al., *Non-invasive urothelial tumours*, in *WHO classification of tumours of the urinary system and male genital organs*, J. Eble, et al., Editors. 2004, IARCC Press: Lyon. p. 110.
24. Okada, R., et al., *Phenotypic classification of human CD4+ T cell subsets and their differentiation.* Int Immunol, 2008. **20**(9): p. 1189-99.
25. Akbar, A.N. and J.M. Fletcher, *Memory T cell homeostasis and senescence during aging.* Curr Opin Immunol, 2005. **17**(5): p. 480-5.
26. Gutcher, I. and B. Becher, *APC-derived cytokines and T cell polarization in autoimmune inflammation.* J Clin Invest, 2007. **117**(5): p. 1119-27.
27. Ponticiello, A., et al., *Analysis of local T lymphocyte subsets upon stimulation with intravesical BCG: a model to study tuberculosis immunity.* Respir Med, 2004. **98**(6): p. 509-14.
28. Shintani, Y., et al., *Intravesical instillation therapy with bacillus Calmette-Guerin for superficial bladder cancer: study of the mechanism of bacillus Calmette-Guerin immunotherapy.* Int J Urol, 2007. **14**(2): p. 140-6.
29. Alberts B, J.A., Lewis J, et al., *Helper T Cells and Lymphocyte Activation*. 4th edition ed. Molecular Biology of the Cell. 4th edition. 2002, New York: Garland Science.
30. Seder, R.A., P.A. Darrah, and M. Roederer, *T-cell quality in memory and protection: implications for vaccine design.* Nat Rev Immunol, 2008. **8**(4): p. 247-58.
31. Abel, B., et al., *The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults.* Am J Respir Crit Care Med, 2010. **181**(12): p. 1407-17.
32. Wilkinson, K.A. and R.J. Wilkinson, *Polyfunctional T cells in human tuberculosis.* Eur J Immunol, 2010. **40**(8): p. 2139-42.
33. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
34. Gaffen, S.L. and K.D. Liu, *Overview of interleukin-2 function, production and clinical applications.* Cytokine, 2004. **28**(3): p. 109-23.
35. Pfeffer, K., *Biological functions of tumor necrosis factor cytokines and their receptors.* Cytokine Growth Factor Rev, 2003. **14**(3-4): p. 185-91.
36. Brunner, C., et al., *Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo.* J Immunol, 2000. **165**(11): p. 6278-86.
37. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
38. Dinarello, C.A., *Overview of the interleukin-1 family of ligands and receptors.* Semin Immunol, 2013. **25**(6): p. 389-93.
39. Choi, P. and H. Reiser, *IL4: role in disease and regulation of production.* Clin Exp Immunol, 1998. **113**(3): p. 317-9.
40. Barnes, T.C., M.E. Anderson, and R.J. Moots, *The many faces of interleukin-6: the role of IL6 in inflammation, vasculopathy, and fibrosis in systemic sclerosis.* Int J Rheumatol, 2011. **2011**: p. 721608.
41. Baggiolini, M. and I. Clark-Lewis, *Interleukin-8, a chemotactic and inflammatory cytokine.* FEBS Lett, 1992. **307**(1): p. 97-101.
42. Ouyang, W., et al., *Regulation and functions of the IL10 family of cytokines in inflammation and disease.* Annu Rev Immunol, 2011. **29**: p. 71-109.
43. Gee, K., et al., *The IL-12 family of cytokines in infection, inflammation and autoimmune disorders.* Inflamm Allergy Drug Targets, 2009. **8**(1): p. 40-52.
44. Wynn, T.A., *IL13 effector functions.* Annu Rev Immunol, 2003. **21**: p. 425-56.

45. Burtis, C., E. Ashwood, and D. Bruns, *TIETZ textbook of clinical chemistry and molecular diagnostics*, ed. t. edition. 2006, St. Louis, MO: Elsevier Saunders.
46. Shapiro, H.M., *References, in Practical Flow Cytometry*. 2003, NJ, USA: John Wiley & Sons, Inc.
47. Waldrop, S.L., et al., *Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency.* J Clin Invest, 1997. **99**(7): p. 1739-50.
48. Li, Y.Y., et al., *Ultrasensitive densitometry detection of cytokines with nanoparticle-modified aptamers.* Clin Chem, 2007. **53**(6): p. 1061-6.
49. Wiemann, B. and C.O. Starnes, *Coley's toxins, tumor necrosis factor and cancer research: a historical perspective.* Pharmacol Ther, 1994. **64**(3): p. 529-64.
50. Parish, C.R., *Cancer immunotherapy: the past, the present and the future.* Immunol Cell Biol, 2003. **81**(2): p. 106-13.
51. Shahani, L., S. Singh, and N.M. Khardori, *Immunotherapy in clinical medicine: historical perspective and current status.* Med Clin North Am, 2012. **96**(3): p. 421-31, ix.
52. M, C.J., *Leon Charles Albert Calmette 1863-1933.* Obituary Notices of Fellows of the Royal Society, 1934. **1**: p. 315-325.
53. Pearl, R., *Cancer and tuberculosis.* American Journal of Hygiene, 1929. **9**: p. 97-159.
54. Old, L.J., D.A. Clarke, and B. Benacerraf, *Effect of Bacillus Calmette-Guerin infection on transplanted tumours in the mouse.* Nature, 1959. **184(Suppl 5)**: p. 291-2.
55. Zbar, B., I.D. Bernstein, and H.J. Rapp, *Suppression of tumor growth at the site of infection with living Bacillus Calmette-Guerin.* J Natl Cancer Inst, 1971. **46**(4): p. 831-9.
56. Gutterman, J.U., et al., *Immunotherapy of human solid tumors with Bacillus Calmette-Guerin: prolongation of disease-free interval and survival in malignant melanoma, breast, and colorectal cancer.* Ann N Y Acad Sci, 1976. **277**(00): p. 135-59.
57. Guinan, P.D., et al., *Adjuvant immunotherapy (BCG) in stage D prostate cancer.* Am J Clin Oncol, 1982. **5**(1): p. 65-8.
58. Hanna, M.G., Jr. and L.C. Peters, *Specific immunotherapy of established visceral micrometastases by BCG-tumor cell vaccine alone or as an adjunct to surgery.* Cancer, 1978. **42**(6): p. 2613-25.
59. Galligioni, E., et al., *Adjuvant immunotherapy treatment of renal carcinoma patients with autologous tumor cells and bacillus Calmette-Guerin: five-year results of a prospective randomized study.* Cancer, 1996. **77**(12): p. 2560-6.
60. Coe, J.E. and J.D. Feldman, *Extracutaneous delayed hypersensitivity, particularly in the guinea-pig bladder.* Immunology, 1966. **10**(2): p. 127-36.
61. Nadler, R.B., et al., *Durability of the tumor-free response for intravesical bacillus Calmette-Guerin therapy.* J Urol, 1994. **152**(2 Pt 1): p. 367-73.
62. Prescott, S., et al., *Intravesical Evans strain BCG therapy: quantitative immunohistochemical analysis of the immune response within the bladder wall.* J Urol, 1992. **147**(6): p. 1636-42.
63. Gontero, P., et al., *The role of bacillus Calmette-Guerin in the treatment of non-muscle-invasive bladder cancer.* Eur Urol, 2010. **57**(3): p. 410-29.
64. Schamhart, D.H., et al., *Urinary cytokines reflecting the immunological response in the urinary bladder to biological response modifiers: their practical use.* Eur Urol, 2000. **37 Suppl 3**: p. 16-23.
65. Burger, M., et al., *ICUD-EAU International Consultation on Bladder Cancer 2012: Non-muscle-invasive urothelial carcinoma of the bladder.* Eur Urol, 2013. **63**(1): p. 36-44.
66. Han, R.F. and J.G. Pan, *Can intravesical bacillus Calmette-Guerin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials.* Urology, 2006. **67**(6): p. 1216-23.

67. Shelley, M.D., et al., *A systematic review of intravesical bacillus Calmette-Guerin plus transurethral resection vs transurethral resection alone in Ta and T1 bladder cancer.* BJU Int, 2001. **88**(3): p. 209-16.
68. Decobert, M., et al., *Maintenance bacillus Calmette-Guerin in high-risk nonmuscle-invasive bladder cancer: how much is enough?* Cancer, 2008. **113**(4): p. 710-6.
69. Koga, H., et al., *Maintenance intravesical bacillus Calmette-Guerin instillation for Ta, T1 cancer and carcinoma in situ of the bladder: randomized controlled trial by the BCG Tokyo Strain Study Group.* Int J Urol, 2010. **17**(9): p. 759-66.
70. Finan, C., et al., *Natural variation in immune responses to neonatal Mycobacterium bovis Bacillus Calmette-Guerin (BCG) Vaccination in a Cohort of Gambian infants.* PLoS One, 2008. **3**(10): p. e3485.
71. Newport, M.J., et al., *Genetic regulation of immune responses to vaccines in early life.* Genes Immun, 2004. **5**(2): p. 122-9.
72. de Reijke, T.M., et al., *Urinary cytokines during intravesical bacillus Calmette-Guerin therapy for superficial bladder cancer: processing, stability and prognostic value.* J Urol, 1996. **155**(2): p. 477-82.
73. Thalmann, G.N., et al., *Interleukin-8 expression in the urine after bacillus Calmette-Guerin therapy: a potential prognostic factor of tumor recurrence and progression.* J Urol, 1997. **158**(4): p. 1340-4.
74. Rabinowitz, R., et al., *Urinary interleukin-8/creatinine level as a predictor of response to intravesical bacillus Calmette-Guerin therapy in bladder tumor patients.* J Urol, 1997. **158**(5): p. 1728-31; discussion 1731-2.
75. Saint, F., et al., *T helper 1/2 lymphocyte urinary cytokine profiles in responding and nonresponding patients after 1 and 2 courses of bacillus Calmette-Guerin for superficial bladder cancer.* J Urol, 2001. **166**(6): p. 2142-7.
76. Watanabe, E., et al., *Urinary interleukin-2 may predict clinical outcome of intravesical bacillus Calmette-Guerin immunotherapy for carcinoma in situ of the bladder.* Cancer Immunol Immunother, 2003. **52**(8): p. 481-6.
77. Schmidt, A.C., et al., *Peripheral blood lymphocyte response in patients with superficial transitional cell carcinoma of the bladder treated with intravesical Bacillus Calmette-Guerin--a useful marker of response?* Br J Urol, 1993. **71**(2): p. 179-82.
78. Taniguchi, K., et al., *Systemic immune response after intravesical instillation of bacille Calmette-Guerin (BCG) for superficial bladder cancer.* Clin Exp Immunol, 1999. **115**(1): p. 131-5.
79. Ardelt, P.U., et al., *Reactive antibodies against bacillus Calmette-Guerin heat-shock protein-65 potentially predict the outcome of immunotherapy for high-grade transitional cell carcinoma of the bladder.* Cancer, 2010. **116**(3): p. 600-9.
80. Haaff, E.O., et al., *Two courses of intravesical bacillus Calmette-Guerin for transitional cell carcinoma of the bladder.* J Urol, 1986. **136**(4): p. 820-4.
81. Catalona, W.J., et al., *Risks and benefits of repeated courses of intravesical bacillus Calmette-Guerin therapy for superficial bladder cancer.* J Urol, 1987. **137**(2): p. 220-4.
82. Allen, T. and G.F. Greene, *BCG Refractory Disease*, in *Bladder Cancer: Diagnosis, Therapeutics, and Management*, C.T. Lee and D.P. Wood, Editors. 2010, Humana Press, a part of Springer Science+Business Media. p. 108-113.
83. Herr, H.W., *Age and Outcome of Superficial Bladder Cancer Treated with Bacille Calmette-Guérin Therapy.* Urology, 2007. **70**(1): p. 65-68.
84. Joudi, F.N., et al., *The impact of age on the response of patients with superficial bladder cancer to intravesical immunotherapy.* J Urol, 2006. **175**(5): p. 1634-9; discussion 1639-40.
85. Fernandez-Gomez, J., et al., *Prognostic factors in patients with non-muscle-invasive bladder cancer treated with bacillus Calmette-Guerin: multivariate analysis of data from four randomized CUETO trials.* Eur Urol, 2008. **53**(5): p. 992-1001.

86. Palou, J., et al., *Female gender and carcinoma in situ in the prostatic urethra are prognostic factors for recurrence, progression, and disease-specific mortality in T1G3 bladder cancer patients treated with bacillus Calmette-Guerin.* Eur Urol, 2012. **62**(1): p. 118-25.
87. van Rhijn, B.W., et al., *A new and highly prognostic system to discern T1 bladder cancer substage.* Eur Urol, 2012. **61**(2): p. 378-84.
88. Lopez-Beltran, A., et al., *Prognostic factors in stage T1 grade 3 bladder cancer survival: the role of G1-S modulators (p53, p21Waf1, p27kip1, Cyclin D1, and Cyclin D3) and proliferation index (ki67-MIB1).* Eur Urol, 2004. **45**(5): p. 606-12.
89. Andius, P. and S. Holmang, *Bacillus Calmette-Guerin therapy in stage Ta/T1 bladder cancer: prognostic factors for time to recurrence and progression.* BJU Int, 2004. **93**(7): p. 980-4.
90. Boorjian, S.A., F. Zhu, and H.W. Herr, *The effect of gender on response to bacillus Calmette-Guerin therapy for patients with non-muscle-invasive urothelial carcinoma of the bladder.* BJU Int, 2010. **106**(3): p. 357-61.
91. Takashi, M., et al., *Multivariate evaluation of factors affecting recurrence, progression, and survival in patients with superficial bladder cancer treated with intravesical bacillus Calmette-Guerin (Tokyo 172 strain) therapy: significance of concomitant carcinoma in situ.* Int Urol Nephrol, 2002. **33**(1): p. 41-7.
92. Lin, W.C., et al., *The role of lymphovascular invasion in predicting the prognosis of clinically localized upper tract urothelial carcinoma (pT1-3cN0M0).* J Urol, 2008. **180**(3): p. 879-84; discussion 884-5.
93. Lotan, Y., et al., *Lymphovascular invasion is independently associated with overall survival, cause-specific survival, and local and distant recurrence in patients with negative lymph nodes at radical cystectomy.* J Clin Oncol, 2005. **23**(27): p. 6533-9.
94. Resnick, M.J., et al., *Longitudinal evaluation of the concordance and prognostic value of lymphovascular invasion in transurethral resection and radical cystectomy specimens.* BJU Int, 2011. **107**(1): p. 46-52.
95. Saito, K., et al., *Lymphovascular invasion is independently associated with poor prognosis in patients with localized upper urinary tract urothelial carcinoma treated surgically.* J Urol, 2007. **178**(6): p. 2291-6; discussion 2296.
96. Zlotta, A.R., et al., *What is the optimal regimen for BCG intravesical therapy? Are six weekly instillations necessary?* Eur Urol, 2000. **37**(4): p. 470-7.
97. Luftenegger, W., et al., *Intravesical Versus Intravesical Plus Intradermal Bacillus Calmette-Guerin: A Prospective Randomized Study In Patient With Recurrent Superficial Bladder Tumors.* The Journal of Urology, 1996. **155**(2): p. 483-487.
98. Ratliff, T.L., et al., *Intravesical Bacillus Calmette-Guerin therapy for murine bladder tumors: initiation of the response by fibronectin-mediated attachment of Bacillus Calmette-Guerin.* Cancer Res, 1987. **47**(7): p. 1762-6.
99. Kavoussi, L.R., et al., *Fibronectin-mediated Calmette-Guerin bacillus attachment to murine bladder mucosa. Requirement for the expression of an antitumor response.* J Clin Invest, 1990. **85**(1): p. 62-7.
100. Mao, Y. and J.E. Schwarzbauer, *Fibronectin fibrillogenesis, a cell-mediated matrix assembly process.* Matrix Biol, 2005. **24**(6): p. 389-99.
101. Ratliff, T.L., L.R. Kavoussi, and W.J. Catalona, *Role of fibronectin in intravesical BCG therapy for superficial bladder cancer.* J Urol, 1988. **139**(2): p. 410-4.
102. Boorjian, S.A., et al., *Fibrin clot inhibitor medication and efficacy of bacillus Calmette-Guerin for bladder urothelial cancer.* J Urol, 2009. **182**(4): p. 1306-12.
103. Suttmann, H., et al., *Neutrophil granulocytes are required for effective Bacillus Calmette-Guerin immunotherapy of bladder cancer and orchestrate local immune responses.* Cancer Res, 2006. **66**(16): p. 8250-7.

104. Simons, M.P., M.A. O'Donnell, and T.S. Griffith, *Role of neutrophils in BCG immunotherapy for bladder cancer.* Urol Oncol, 2008. **26**(4): p. 341-5.
105. Saint, F., et al., *Prognostic value of a T helper 1 urinary cytokine response after intravesical bacillus Calmette-Guerin treatment for superficial bladder cancer.* J Urol, 2002. **167**(1): p. 364-7.
106. Kaempfer, R., et al., *Prediction of response to treatment in superficial bladder carcinoma through pattern of interleukin-2 gene expression.* J Clin Oncol, 1996. **14**(6): p. 1778-86.
107. Saint, F., et al., *Urinary IL2 assay for monitoring intravesical bacillus Calmette-Guerin response of superficial bladder cancer during induction course and maintenance therapy.* Int J Cancer, 2003. **107**(3): p. 434-40.
108. Kumar, A., et al., *Urinary interleukin-8 predicts the response of standard and low dose intravesical bacillus Calmette-Guerin (modified Danish 1331 strain) for superficial bladder cancer.* J Urol, 2002. **168**(5): p. 2232-5.
109. Sagnak, L., et al., *Predictive value of urinary interleukin-8 cutoff point for recurrences after transurethral resection plus induction bacillus Calmette-Guerin treatment in non-muscle-invasive bladder tumors.* Clin Genitourin Cancer, 2009. **7**(2): p. E16-23.
110. Thalmann, G.N., et al., *Urinary Interleukin-8 and 18 predict the response of superficial bladder cancer to intravesical therapy with bacillus Calmette-Guerin.* J Urol, 2000. **164**(6): p. 2129-33.
111. Bunde, T., et al., *Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells.* J Exp Med, 2005. **201**(7): p. 1031-6.
112. Lachmann, R., et al., *Polyfunctional T cells accumulate in large human cytomegalovirus-specific T cell responses.* J Virol, 2012. **86**(2): p. 1001-9.
113. McLaughlin, B.E., et al., *Nine-color flow cytometry for accurate measurement of T cell subsets and cytokine responses. Part I: Panel design by an empiric approach.* Cytometry A, 2008. **73**(5): p. 400-10.
114. Maecker, H. and J. Trotter, *[Selecting reagents for multicolor BD flow cytometry].* Postepy Biochem, 2009. **55**(4): p. 461-7.
115. Perfetto, S.P., et al., *Amine-reactive dyes for dead cell discrimination in fixed samples.* Curr Protoc Cytom, 2010. **Chapter 9**: p. Unit 9 34.
116. Spaulding, A.R., et al., *Staphylococcal and streptococcal superantigen exotoxins.* Clin Microbiol Rev, 2013. **26**(3): p. 422-47.
117. Sallusto, F. and A. Lanzavecchia, *Exploring pathways for memory T cell generation.* J Clin Invest, 2001. **108**(6): p. 805-6.
118. Campbell, J.J., et al., *CCR7 expression and memory T cell diversity in humans.* J Immunol, 2001. **166**(2): p. 877-84.
119. Martorell, J., et al., *CD27 induction on thymocytes.* J Immunol, 1990. **145**(5): p. 1356-63.
120. Ogawa, S., et al., *CD28 signaling in primary CD4(+) T cells: identification of both tyrosine phosphorylation-dependent and phosphorylation-independent pathways.* Int Immunol, 2013. **25**(12): p. 671-81.
121. Myrianthefs, P., et al., *Seasonal variation in whole blood cytokine production after LPS stimulation in normal individuals.* Cytokine, 2003. **24**(6): p. 286-92.
122. Lee, J.W., et al., *Fit-for-purpose method development and validation for successful biomarker measurement.* Pharm Res, 2006. **23**(2): p. 312-28.
123. Andius, P., O. Damm, and S. Holmang, *Prognostic factors in patients with carcinoma in situ treated with intravesical bacille Calmette-Guerin.* Scand J Urol Nephrol, 2004. **38**(4): p. 285-90.
124. Sylvester, R.J., et al., *Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials.* Eur Urol, 2006. **49**(3): p. 466-5; discussion 475-7.

125. Goronzy, J.J. and C.M. Weyand, *Understanding immunosenescence to improve responses to vaccines.* Nat Immunol, 2013. **14**(5): p. 428-36.
126. Fernandez-Gomez, J., et al., *Predicting nonmuscle invasive bladder cancer recurrence and progression in patients treated with bacillus Calmette-Guerin: the CUETO scoring model.* J Urol, 2009. **182**(5): p. 2195-203.
127. Losa, A., R. Hurle, and A. Lembo, *Low dose bacillus Calmette-Guerin for carcinoma in situ of the bladder: long-term results.* J Urol, 2000. **163**(1): p. 68-71; discussion 71-2.
128. Hurle, R., et al., *Intravesical bacille Calmette-Guerin in Stage T1 grade 3 bladder cancer therapy: a 7-year follow-up.* Urology, 1999. **54**(2): p. 258-63.
129. Ovesen, H., T. Horn, and K. Steven, *Long-term efficacy of intravesical bacillus Calmette-Guerin for carcinoma in situ: relationship of progression to histological response and p53 nuclear accumulation.* J Urol, 1997. **157**(5): p. 1655-9.
130. Pansadoro, V., et al., *Long-term follow-up of G3T1 transitional cell carcinoma of the bladder treated with intravesical bacille Calmette-Guerin: 18-year experience.* Urology, 2002. **59**(2): p. 227-31.
131. Davis, J.W., et al., *Superficial bladder carcinoma treated with bacillus Calmette-Guerin: progression-free and disease specific survival with minimum 10-year followup.* J Urol, 2002. **167**(2 Pt 1): p. 494-500; discussion 501.
132. Cookson, M.S. and M.F. Sarosdy, *Management of stage T1 superficial bladder cancer with intravesical bacillus Calmette-Guerin therapy.* J Urol, 1992. **148**(3): p. 797-801.
133. Lebret, T., et al., *Recurrence, progression and success in stage Ta grade 3 bladder tumors treated with low dose bacillus Calmette-Guerin instillations.* J Urol, 2000. **163**(1): p. 63-7.
134. Martinez-Pineiro, J.A., et al., *Long-term follow-up of a randomized prospective trial comparing a standard 81 mg dose of intravesical bacille Calmette-Guerin with a reduced dose of 27 mg in superficial bladder cancer.* BJU Int, 2002. **89**(7): p. 671-80.
135. Kelley, D.R., et al., *Prognostic value of purified protein derivative skin test and granuloma formation in patients treated with intravesical bacillus Calmette-Guerin.* J Urol, 1986. **135**(2): p. 268-71.
136. Luftenegger, W., et al., *Intravesical versus intravesical plus intradermal bacillus Calmette-Guerin: a prospective randomized study in patients with recurrent superficial bladder tumors.* J Urol, 1996. **155**(2): p. 483-7.
137. Herr, H.W., et al., *Effect of intravesical Bacillus Calmette-Guerin (BCG) on carcinoma in situ of the bladder.* Cancer, 1983. **51**(7): p. 1323-6.
138. Shinka, T., et al., *Clinical study of prognostic factors of superficial bladder cancer treated with intravesical bacillus Calmette-Guerin.* Br J Urol, 1990. **66**(1): p. 35-9.
139. Okamura, T., et al., *Clinicopathological evaluation of repeated courses of intravesical bacillus Calmette-Guerin instillation for preventing recurrence of initially resistant superficial bladder cancer.* J Urol, 1996. **156**(3): p. 967-71.
140. Zlotta, A.R., et al., *Evolution and clinical significance of the T cell proliferative and cytokine response directed against the fibronectin binding antigen 85 complex of bacillus Calmette-Guerin during intravesical treatment of superficial bladder cancer.* J Urol, 1997. **157**(2): p. 492-8.
141. Boehm, U., et al., *Cellular responses to interferon-gamma.* Annu Rev Immunol, 1997. **15**: p. 749-95.
142. Newport, M.J., et al., *A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection.* N Engl J Med, 1996. **335**(26): p. 1941-9.
143. Jouanguy, E., et al., *A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection.* Nat Genet, 1999. **21**(4): p. 370-8.
144. Dighe, A.S., et al., *Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors.* Immunity, 1994. **1**(6): p. 447-56.

145. Chin, Y.E., et al., *Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1.* Science, 1996. **272**(5262): p. 719-22.
146. Detjen, K.M., et al., *Interferon gamma inhibits growth of human pancreatic carcinoma cells via caspase-1 dependent induction of apoptosis.* Gut, 2001. **49**(2): p. 251-62.
147. Xu, X., et al., *IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression.* Cancer Res, 1998. **58**(13): p. 2832-7.
148. Coughlin, C.M., et al., *Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis.* J Clin Invest, 1998. **101**(6): p. 1441-52.
149. Qin, Z. and T. Blankenstein, *CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells.* Immunity, 2000. **12**(6): p. 677-86.
150. Elsasser, J., et al., *Antigen-specific CD4 T cells are induced after intravesical BCG-instillation therapy in patients with bladder cancer and show similar cytokine profiles as in active tuberculosis.* PLoS One, 2013. **8**(9): p. e69892.
151. Barnetson, R.S. and G.M. Halliday, *Regression in skin tumours: a common phenomenon.* Australas J Dermatol, 1997. **38 Suppl 1**: p. S63-5.
152. Atkins, M.B., M. Regan, and D. McDermott, *Update on the role of interleukin 2 and other cytokines in the treatment of patients with stage IV renal carcinoma.* Clin Cancer Res, 2004. **10**(18 Pt 2): p. 6342S-6S.
153. van Horssen, R., T.L. Ten Hagen, and A.M. Eggermont, *TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility.* Oncologist, 2006. **11**(4): p. 397-408.
154. Lejeune, G., *[Possibilities of the application of homografts in human clinical practice].* Rev Med Liege, 1960. **15**: p. 810-1.
155. Agarwal, A., et al., *Flow cytometric analysis of Th1 and Th2 cytokines in PBMCs as a parameter of immunological dysfunction in patients of superficial transitional cell carcinoma of bladder.* Cancer Immunol Immunother, 2006. **55**(6): p. 734-43.
156. Korniluk, A., H. Kemona, and V. Dymicka-Piekarska, *Multifunctional CD40L: pro- and anti-neoplastic activity.* Tumour Biol, 2014. **35**(10): p. 9447-57.
157. French, L.E. and J. Tschopp, *Defective death receptor signaling as a cause of tumor immune escape.* Semin Cancer Biol, 2002. **12**(1): p. 51-5.
158. Sandin, L.C., et al., *Locally delivered CD40 agonist antibody accumulates in secondary lymphoid organs and eradicates experimental disseminated bladder cancer.* Cancer Immunol Res, 2014. **2**(1): p. 80-90.
159. Cooper, A.M., *Editorial: Be careful what you ask for: is the presence of IL-17 indicative of immunity?* J Leukoc Biol, 2010. **88**(2): p. 221-3.
160. Khader, S.A., et al., *IL23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge.* Nat Immunol, 2007. **8**(4): p. 369-77.
161. Takeuchi A, Dejima T, Yamada H, Shibata K, Nakamura R, Eto M, Nakatani T, Naito S, Yoshikai Y. IL-17 production by γδ T cells is important for the antitumor effect of Mycobacterium bovis bacillus Calmette-Guérin treatment against bladder cancer. Eur J Immunol. 2011 Jan;41(1):246-51.
162. Sester, U., et al., *Whole-blood flow-cytometric analysis of antigen-specific CD4 T-cell cytokine profiles distinguishes active tuberculosis from non-active states.* PLoS One, 2011. **6**(3): p. e17813.
163. Streitz, M., et al., *The phenotypic distribution and functional profile of tuberculin-specific CD4 T-cells characterizes different stages of TB infection.* Cytometry B Clin Cytom, 2012. **82**(6): p. 360-8.

164. Jallad, S., et al., *Prognostic value of inflammation or granuloma after intravesival BCG in non-muscle-invasive bladder cancer.* BJU Int, 2014. **113**(5b): p. E22-7.
165. Saint, F., et al., *Leukocyturia as a predictor of tolerance and efficacy of intravesical BCG maintenance therapy for superficial bladder cancer.* Urology, 2001. **57**(4): p. 617-21; discussion 621-2.
166. Haaff, E.O., W.J. Catalona, and T.L. Ratliff, *Detection of interleukin 2 in the urine of patients with superficial bladder tumors after treatment with intravesical BCG.* J Urol, 1986. **136**(4): p. 970-4.
167. Mattern, T., H.D. Flad, and A.J. Ulmer, *Stimulation of human T lymphocytes by lipopolysaccaride (LPS) in the presence of autologous and heterologous monocytes.* Prog Clin Biol Res, 1998. **397**: p. 243-54.

10. Appendices

10.1 Appendix 1

Study	No. of patients	Type of factor	Factor	End point	Results
Herr *et al* [83]	805	Clinical	• Age	Initial response and recurrence free survival	27% 5-year cancer free survival if >70 compared to 37% for <70
Joudi *et al* [84]	412	Clinical	• Age	Recurrence-free survival	22% reduction in recurrence free survival when >80 years
Fernandez-Gomez *et al* [85]	1062	Clinical	• Gender • Recurrence • Mutlifocal • CIS	Recurrence-free and progression-free survivals	Female gender (HR=1.71), Recurrent tumours (HR= 1.9), Multifocal disease (HR=1.54) and CIS (HR=1.54) increased risk of recurrence. Recurrent tumours (HR=1.62), Higher grade (HR=5.64), Higher stage (HR=2.15), and recurrence at 3 months (HR=4.6) increased risk of progression
Palou *et al* [86]	146	Clinical	• Gender • CIS	recurrence, progression , and disease-related mortality	Female gender and presence of CIS in the prostatic urethra were associated with an increased risk of recurrence (*P* value=0.0003, HR: 2.53), progression (*P* value =0.001, HR: 3.59), and death from cancer (*P* value =0.004, HR: 3.53)
Van Rhijn *et al* [87]	134	Clinical	• T stage • CIS • Gender	progression and disease-specific survival (DDS)	Extensive T1 compared to micro-invasion sub-stage (was significant predictor for progression (*P* value=0.001) and DSS (*P* value=0.032) Female gender (*P* value =0.006) and carcinoma in situ (*P* value =0.034) were also significant predictors of progression

Lopez-Beltran *et al* [88]	159	Clinical	• Tumour size • Grade	Progression free survival	Univariate analysis revealed association of tumour size (*P* value = .0353) and grade in stage Ta tumours (*P* value =0.0074) with progression free survival
Andius *et al* [89]	236	Clinical	• BCG course • +ve 1st cystoscopy	Time to recurrence and progression	Negative first cystoscopy and maintenance BCG had a significantly longer time to recurren ce (*P* value < 0.001). Positive first cystoscopy (*P* value <0.001), tumour grade (*P* value =0.003) and six or fewer initial instillations (*P* value =0.002) were significantly associated with progression
Boorjian *et al* [90]	1021	Clinical	• Gender	Initial response to BCG and the time to disease recurrence and progression	Multivariate analysis no significant association between gender and recurrence (*P* value = 0.44) or progression (1.18, 0.85-1.63; *P* value = 0.33) after BCG
Takashi *et al* [91]	156	Clinical	• CIS • Age • Previous TCC •	Recurrence, Progression and survival	CIS was significantly associated with disease progression on univariate analysis (*P* value = 0.002). Age, history of bladder cancer, and concomitant CIS were significant on multivariate analysis
Lin *et al* [92]	106	Clinical	• Lympho-vascular invasion	Recurrence-free survival	Lymphovascular invasi on was associated with reduced 5-year recurrence-free survival (*P* value <0.001).
Lotan *et al* [93]	958	Clinical	• Lympho-vascular invasion	Recurrence and overall, and disease specific survivals.	Lymphovascular invasion was an independent predictor of local (HR = 2.03, *P* value =0.049), distant (HR = 2.60, *P* value = 0.0011), and overall (HR = 2.02, *P* value = 0.0003) recurrence in node-negative patients

					Lymphovascular invasion was an independent predictor of overall (HR = 1.84, *P* value = 0.0002) and cause-specific (HR =2.07, *P* value = 0.0012) survival in node-negative patients
Resnick *et al* [94]	487	Clinical	• Lympho-vascular invasion	Presence of nodal disease at cystectomy. Overall and recurrence-free survivals.	Nodal disease was higher in those patients with LVI *P* value < 0.001. Significant decrement in overall and recurrence-free survival among those with LVI
Saito *et al* [95]	135	Clinical	• Lympho-vascular invasion	Recurrence-free survival and disease-specific survivals.	Recurrence-free and disease specific survival rates lower with lymphovascular invasio n (*P* value = 0.001)
Luftenegger *et al* [97]	76	Clinical	• Symptoms after BCG	Time to recurrence. Recurrence rate in patients having intravesical alone compared to combined intradermal and intravesical BCG.	No difference between the 2 groups regarding interval to initial recurrence or recurrence rates after BCG treatment Changes in the purified protein derivative skin test performed before and after BCG therapy were not useful to predict response Development of fever was associated with better response (*P* value = 0.009)
Andius *et al* [123]	173	Clinical	• CIS • T stage	Times to recurrence and progression	CIS and T1 stage, had no prognostic value in terms of time to recurrence or progression.
Sylvester *et al* [124]	2596	Clinical	• Number of tumours • Tumour size • T stage • CIS • Grade	Short and long term recurrence and progression risk.	Number of tumours, tumour size, prior recurrence rate, T category, carcinoma in situ, and grade were all associated with increased recurrence and progression.

Fernandez-Gomez *et al* [126]	1062	Clinical	• Gender • Age • Grade • Multifocal • CIS	Recurrence and progression risks in patients treated with BCG.	Gender, age, grade, tumour status, multifocality and CIS were associated with recurrence. Age, grade, tumour status, T stage, multifocality and CIS were associated with progression.
Losa *et al* [127]	70	Clinical	• CIS	Disease recurrence and disease progression.	Risk of treatment failure was significantly greater for carcinoma in situ with stage T1 papillary tumour (*P* value = 0.0001) or severe dysplasia (*P* value = 0.0005), and the risk of disease progression was significantly greater for carcinoma in situ with stage T1 papillary tumour (*P* value = 0.0001)
Hurle *et al* [128]	51	Clinical	• Tumour size • CIS	Progression-free survival	The risk of disease progression was significantly higher for patients with a tumour measuring 3 cm or more and those with tumour associated with carcinoma in situ (CIS) in multivariate analyses
Pansadoro *et al* [130]	670	Clinical	• CIS	Recurrence and progression	No significant association with CIS
Davis *et al* [131]	98	Clinical	• CIS	Progression-free and disease specific survivals.	No significant association with CIS
Cookson *et al* [132]	86	Clinical	• Grade • T stage • CIS • Multifocal	Disease recurrence and progression.	Grade of the stage T1 tumour, concurrent carcinoma in situ and tumour multiplicity before BCG did not predict tumour recurrence or progression.
Lebert *et al* [133]	605	Clinical	• Multifocal • CIS	Recurrence and progression on rates, and the success of BCG therapy	The number of tumours at primary resection or CIS did not appear to be predictive factors of progression
Martinez-	500	Clinical	• Multifocal	Disease recurrence and	Reduced dose BCG

Pineiro *et al* [134]			disease	progression in reduced dose and full dose BCG treatment	treatment had similar results to full standard dose BCG treatment except for multifocal disease (*P* value =0.048) with regards to progression.
Shinka *et al* [138]	96	Clinical	• PPD skin test	Recurrence rate	No correlation between PPD responsiveness and outcome
Okamura *et al* [139]	76	Clinical	• Previous TB infection • Number of tumours • Mutlifocal disease • Previous treatment	Recurrence after treatment	No association between previous TB infection and response to intravesical BCG therapy. The number of lesions, i.e. multifocal disease (*P* value <0.05), and previous intravesical treatment (*P* value = 0.005) were significantly associated with recurrence.
Kelley *et al* [135]	62	Clinical and post treatment immuno-histological marker	• PPD conversion • Granuloma	Disease recurrence in relation to PPD skin conversion and granuloma in bladder samples.	PPD conversion (*P* value =0.0006) and Granuloma in post treatment histology (*P* value <0.003) significantly correlated with recurrence-free status.
Ovesen *et al* [129]	60	Clinical and post treatment immuno-histological marker	• Previous TCC • P53 nuclear activity	Complete response and progression rate.	BCG was more effective for treatment of primary than secondary carcinoma in situ. Post treatment p53 nuclear activity was associated with recurrence and progression.
Takeuchi *et al* [161]	10	During treatment immuno-histological marker	• IL17	Response to intravesical BCG (animal study)	Il17a production preceded neutrophils infiltration when starting BCG therapy. IL17 deficient mice did not respond to BCG therapy.
Jallad *et al* [164]	215	Post treatment immuno-histological marker	• Granuloma	Recurrence-free and progression-free survivals after BCG therapy.	Recurrence-free survival and progression-free survival were significantly higher in the presence of granuloma and/or inflammation groups (*P* value <0.001)
Saint *et al*	72	Post	• Leukocyturi	Recurrence-free status	High leukocyturia

[165]		treatment urine immune marker	a		during BCG therapy (cut off 1.65 x 10^5/ml) correlated with recurrence-free status (*P* value= 0.009)
Saint *et al* [105]	37	Post treatment Urine immune markers	• Urine IFNγ • Urine IL10 • Urine IL2	Prognostic value of urine Th1 cytokines after BCG on recurrence and progression	No correlation to IFNγ and IL10. IL-2 less than 27 pg./micromole was strongly associated with recurrence (*P* value =0.0009)
Watanabe *et al* [76]	20	Post treatment Urine immune markers	• Urine IL2 • Urine IL6 • Urine IL8 • Urine IL10 • Urine TNF	Recurrence after induction treatment.	Significantly higher levels of IL-2, IL-6, IL-8, IL-10, and TNF-alpha was observed in the responder group (*P* value <0.05). Multivariate analysis revealed IL-2 as an independent prognostic marker
Saint *et al* [75]	19	Post treatment Urine immune markers	• Urine Th1 cystokines • Maintena-nce BCG	Recurrence	T helper 1 lymphocyte urinary cytokine profile was associated with a clinical response to BCG. A repeat BCG course induces a favourable immune response suggesting that maintenance therapy may be beneficial
Haaf *et al* [166]	18	Post treatment Urine immune markers	• Urine IL2	Response to treatment vs IL2 levels	Differences in IL2 observed with different instillations and were higher in responders
De Reijke *et al* [72]	23	Post treatment Urine immune markers	• Urine IL2	Recurrence	A significant correlation between urinary interle ukin-2 and tumour recurrence was found (*P* value =0.003)
Shintani *et al* [28]	20	Post treatment Urine immune markers	• Urine TNF	Recurrence and recurrence-free survival	TNF-alpha level 4 h after the sixth week's instillation had a strong tendency towards the absence of recurrence
Rabinowitz *et al* [74]	46	During/ post treatment Urine immune markers	• Urine IL8	Tumour free after BCG induction	IL-8 levels obtained before intravesical BC G therapy and after instillations were not helpful in predicting recurrence.
Thalmann *et al* [73]	20	Post treatment	• Urine IL8	Treatment response (recurrence after	Patients secreting less than 4,000 ng. IL-8

		Urine immune markers		induction)	into the urine during the first 6 hours after BCG therapy had a significantly higher risk of tumour recurrence and progression (*P* value <0.0002)
Kumar *et al* [108]	26	Post treatment Urine immune markers	• Urine IL8	Treatment response (recurrence after induction)	Il-8 levels 4 hours after BCG treatment were significantly higher in responders than in non-responders (*P* value = 0.001)
Sagnak *et al* [109]	41	During treatment Urine immune markers	• Urine IL8	Recurrence after induction treatment	The change in IL-8 levels at 2 hours after the first BCG compared with the levels before BCG instillation was found to be significantly predictive of recurrence (*P* value = 0.047)
Schmidt *et al* [77]	10	During/ Post treatment peripheral blood immune markers	• Peripheral blood PBMC stimulation index	Recurrence after induction treatment	In vitro response of peripheral blood lymphocytes (stimulation index "SI") was higher in responders SI>5 compared to non-responders
Taniguchi *et al* [78]	21	During/ Post treatment Peripheral blood and urine immune markers	• Peripheral blood NK activity	Recurrence after treatment	Peripheral blood NK cell activity was higher in responders than non-responders
Ardelt *et al* [79]	16	During / Post treatment peripheral blood immune markers	• Peripheral blood anti-HSP-65	Recurrence after treatment	Increasing IgA and IgG anti-HSP-65 titres specifically predicted a positive patient
Zlott *et al* [140]	29	Post treatment Peripheral blood immune markers	• Peripheral blood immunity against BCG	Immune response and recurrence after treatment	Intravesical BCG instillations induce a transient (less than 6 months) peripheral immune activation against sever al purified BCG antigens. No correlation could be found between the

					immunological response
Elsasser *et al* [150]	72	During treatment Peripheral blood immune markers	• IFN-γ producing PPD-specific T cells	Change in cytokine profile with BCG therapy.	IFN-γ producing PPD-specific T cells showed 8 fold increase after the 5th instillation. Systemic T cell responses were induced after BCG-therapy, and their kinetics and cytokine profile depended on pre-existing immunity No correlation with outcome observed

Table 1: Summary table of published clinical and biomarkers factors relating to the response to intravesical BCG for bladder cancer.

10.2 Appendix 2

The time scale of case recruitment and sample collections; Patients who initially had trans-urethral resection of bladder tumour and **diagnosed** with high-risk non-muscle invasive bladder cancer and due to receive intravesical BCG treatment for non-muscle invasive bladder cancer were invited to participate in the study. Patients followed a routine BCG treatment (12.5mg intravesical OncoTICE® from Organon N.V) schedule over 1 year; this started with a 6 weeks **BCG induction** course with 6 instillations. After completing each cycle, patient undergone **cystoscopic** surveillance of their bladder and then had further **maintenance** boosters of 3 instillations every 4 months.

Following written informed **consent**, relevant clinical information was collected from individuals and their hospital records. **Urine and blood samples** were collected before starting treatment. Another **urine** sample collected 4 hours after completing BCG treatment. The **second blood** sample was collected 8 weeks after completing induction BCG treatment and before the next **maintenance BCG.** See figure below.

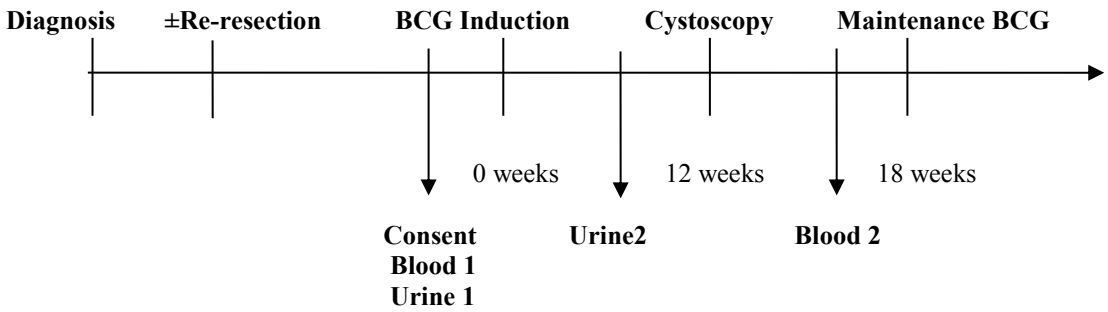

Figure 1: Time scale of patient recruitment and sample collection

10.3 Appendix 3

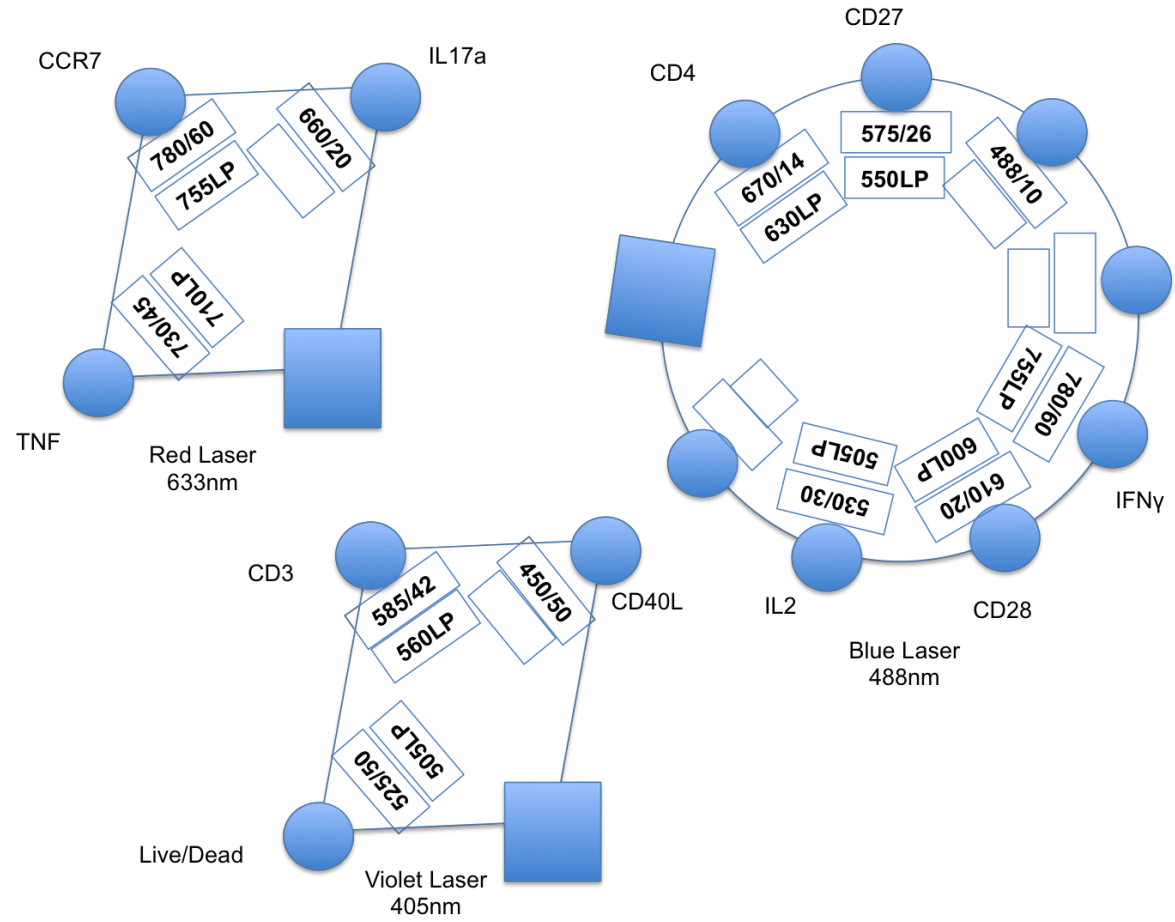

Figure 2: Flow-cytometry filter panel and filter layout

10.4 Appendix 4

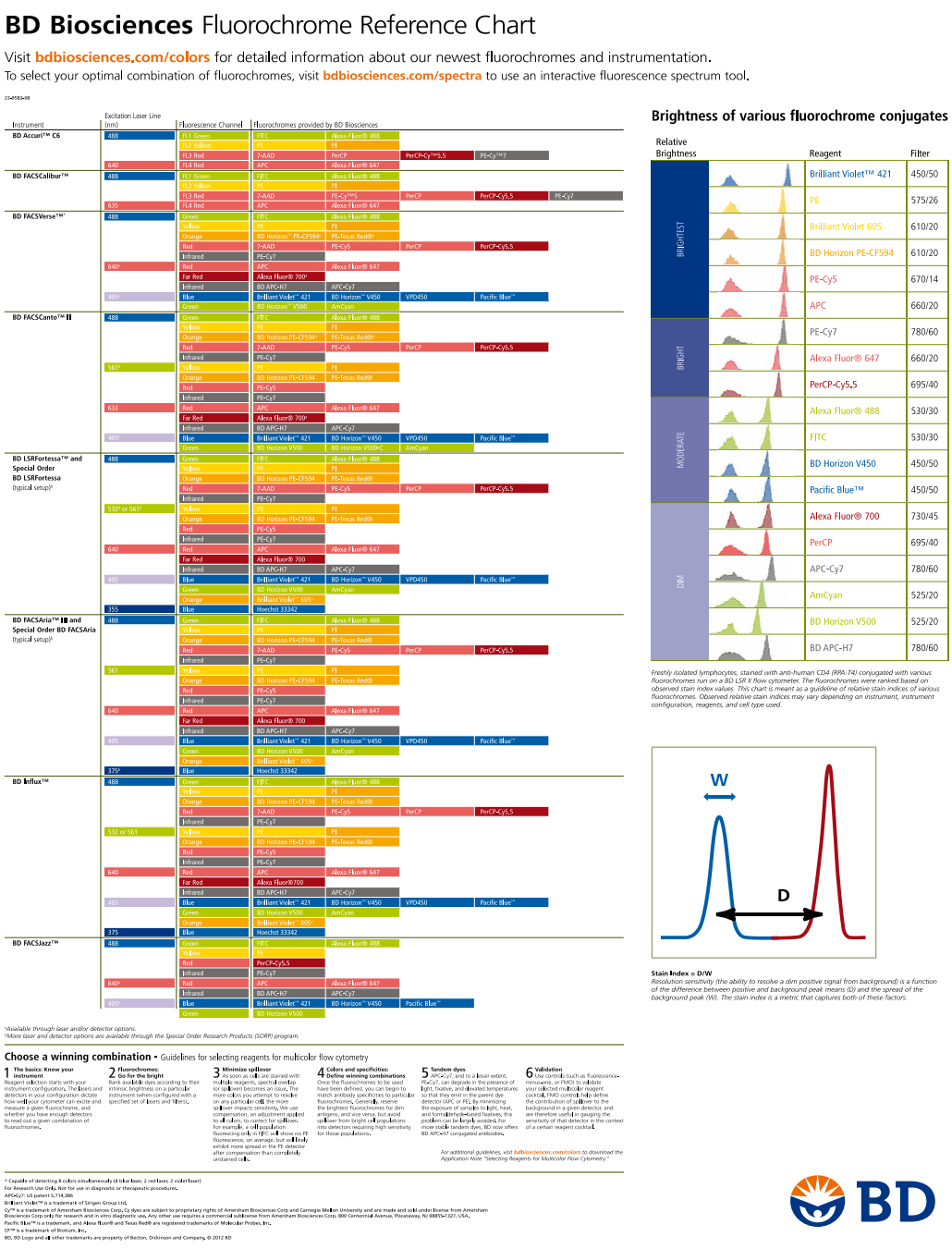

Figure 3: Flurochrome Reference Chart from BD bioscience

Further information can be obtained from the link below:

1. Flourochrome selection guide. https://www.thermofisher.com/content/dam/LifeTech/migration/en/filelibrary/cell-tissue-analysis/pdfs.par.13383.file.dat/fluorophore-selection-guide-flow-cytometry.pdf . Accessed 2012.
2. BD Bioscience. Multicolor Flow Cytometry Designer. http://www.bdbiosciences.com/us/applications/research/multicolor-flow/m/745795/resourcestools?cc=US . Accessed 2012.

10.5 Appendix 5

CCR7+ CD27+ CD28+						
CD40L	IFNγ	IL2	IL17a	TNF	Recurrence-free Median (range)	Recurrence Median (range)
+	+	+	+	+	0% (0 –0.04%)	0% (0 – 0%)
+	+	+	+		0% (0 – 0%)	0% (0 – 0%)
+	+	+		+	0.03% (0 - 0.97%)	0.01%(0 – 0.12%)
+	+	+			0% (0 –0.03%)	0% (0 – 0%)
+	+		+	+	0% (0 – 0%)	0% (0 – 0%)
+	+		+		0% (0 – 0%)	0% (0 – 0%)
+	+			+	0% (0 –0.08%)	0% (0 – 0.02%)
+	+				0% (0– 0.02%)	0% (0 – 0.02%)
+		+	+	+	0% (0– 0.09%)	0% (0 – 0.02%)
+		+	+		0% (0– 0.02%)	0% (0 – 0.01%)
+		+		+	0% (0 - 0.53%)	0% (0 – 0.37%)
+		+			0% (0 - 0.23%)	0.02%(0 – 0.13%)
+			+	+	0% (0– 0.04%)	0% (0 – 0%)
+			+		0% (0– 0.05%)	0% (0 – 0.04%)
+				+	0.02% (0– 0.36%)	0.01%(0 – 0.62%)
+					0.26% (0– 1.32%)	0.17%(0 – 2.25%)
	+	+	+	+	0% (0 – 0%)	0% (0 – 0%)
	+	+	+		0% (0 – 0%)	0% (0 – 0%)
	+	+		+	0% (0– 0.02%)	0% (0 – 0%)
	+	+			0% (0 – 0%)	0% (0 – 0.01%)
	+		+	+	0% (0 – 0%)	0% (0 – 0%)
	+		+		0% (0– 0.04%)	0% (0 – 0.05%)
	+			+	0% (0– 0.01%)	0% (0 – 0%)
	+				0.05% (0– 0.21%)	0.02%(0 – 0.16%)
		+	+	+	0% (0 – 0%)	0% (0 – 0%)
		+	+		0% (0–0.04%)	0% (0 – 0.01%)
		+		+	0% (0 – 0%)	0% (0 – 0%)
		+			0.03% (0 – 0.1%)	0.01%(0 – 0.12%)
			+	+	0% (0 –0.02%)	0% (0 – 0.01%)
			+		0.12% (0- 2.53%)	0% (0 – 0.25%)
				+	0.01% (0 - 0.39%)	0% (0 – 0.19%)

Table 1: the percentage of functional subsets within the CCR7+CD27+CD28+ CD4 lymphocytes subset in the recurrence-free and recurrence groups before starting intravesical BCG treatment for patients with high-grade non-muscle invasive bladder cancer.

CCR7- CD27+ CD28+						
CD40L	IFNγ	IL2	IL17a	TNF	Recurrence-free Median (range)	Recurrence Median (range)
+	+	+	+	+	0% (0 – 0.04%)	0% (0 – 0%)
+	+	+	+		0% (0 – 0%)	0% (0 – 0%)
+	+	+		+	0.08% (0 – 1.04%)	0.03% (0 – 0.47%)
+	+	+			0% (0– 0.06%)	0% (0 – 0.05%)
+	+		+	+	0% (0 – 0%)	0% (0 – 0%)
+	+		+		0% (0 – 0%)	0% (0 – 0%)
+	+			+	0.02% (0 - 0.27%)	0.02% (0 – 0.17%)
+	+				0% (0 - 0.12%)	0.1% (0 – 0.07%)
+		+	+	+	0% (0 –0.07%)	0% (0 – 0.03%)
+		+	+		0% (0 – 0.03%)	0% (0 – 0%)
+		+		+	0.03% (0– 0.6%)	0.04% (0 – 0.92%)
+		+			0.01% (0– 0.4%)	0.03% (0 – 0.14%)
+			+	+	0% (0– 0.03%)	0% (0 – 0.03%)
+			+		0% (0– 0.01%)	0% (0 – 0.02%)
+				+	0.03% (0– 0.32%)	0.04% (0 – 0.48%)
+					0.12% (0– 0.96%)	0.26% (0 – 0.78%)
	+	+	+	+	0% (0– 0%)	0% (0 – 0%)
	+	+	+		0% (0– 0%)	0% (0 – 0%)
	+	+		+	0% (0 – 0.05%)	0% (0 – 0%)
	+	+			0% (0– 0%)	0% (0 – 0%)
	+		+	+	0% (0– 0%)	0% (0 – 0%)
	+		+		0% (0– 0.01%)	0% (0 – 0%)
	+			+	0% (0 – 0.06%)	0% (0 – 0.03%)
	+				0% (0 – 0.18%)	0% (0 – 0.06%)
		+	+	+	0% (0 – 0%)	0% (0 – 0%)
		+	+		0% (0 – 0%)	0% (0 – 0%)
		+		+	0% (0 – 0%)	0% (0 – 0%)
		+			0.01% (0 – 0.01%)	0% (0 – 0.09%)
			+	+	0% (0 – 0.02%)	0% (0 – 0%)
			+		0% (0 – 0.28%)	0.02% (0 – 0.09%)
				+	0% (0 – 0.29%)	0% (0 – 0.04%)

Table 2: the percentage of functional subsets within the CCR7-CD27+CD28+ CD4 lymphocytes subset in the recurrence-free and recurrence groups before starting intravesical BCG treatment for patients with high-grade non-muscle invasive bladder cancer.

CCR7- CD27- CD28+						
CD40L	IFNγ	IL2	IL17a	TNF	Recurrence-free Median (range)	Recurrence Median (range)
+	+	+	+	+	0% (0 – 0.02%)	0% (0 – 0%)
+	+	+	+		0% (0 – 0%)	0% (0 – 0%)
+	+	+		+	0.07% (0 - 0.8%)	0% (0 – 0.2%)
+	+	+			0% (0– 0.03%)	0.02% (0 – 0%)
+	+		+	+	0% (0 – 0.01%)	0% (0 – 0%)
+	+		+		0% (0 – 0%)	0% (0 – 0%)
+	+			+	0.03% (0 – 0.17%)	0% (0 – 0.06%)
+	+				0% (0 – 0.03%)	0% (0 – 0.02%)
+		+	+	+	0% (0 – 0.06%)	0% (0 – 0.02%)
+		+	+		0% (0 – 0%)	0% (0 – 0%)
+		+		+	0.01% (0 – 0.17%)	0.01% (0 – 0.28%)
+		+			0% (0 – 0.05%)	0% (0 – 0.03%)
+			+	+	0% (0 – 0.05%)	0% (0 – 0.02%)
+			+		0% (0 – 0.03%)	0% (0 – 0%)
+				+	0% (0 – 0.15%)	0% (0 – 0.09%)
+					0.03% (0 – 0.31%)	0.06% (0 – 0.18%)
	+	+	+	+	0% (0 – 0%)	0% (0 – 0%)
	+	+	+		0% (0 – 0%)	0% (0 – 0%)
	+	+		+	0% (0 – 0.09%)	0% (0 – 0%)
	+	+			0% (0 – 0%)	0% (0 – 0%)
	+		+	+	0% (0 – 0%)	0% (0 – 0%)
	+		+		0% (0 – 0%)	0% (0 – 0%)
	+			+	0% (0 – 0.08%)	0% (0 – 0%)
	+				0% (0 – 0.02%)	0% (0 – 0.02%)
		+	+	+	0% (0 – 0.02%)	0% (0 – 0%)
		+	+		0% (0 – 0%)	0% (0 – 0%)
		+		+	0% (0 – 0.01%)	0% (0 – 0%)
		+			0% (0 – 0.03%)	0% (0 – 0.05%)
			+	+	0% (0 – 0%)	0% (0 – 0%)
			+		0% (0 – 0.06%)	0% (0 – 0.04%)
				+	0% (0 – 0.06%)	0% (0 – 0.01%)

Table 3: the percentage of functional subsets within the CCR7-CD27-CD28+ CD4 lymphocytes subset in the recurrence-free and recurrence groups before starting intravesical BCG treatment for patients with high-grade non-muscle invasive bladder cancer.

CCR7- CD27- CD28-						
CD40L	IFNγ	IL2	IL17a	TNF	Recurrence-free Median (range)	Recurrence Median (range)
+	+	+	+	+	0% (0 – 0%)	0% (0 – 0%)
+	+	+	+		0% (0 - 0%)	0% (0 - 0%)
+	+	+		+	0% (0 – 0.39%)	0% (0 – 0.06%)
+	+	+			0% (0 – 0%)	0% (0 – 0%)
+	+		+	+	0% (0 – 0%)	0% (0 – 0%)
+	+		+		0% (0 – 0%)	0% (0– 0%)
+	+			+	0% (0 –1. 53%)	0% (0 – 0.86%)
+	+				0% (0 – 0.02%)	0% (0 – 0.05%)
+		+	+	+	0% (0 – 0%)	0% (0 – 0%)
+		+	+		0% (0 – 0%)	0% (0 – 0%)
+		+		+	0% (0 – 0%)	0.% (0 – 0%)
+		+			0% (0 – 0.01%)	0% (0 – 0%)
+			+	+	0% (0 – 0%)	0% (0 – 0%)
+			+		0% (0 – 0%)	0% (0 – 0%)
+				+	0% (0 – 0.04%)	0% (0– 0%)
+					0% (0 – 0.03%)	0% (0 – 0.11%)
	+	+	+	+	0% (0 – 0.01%)	0% (0 – 0%)
	+	+	+		0% (0– 0%)	0% (0 – 0%)
	+	+		+	0% (0 – 0.04%)	0% (0 – 0%)
	+	+			0% (0 – 0%)	0% (0 – 0%)
	+		+	+	0% (0 – 0%)	0% (0 – 0%)
	+		+		0% (0– 0%)	0% (0 – 0%)
	+			+	0% (0 – 0.32%)	0% (0 – 0.19%)
	+				0% (0 – 0.04%)	0% (0 – 0.04%)
		+	+	+	0% (0 - 0%)	0% (0 – 0%)
		+	+		0% (0 – 0%)	0% (0 – 0%)
		+		+	0% (0 – 0%)	0% (0 – 0%)
		+			0% (0 – 0.03%)	0% (0 – 0.04%)
			+	+	0% (0 – 0%)	0% (0 – 0%)
			+		0% (0 – 0.01%)	0% (0 – 0.03%)
				+	0% (0 – 0.15%)	0% (0 – 0%)

Table 4: the percentage of functional subsets within the CCR7-CD27-CD28- CD4 lymphocytes subset in the recurrence-free and recurrence groups before starting intravesical BCG treatment for patients with high-grade non-muscle invasive bladder cancer.

10.6 Appendix 6

Sylvester et al [124] published a meta-analysis on 7 EORTC randomised controlled trials involving 2596 patients in order to calculate the short term and long term risks of recurrence and progression after transurethral resection of non-muscle invasive bladder cancer. See tables 1,2 and 3 below.

Factor	Recurrence	Progression
Number of tumours		
Single	0	0
2 to 7	3	3
≥ 8	6	3
Tumour diameter		
< 3 cm	0	0
≥ 3 cm	3	3
Prior recurrence rate		
Primary	0	0
≤ 1 recurrence / year	2	2
> 1 recurrence / year	4	2
Category		
Ta	0	0
T1	1	4
Concomitant CIS		
No	0	0
Yes	1	6
Grade		
G1	0	0
G2	1	0
G3	2	5
Total	0-17	0-23

Table 5: Calculation of recurrence and progression scores

Recurrence score	Prob. of recurrence 1 year	Prob. of recurrence 5 years	Recurrence risk groups
0	15%	31%	Low risk
1-4	24%	46%	Intermediate risk
5-9	38%	62%	Intermediate risk
10-17	61%	78%	High risk

Table 6: Probability of recurrence

Progression score	Prob. of progression 1 year	Prob. of progression 5 years	Progression risk group
0	0.2%	0.8%	Low risk
2-6	1%	6%	Intermediate risk
7-13	5%	17%	Intermediate risk
14-23	17%	45%	High risk

Table 7: Probability of progression

Fernandez-Gomez et al [85] reported on 4 Spanish Urological Club for Oncological Treatment (CUETO) in a meta-analysis of randomised controlled trials involving 1,062 patients who were treated with intravesical BCG. Aiming to develop a risk stratification model to provide estimates on recurrence and progression in patients who had BCG treatments. See tables 4 and 5 below.

Factor	Recurrence score	Progression score
Gender:		
M	**0**	**0**
F	**3**	**0**
Age:		
Less than 60	**0**	**0**
60-70	**1**	**0**
Greater than 70	**2**	**2**
Recurrent tumour:		
No	**0**	**0**
Yes	**4**	**2**
No. tumours:		
3 or less	**0**	**0**
Greater than 3	**2**	**1**
T category:		
Ta	**0**	**0**
T1	**0**	**2**
Associated Tis:		
No	**0**	**0**
Yes	**2**	**1**
Grade:		
G1	**0**	**0**
G2	**1**	**2**
G3	**3**	**6**
Total score	**0-16**	**0-14**

Table 4: Factors by weight to calculate recurrence and progressions scores.

	% 1 year		% 2 year		% 5 years	
Score	Recurrence	Progression	Recurrence	Progression	Recurrence	Progression
0-4	8.42	1.17	12.6	2.16	20.98	3.76
5-6	12.07	3	22.28	4.97	35.57	11.69
7-9	25.36	5.55	39.61	11.95	47.65	21.26
10 or greater	41.79	13.97	52.55	24.81	67.61	33.57

Table 5: Recurrence and progression probabilities at 1,2 and 5 years

11. Presentations

1. (Oral presentation) British Association of Urological Surgeons Conference – Liverpool 2014
2. (Poster presentation) 1st Immunotherapy of Cancer Conference – Munich 2014
3. (Oral presentation) Society of Academic and Research Surgery (SARS) – Cambridge 2014
4. (Poster presentation) Society of Basic Urological Research – Nashville 2013
5. (Oral presentation) BSMS 10 year anniversary conference – 2013
6. (Poster presentation) BSMS Postgraduate Research Student Symposium – 2013

12. Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

01/05/2015