Universitat Autònoma
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Improving bladder cancer treatment:

A new formulation containing an environmental mycobacterium

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Vist-i-plau de la directora de la Tesi,

Dra. Esther Julián Gómez

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Abstract

Bladder cancer (BC) is one of the most common cancers in Europe. Fortunately, most of the cases are diagnosed at early stages of the disease, when tumours are still confined to the mucosa. These are called non-muscle invasive bladder cancer (NMIBC). The treatment consists on resecting the tumour and, then, on the intravesical administration of the attenuated stain of *Mycobacterium bovis, M. bovis* BCG in its live form. The benefits of BCG in NMIBC patients are clear; BCG avoids recurrence and progression of BC which improves the survival rate of the patients.

Nevertheless, the drawbacks cannot be forgotten. Most treated patients experience mild to severe side effects and many patients have to abort the BCG treatment due to its toxicity. Thus, safer alternatives are needed.

It has been recently described the antitumour capacity of *Mycobacterium brumae*, an environmental mycobacterium. *M. brumae* showed *in vitro* a similar antitumour activity to BCG on different BC cell lines. Moreover, these species showed to be able to activate murine macrophages and to activate peripheral blood mononuclear cells' cytotoxicity against BC cells. Thus, the next step was to test *M. brumae*'s antitumour capacity and its ability to activate an immune response in an animal model of BC.

Moreover, another important issue was addressed on the way. It is known that mycobacteria, due to the high lipidic content of their cell wall, are very hydrophobic and have the tendency to aggregate in aqueous solutions. These clumps might difficult the interaction between the bacteria and the BC cells. In this thesis, different emulsions were assayed in order to diminish the size of these aggregates. In addition, the chosen emulsion had to maintain *M. brumae* viability, to maintain its antitumour activity and its capacity to induce an immune response *in vitro*. Once one emulsion was chosen, different treatments were tested in the animal model. Survival rates of the treated tumour-bearing mice were recorded and the local and systemic immune response was also studied by using different techniques.

In conclusion, emulsified *M. brumae* showed promising results in the BC mouse model which means it could be a safer alternative to BCG for NMIBC patients.

Resum

El càncer de bufeta (BC) és un dels càncers més comuns a Europa. Afortunadament, la majoria de pacients es diagnostiquen en els estadis primerencs de la malaltia, quan el tumor es troba a la mucosa. D'aquest estadi en diem càncer de bufeta no invasiu (NMIBC). El tractament consisteix en la resecció del tumor i, posteriorment, a administrar intravesicalment la soca atenuada de *Mycobacterium bovis*, *M. bovis* BCG viu. Els beneficis del BCG com a teràpia per a pacients de NMIBC són clars: el BCG evita la recurrència i la progressió del BC, la qual cosa millora la supervivència dels pacients.

Tot i així, cal no oblidar els desavantatges. La majoria de pacients pateixen efectes secundaris lleus i, fins i tot, severs i molts pacients han d'abandonar el tractament a causa de la seva toxicitat. Per tant, cal trobar alternatives més segures.

Recentment s'ha descrit la capacitat antitumoral del micobacteri ambiental *Mycobaterium brumae*. *M. brumae* ha demostrat tenir una activitat antitumoral semblant al BCG sobre diferents línees cel·lulars de BC humanes. A més a més, aquesta espècie és capaç d'activar macròfags de ratolí i, també, d'activar cèl·lules mononuclears de sang perifèrica per a que siguin citotòxiques per a les cèl·lules de BC. Per tant, el següent pas seria estudiar la capacitat antitumoral de *M. brumae* i la seva capacitat estimuladora del sistema immunitari en el model animal de BC.

Però primer, caldria abordar un altre qüestió important. Es coneix que els micobacteris, degut a l'alt contingut lipídic de la seva paret, són molt hidrofòbics i tenen tendència a formar agregats quan es troben en solucions aquoses. Aquests agregats dificultarien la interacció dels micobacteris amb les cèl·lules canceroses. En aquesta investigació, es van estudiar diferents emulsions amb l'objectiu de disminuir la mida d'aquests agregats. A més a més, l'emulsió escollida havia de mantenir la viabilitat d'*M. brumae*, de mantenir la seva capacitat antitumoral i de induir una resposta immunitària *in vitro*. Quan finalment es va escollir, es van aplicar diferents tractaments en el model animal. La supervivència dels animals amb tumor va ser registrada i la resposta immunitària generada es va estudiar mitjançant diverses tècniques.

M. brumae ha mostrat resultats prometedors en el model animal i, per tant, podria ser considerat una alternativa segura al BCG per al tractament de pacients de NMIBC.

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Acronyms

AJCC	American Joint Committee on Cancer
ATCC	American Type Culture Collection
BC	Bladder Cancer
BCG	*Mycobacterium bovis* Bacillus Calmette-Guèrin
BNN	N-butyl-N-(4-hydroxybutyl)-nitrosamine
CD	Cluster Diferentiation
CF	Cord Factor
CFU	Colony Forming Units
CIS	Carcinoma *in situ*
Con A	Concanavalin A
CT	Computed Tomography
CWE	Cell Wall Extracts
CWS	Cell Wall Skeleton
EAU	European Association of Urology
ER	Early Recurrence
FAP	Fibronectin Attachment Protein
FDA	Federal Drug Administration
GAG	Glycosaminoglycan
G-CSF	Granulocyte–Colony-Stimulating Factor
GEMM	Genetically Engineered Mouse Models
GM-CSF	Granulocyte–Macrophage Colony-Stimulating Factor
h-k	Heat-killed
IFN	Interferon

IL	Interleukin
INH	isoniazid (Isonicotinylhydrazine)
IP	Interferon-inducible Protein
IP-10	interferon-γ-inducible Protein-10
ISUP	International Society of Urologic Pathology
IVU	Intravenous Urography
KBMA	Killed But Metabolically Active
KC	Keratinocyte Chemoattractant
LUTS	Lower Urinary Tract Symptoms
MATH	Microbial Adhesion To Hydrocarbons
MBT-2	Mouse Bladder Tumour-2
MCP	Monocyte Chemoattractant Protein
MCP-1	Monocyte Chemoattractant Protein-1
MCWE	Mycobacterium phlei Cell Wall Extract
MDC	Macrophage-Derived Chemokine
MIP	Macrophage Inflammatory Protein
MMC	Mitomycin C
MMP-9	Matrix Metaloproteinase 9
MOI	Mutiplicity of Infection
MRI	Magnetic Resonance Imaging
MTB	*Mycobacterium tuberculosis*
NMIBC	Non-Muscle Invasive Bladder Cancer
OADC	Oleic-Albumin-Dextrose-Catalase
PBMC	Peripheral Blood Mononuclear Cells
PPD	Purified Protein Derivative
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RFI	Recurrence Free Interval

RT	Room Temperature
SWOG	South-West Oncology Group
TB	Tuberculosis
TBS	Tris-Buffered Saline
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TNM	Tumour Node Metastasis staging system
TRAIL	Tumour necrosis factor Related Apoptosis-Inducing Ligand
TUR	Transurethral Resection
UAB	Universitat Autònoma de Barcelona
UICC	International Union for Cancer Control
Um	Umbrella Cells
UTI	Urinary Tract Infection
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WLC	White Light Cytoscopy
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RFI	Recurrence Free Interval
RT	Room Temperature
SWOG	South-West Oncology Group
TB	Tuberculosis
TBS	Tris-Buffered Saline

A. General introduction

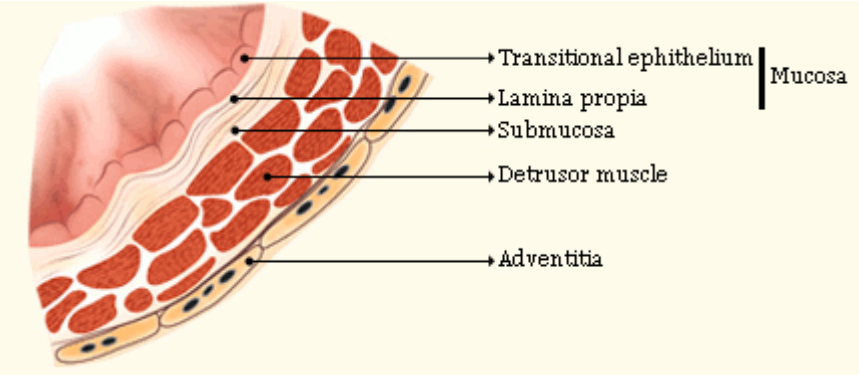
A.1. Bladder cancer (BC): general considerations

This introductory section provides an overview of BC towards better understanding its social impact and magnitude and, furthermore, to summarize the currently accepted guidelines for the diagnosis, staging and treatment of BC.

The bladder

The bladder is a hollow distensible muscular organ located in the pelvic cavity which is part of the urinary track. Urine, a waste product produced in the kidneys by filtration of the blood, travels down the ureters to the bladder where it is stored until micturition when muscular layers of the bladder contract and urine is pushed through the urethra to be discharged from the body [1,2].

Figure A.1-1: tissue layers of the healthy human bladder



Adapted from https://sites.google.com/site/msubladdercancer/pathophysiology

Many parts of the urinary track (calyces, renal pelvis, ureters and urethra) have a similar histologic structure to the bladder which basically consists, from the inner to the outer part, of epithelium, lamina propia, submucosa, muscular layers and adventitia (see Figure A.1-1). The epithelium is a specialized one named urothelium or transitional epithelium. The urothelium is surrounded by the highly vascularized lamina propia and submucosa. Covering them there are smooth muscle layers, collectively called detrusor muscle which is responsible for the contraction of the bladder to void the urine. The outermost layer of connective tissue that covers the bladder is the adventitia except for the upper part which is covered by the serosa of the

peritoneum. [3,4]. Blood vessels and immune system elements are embedded in these structures, as are sensory and motor neurons [5]. These neurons work and coordinate together with the different structures of the bladder in the two phases of the bladder: the filling and storage phase and the emptying phase.

At a cytological level, a healthy urothelium is stratified into three to six layers of cells depending on the grade of distension of the bladder. These strata of cells lay on a basement membrane and consist of first, basal-layer cells that are compact and cuboidal, second, intermediate-layer cells that are more columnar and third, the surface cells which work to avoid permeabilization of urine even at the maximum distension. These superficial large cells are called Umbrella cells (Um) or dome cells have a clearly eosinophilic cytoplasm and are able to execute their function due to their ability to change from an ovoid shape when the bladder is empty to a flatter shape when it is full. Moreover, to fulfil their function properly Um's apical membrane is thicker and it is covered by plaques [5]. Plaques are formed by connecting cytoplasmic vesicles with a cytoskeletal network. The plaques of the Um are, in turn, covered by a hydrophilic GlycosAminoGlycan (GAG)-rich mucin layer that completely blocks the penetration of any solution, solute or particle. The detrusor muscle is also stratified into inner, middle and outer layers. Finally, serosa is composed of connective tissue covered by a layer of mesothelial cells that form a simple squamous epithelium [5,6].

There are two main bladder disorders related to a failure of the signals from the bladder to the nervous system. These are an overactive bladder and incontinence and they are usually associated with one another [7]. Patients affected by these diseases usually present a characteristic combination of symptoms that are known as Lower Urinary Tract Symptoms (LUTS). Overactive bladder is the urge to urinate when the bladder is not full. The main cause of an overactive bladder is an inappropriate detrusor muscle contraction. The most common symptoms are frequent urination and nocturia (waking up to urinate in the night).Incontinence consists of the uncontrolled leak of urine. It has many causes including an overactive bladder, stress, the inability to completely empty the bladder usually as a result of an obstruction of the urethra that may cause bladder stones or overflow due to malfunction of the nervous system or bladder.

One of the most common bladder disorders is the Urinary Tract Infections (UTI) which lead to the inflammation of the bladder (cystitis), haematuria (blood presence in the urine) and dysuria (discomfort when urinating) [7].

Finally, BCs which are the most common malignancies of the urinary tract have similar symptoms as UTI. In the following sections BCs and their characteristics are described in detail.

A.1.1. RISK FACTORS

BC risk factors have to be differentiated into genetic predispositions and external exposures to carcinogens [8]. As the guidelines of the European Association of Urology (EAU) for BC state, the risks factors are the following [9]:

Genetic predisposition

Increasing evidence suggests the inherited genetic factors have a significant influence on the incidence of BC but always as enhancers of the susceptibility to external factors; it is known that first-degree relatives of BC patients have a two-fold higher risk of developing BC [8].

Exposure to carcinogens

The most important risk factor for BC is tobacco smoking. Approximately half of the cases are attributed to it and this is because the aromatic amines and polycyclic aromatic hydrocarbons that the tobacco smoke contains are excreted via renal routes.

Occupational exposure to carcinogens is the second most important risk factor for BC. People who work in industries related to paint, dye, metal and petroleum derivative products are usually in contact with these two compound types present in tobacco smoke together with chlorinated hydrocarbons.

Exposure to other carcinogens or medical conditions have uncertain significance. Some examples are: (1) dietary factors such as drinking water that contains arsenic and varying levels of trihalomethanes, due to the chlorination of the water; (2) personal hair dye use; (3) exposure to ionizing radiation, and (4) schistosomiasis caused by *Schistosoma haematobium* a parasitic trematode that induces chronic cystitis [8,10].

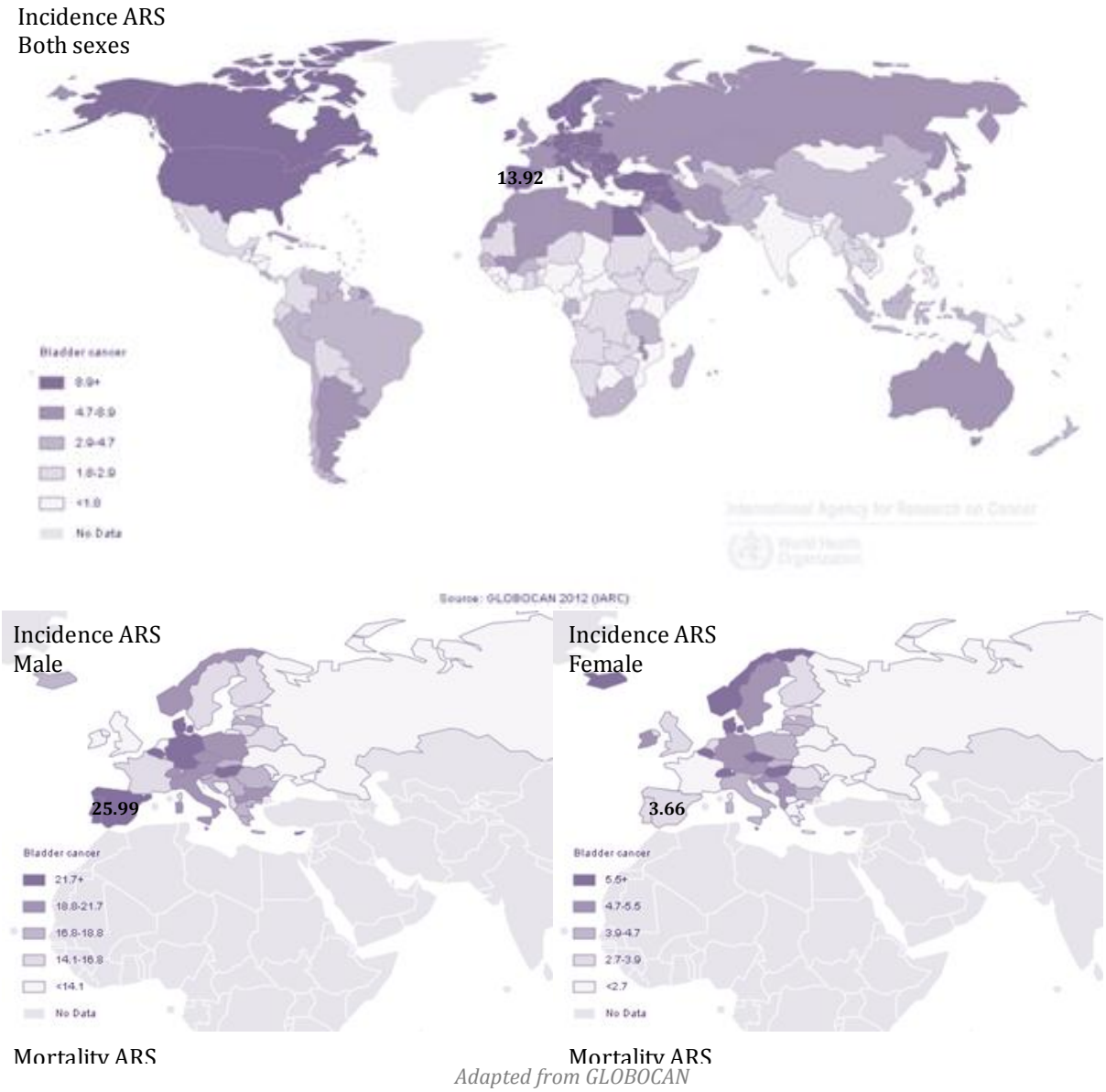
A.1.2. EPIDEMIOLOGY STATISTICS

BC is the 7th most common cancer in men and is the 17th in women worldwide. In data per 100,000 people, the incidence in men is 9 and in women 2 worldwide. However, in Europe, is 27 and 6, respectively [11]. One possible explanation to this fact is the historical exposure to tobacco smoke and occupational carcinogens in men. Unfortunately, Spain stands out in BC incidence and mortality statistics in Europe being one of the countries with more cases per 100,000 inhabitants (see Figure A.1-2) [9,12]. It has to be understood some bias due to the different methodology used in different national registers.

In Catalonia (2003-2007), data on incidence of BC is above the European average. In men, BC is the 4th in incidence with 11.2% of the diagnosed cancers at a median age of 71.2 years old. In the first place there is prostate cancer, in the second lung cancer, colon and rectum are in the third place and in fifth place oral cavity and pharynx cancers. In women, is the 8th being 3.1% of

the diagnosed cancers being the median age 75.3 years old. Moreover, in terms of mortality, BC is also in the top five in Catalonia. Thus, as BC is one of the most incident cancers in Catalonia, there is a primary outpatient programme for its prevention based on encouraging the smoking population to stop smoking [13].

Figure A.1-2: incidence of BC in the world and in Europe 2012



Adapted from GLOBOCAN

A.1.3. CLASSIFICATION: STAGES AND GRADES

To determine the cancer stage at the time of diagnosis is crucial in order to know the prognosis and the appropriate treatment for each patient. There are several staging systems, but the system most accepted worldwide is the Tumour Node Metastasis (TNM) system. The American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) support this system. Furthermore, it has clinical usefulness because it is applicable to any

part of the body and consists of determining the size and extent of the primary tumour, the involvement of the regional lymph nodes, and the presence or absence of metastases [14].

There are other descriptors that allow clinicians to classify tumours. The World Health Organization (WHO) proposed the histopathologic type classification which tells us the tissue type or cell type that most resembles the tumour. The grade is another classification based on the degree of differentiation of the tumour[14].

In accordance with the last update of the AJCC Cancer Staging Manual [14], below there is the classification of the urinary bladder tumours:

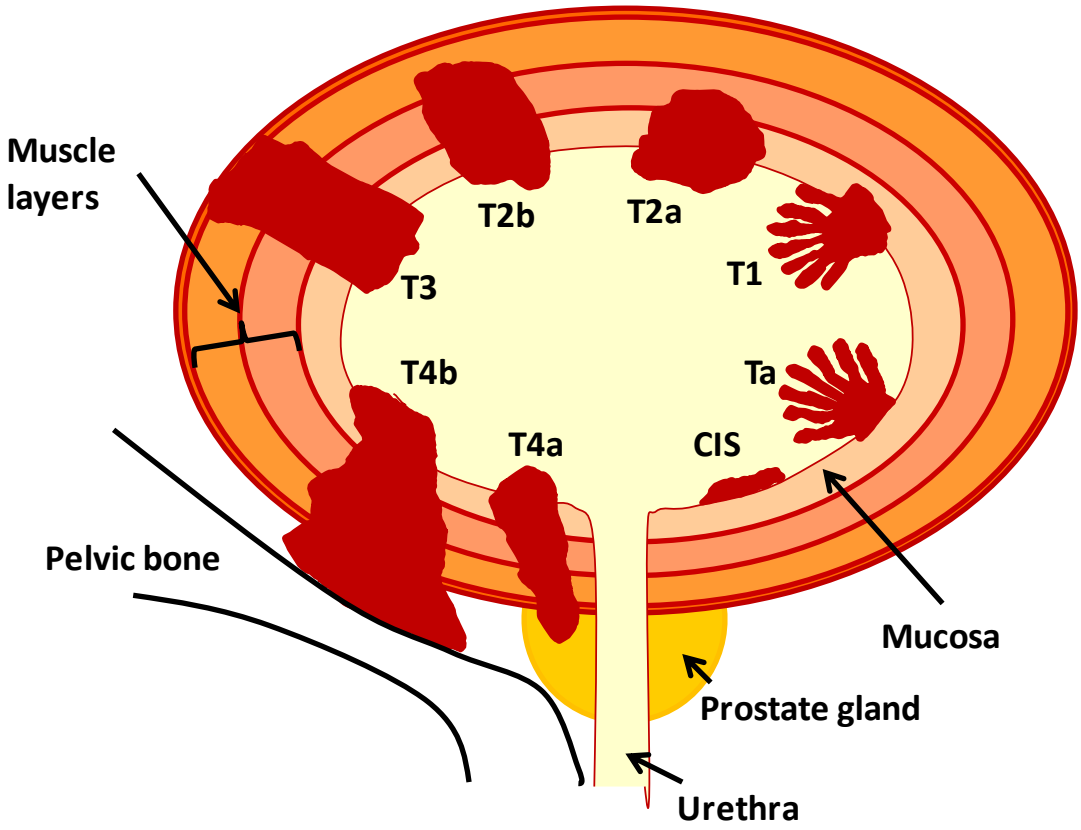
Definitions of TNM

Table A.1-1: classification of primary tumours

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma in situ: "flat tumour"
T1	Tumour invades lamina propia
T2	Tumour invades muscular layers
pT2a	Tumour invades superficial muscular layers (inner half)
pT2b	Tumour invades deep muscular layers (outer half)
T3	Tumour invades perivesical tissue
pT3a	Microscopically
pT3b	Macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumour invades prostatic stroma, uterus, vagina
T4b	Tumour invades pelvic wall, abdominal wall

The different grades of the tumours depending on the invasion are depicted graphically in Figure A.1-3.

Figure A.1-3: primary tumour stages



Depicted bladder where there are represented the different grades of the tumours depending on the invasion

Table A.1-2: grades depending on regional lymph nodes affection

Regional lymph Nodes (N) Regional limph nodes include both primary and secondary drainage regions. All other nodes above the aortic bifurcation are considered distant lymph nodes.	
NX	Lymph nodes cannot be assessed
N0	No lymph node metastasis
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node)
N2	Multiple regional lymph node metastasis in the true pelvis hypogastric, obturator, external iliac, or presacral lymph node)
N3	Lymph node metastasis to the common iliac lymph nodes

Table A.1-3: grades depending on the presence or absence of metastasis

Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Tables A.1-1 to A.1-3 adapted from the AJCC Cancer Staging Manual [14].

Anatomic stage/prognostic group

Table A.1-4: bladder tumours stage

Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a	N0	M0
	T2b	N0	M0
Stage III	T3a	N0	M0
	T3b	N0	M0
	T4A	N0	M0
Stage IV	T4b	N0	M0
	Any T	N1-3	M0
	Any T	Any N	M1

Table adapted from the AJCC Cancer Staging Manual [14]. T=primary tumour; N=Regional Lymph Nodes; M=Distant Metastasis; is (suffix)=Carcinoma in situ; no prefix=clinical classification, based data acquired on the clinical history, biopsy, surgical exploration, etc.; numbers=extent of the cancer.

If the grading system is not specified, the following is used:

Table A.1-5: bladder tumour grades

GX	Grade cannot be assessed
G1	Well differenciated
G2	Moderately differenciated
G3	Poorly differenciated
G4	Undifferenciated

Table adapted from the AJCC Cancer Staging Manual [14].

Histopathologic type

The most common histopathologic type is urothelial (transitional cell) carcinoma. The other histopathologic types are rare.

Non-Muscle Invasive Bladder Cancer

Non-Muscle Invasive Bladder Cancer (NMIBC), also known as superficial BC is a tumour confined to the mucosa (transitional epithelium and/or lamina propia). This means that no malignant cells are found in the muscle layer, the perivesical tissue or neighbouring organs. In the TNM staging, NMIBC is classified as one of the three stages: Ta, T1 or Tis [15]. At the time of diagnosis about 70% of patients present superficial tumours [16,17].

Histologic grade (G) of non-muscle-invasive bladder tumours

Histologic grade express the similarity between the tumour cell and the original epithelial cell. Since 2004, the WHO/International Society of Urologic Pathology (ISUP) recommendation is to use the grading system in their web site; the detailed histopathology of each grade is described there [18]. The aim of introducing this new grading system was to decrease the interobserver variability that existed with the previous system (WHO 1973). It has to be pointed out that WHO 2004 has been accepted without solid data about its predictive value, and the interobserver variability is still high [19]. Thus, there is much controversy about the clinical usefulness of the more recent grading system and more recent molecular grading systems will have to substitute those morphological systems [20–22].

The best reported way to determine a suitable treatment for each NMIBC patient is dividing the tumours into three risk groups depending on the expected prognosis. This classification, established by Millán-Rodríguez and collaborators, defines recurrence, progression and mortality rates in superficial BC [23].

Table A.1-6: classification to determine the suitable treatment for NMIBC

Low risk
Grade 1 stage Ta
Grade 1 stage T1, single tumour
Intermediate risk
Grade 1 stage T1, multiple tumours
Grade 2 stage Ta
Grade 2 stage T1, single tumour
High risk
Grade 2 stage T1, multiple tumours
Grade 3 stage Ta
Grade 3 stage T1
CIS

Table adapted from the original article of Millán-Rodríguez et al. [24].

A.1.4. DIAGNOSIS

One of the most common symptoms of BC is haematuria. Haematuria may be detected by a change in the urine colour and, if not, it can often be detected in the urinalysis of patients who undergo a general health check-up [1]. Unlike Carcinoma *in situ* (CIS), that may cause pain or LUTS, Ta and T1 are almost asymptomatic diseases [9].

When a patient is likely to present a bladder malignancy given their symptomatology, the first steps are to study the patient clinical history and make a physical examination, which

consists of assessing the primary tumour by bimanual examination under anaesthesia. But physical examination is useful when tumours are big enough so it does not reveal Ta, T1 or CIS.

Imaging techniques are also used for the evaluation of the patient: (1) IntraVenous urography (IVU), for which a special dye for x-ray is injected intravenously. This dye is removed from the body through the urinary system so this technique is used to detect filling defects that may be due to tumour masses obstructing the urethra; (2) a less commonly used technique is retrograde pyelogram which is similar to IVU but the dye is injected through a catheter placed in the urethra of the patient; (3) computed tomography (CT) gives information about size, shape and position of tumours located in the urinary tract and also in limph nodes and neighbouring organs but the patient undergoes higher radiation exposure; (4) Magnetic Resonance Imaging (MRI) scan is not only useful to detect urinary system tumours, but also can reveal tumours in the neighbouring organs; (5) ultrasound is often used to initially examine the urinary tract; (6) white light cytoscopy (WLC) is crucial for the detection of papillary or CIS tumours which cannot be detected with the other imaging methods. However, it should only be performed if the tumour has not been detected with any of the other imaging methods because patients will undergo TransUrethral Resection (TUR) regardless [25]. TUR will be explained in detail in Section A.1.5.1. To perform a cytoscopy a flexible tubular instrument with a video camera on the tip, a cytoscope, is introduced through the patient's urethra. Findings need to be described accurately. Recently, improved imaging techniques have been developed, for example narrow-band imaging or fluorescence cytoscopy which is significantly more sensitive than WLC in the detection of CIS, low-grade papillary lesions and multifocal NMIBC [9,19].

Other relevant techniques might be combined with the already mentioned. Urinary cytology is the analysis of the voided cells in the urine of a patient; it is a good technique for the detection of HG tumours, such as CIS but it has low sensitivity in LG tumours [9]. Ideally, detection of urinary molecular markers would be useful to detect LG tumours in order to compensate for the limitations of cytology. Although many markers have been studied none of them are currently accepted as standard for diagnosis or follow-up. Moreover, random biopsies are recommended when a patient presents a normal cytoscopy but an abnormal urinary cytology in order to detect CIS.

TUR of Ta and T1 bladder tumours has two purposes: the treatment and the elaboration of the subsequent pathological report of the tumour. It is important to resect the tumour plus a part of the surrounding muscle tissue to grade the tumour invasivity [19]. A detailed pathological report is key to choosing a proper treatment for and to determining the prognosis of the patient.

A.1.5. TREATMENT FOR BLADDER MALIGNANCIES

Usually, the first step to treating BC is surgery (TUR). Radical and partial cystectomy, that mean, removing the entire bladder or part of it is recommended when the tumour is invasive or when tumours are recurring [9]. When there is only one tumour and it is not very large, partial cystectomy should be enough. The main side effect of this surgery procedure is the reduction of capacity of the patient's bladder. When the tumours are larger or there are many of them, the entire bladder is removed (radical cystectomy). Moreover, the limph nodes and the prostate (in men) and the ovaries, fallopian tubes, uterus and a small portion of the vagina (in women) might also be removed [1].

The second step is often systemic therapy or local therapy. In the case of invasive BC, high stage or metastatic diseases it is better to receive drugs systemically, usually into a vein or in a pill form, while, local therapy is recommended to NMIBC patients because it is not able to reach deeper layers of the bladder, [26,27].

Intravesical therapy

The local therapy, in which only a part of the body is in contact with the therapeutic agent, in this case the bladder, is called intravesical therapy and each dose is called intravesical instillation.

The bladder is the perfect organ for local therapy because the access is relatively easy through a catheter and for all the characteristics mentioned in Section A.1 [28].

Table A.1-7: intravesical therapy compared to systemic therapy

Intravesical therapy	Systemic therapy
Directly reaches the target	The drug is present in all the body
Higher exposure to the drug	Only a few amount reaches the target
The drug is diluted in the urine	Requires large doses of the drug
The drug is voided along with urine	Undesired distribution of the drug
Minimizes side effects	Higher side effects
The bladder has low permeability	There is no barriers
Repeated instillations are needed	Do not imply catheterization

Adapted from the original article of GuhaSarkar, S. & Banerjee [28]

A.1.5.1. Treatments for non-muscle-invasive bladder cancer

Since NMIBC is a heterogeneous disease, the clinicians have the difficult task of choosing an appropriate therapy for each patient. As said before, TUR is the first treatment for BC patients. TUR is a similar procedure to cytoscopy, but this time the cytoscope is rigid and in addition to

the video camera, it has a wire loop at its tip to remove tumours. This instrument is called resectoscope. For this operation, the patient is under general or local anaesthesia [1].

TUR is usually combined with laser or fulguration which are procedures similar to cauterization. Often, those techniques are used in the base of the tumour to eliminate remaining tumour cells. Small tumours can be burned with laser or fulguration but is not recommended as, unlike TUR, the tumour tissue cannot be examined by a pathologist [9,17].

However, in addition to TUR, further therapies are needed due to the unacceptably high rate of recurrence [29]. In the following five years after undergoing TUR, between 30-80% of BCs will recur and 1-45% will progress to more invasive stages within 5 years [19,30] Table A.1-8, that has been adapted from the work of Millán-Rodríguez *et al.,* compiles recurrence, progression and mortality rates 1 year and 3 years after TUR in each risk grup.

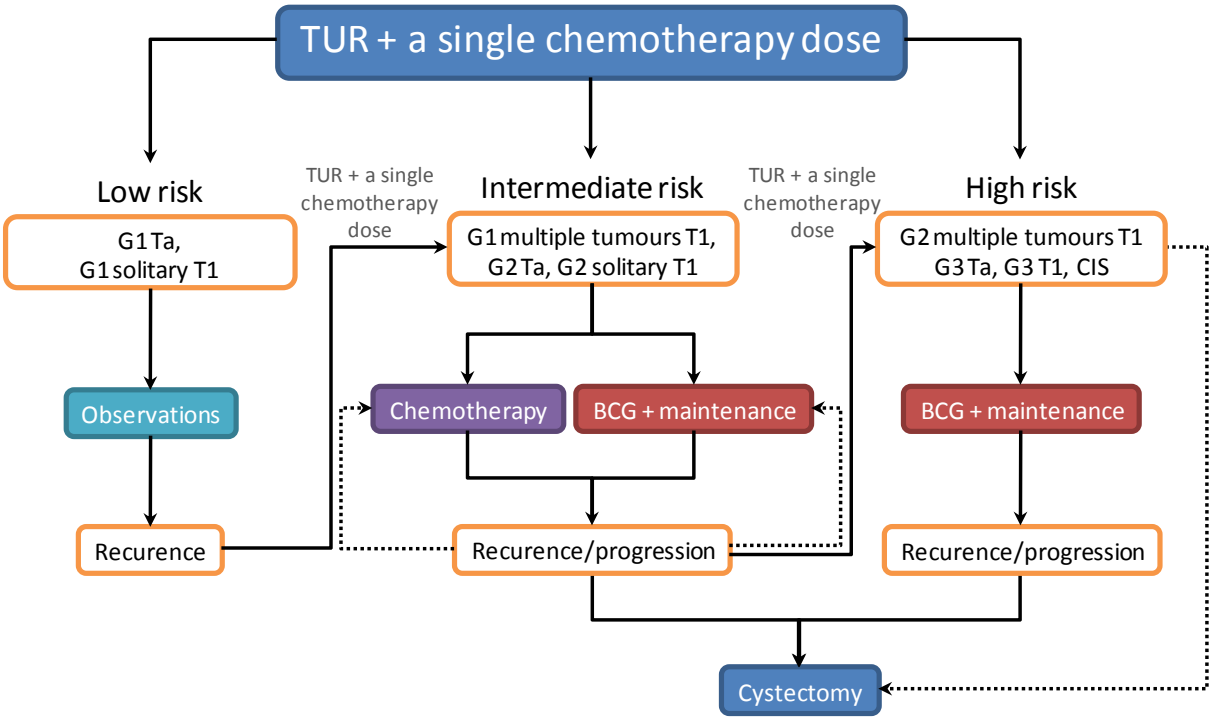
Table A.1-8: risk of recurrence, progression and mortality

Risk	**% recurrence**		***% progression***		***% Mortality***	
	1 yr	**3 yr**	**1 yr**	**3 yr**	**1 yr**	**3 yr**
Low	15	30	-	-	-	-
Intermediate	*26*	*45*	*0.4*	*1.8*	*0.4*	*0.7*
High	*39*	*56*	*8*	*16*	*1*	*7*

Table adapted from the original article of Millán-Rodríguez et al. [24].

According to these data treatment options for the three risk groups were determined. Figure A.1-4 shows a schematic diagram of the appropriate treatments for NMIBC. Thus, EAU Guidelines recommend TUR followed by a single dose of intravesical chemotherapy as the initial step for the treatment for any risk group of NMIBC because several studies have revealed it diminishes recurrence rates as compared to TUR alone [19,29,31]. It has been described that a single dose of local chemotherapy with either epirubicin or mitomycin C (MMC) prolong recurrence free interval (RFI) and reduce early recurrences (ERs) [32,33]. However, further studies are necessary with better randomization and blinding to evaluate the usefulness of this therapy in NMIBC patients [19,32]. However, when TUR results in inter or intraperitoneal perforation or there is suspicion of it, this treatment is not recommended [9,34]

Figure A.1-4: treatments for NMIBC based on risk groups



Adapted from the original article of Lamm, D. L. et al [35].

The following steps depend on the risk group. Low risk NMIBC patients only need to be periodically examined in order to detect any sign of recurrence, while higher risk patients need specific treatments, called adjuvant treatments, to avoid recurrences, tumour progression and mortality (see Figure A.1-4) Due to the high rate of recurrence, follow-up strategy must be well defined and patients might need repeated treatments.

Some patients require posterior TUR. When muscularis has not been obtained in the first TUR, if a second TUR is not performed, the tumour is understaged in more than 50% of cases [17]. It is also recommended in all T1 and high risk NMIBC patients except primary CIS [9]. Furthermore, when the tumour is not small enough to be removed all at once or, even after a successful treatment, cancer recurs.

A.1.5.1.1. Further chemotherapy

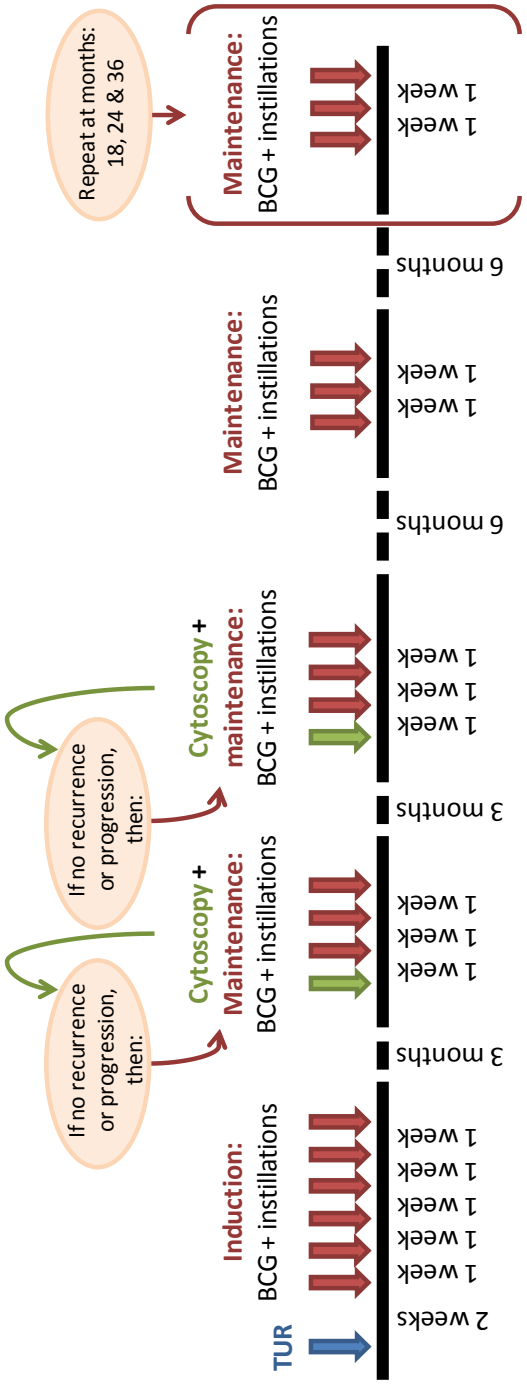
Chemotherapy can be administered intravesically or systemically. There are many chemotherapeutic agents in use on the market. Drugs are used alone or in combination. The most commonly used for intravesical treatment are MMC and thiotepa [1].

A.1.5.1.2. BGC immunotherapy

Cancer immunotherapy is a kind of therapy in which the agent does not directly target the tumour cells but it incuces the activation patient's immune system to work in order to help to cure the tumour.

In the case of BC, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), an attenuated bacterium, is administered intravesically, in its live form, into the bladder of some intermediate and high risk BC patients.

Figure A.1-5: southwest Oncology Group's schedule for BCG induction and maintenance



Recently, A M Kamat and other expert urologists reach a consensus on the appropriate treatment for NMIBC based on several clinical trials and their own personal experience. The treatment of choice for high risk NMIBC patients should be the protocol designed by the Southwest Oncology Group (SWOG) with some modern modifications [36,37]. This protocol has two parts: induction and maintenance (continued treatment). In detail, the induction is a course of six weekly BCG instillations and the BCG maintenance consists of three weekly instillations at 3, 6, 12, 18, 24, 30 and 36 months after TUR [36]. In Figure A.1-5 the schedule designed by the SWOG is outlined.

The efficacy of BCG for the treatment of NMIBC will be assessed in detail in Section A.2.3.

A.1.5.1.3. Partial and radical cystectomy

As Figure A.1-4 shows, some intermediate and high risk patients undergo cystectomy. Immediate cystectomy after TUR is recommended to high risk NMIBC patients likely to experience disease progression to muscle-invasive BC and also when bladder is not functioning. Delayed cystectomy is performed in NMIBC patients after BCG failure [9]. Excellent disease-free survival is achieved when radical cystectomy is performed at the time of diagnosis but more than 50% of patients are overtreated.

The case of CIS

Although CIS is considered high risk NMIBC the way to treat it is controversial. There is no consensus in whether CIS should be treated with intravesical adjuvant treatment or with radical cystectomy. Radical cystectomy is effective in terms of survival rates but, again, half of patients might be overtreated [9]. Retrospective studies show that 48% of patients who underwent intravesical chemotherapy showed complete response to treatment and 72-93% with BCG, so cystectomy might be avoided [9].

A.2. MYCOBACTERIA AND CANCER

In this second section, the relationship between mycobacteria and cancer will be addressed with special focus on BCG and BC.

A.2.1. *MYCOBACTERIUM*

The genus *Mycobaterium* is the only member of the family of *Mycobacteriaceae* within the order *Actinomycetales* [38].

Mycobacterium is comprised by acid-fast aerobic bacilli with high G+C genomic DNA content that usually form curved or straight nonmotile rods. *Mycobacterium* are mycolic-acid producers, like *Nocardia* and *Corynebacterium* which also belong to *Actinomycetales* [38]. In the taxonomic

classification of this genus two groups of species can be differentiated: rapid and slow growers. This genus presents variety of colonial morphology and among the rapid growers, pigmentation ranges from light yellow to orange [39].

The one-of-a-kind mycobacteria's characteristic is the high lipid content of their cell wall which represents more than half of the dry weight of the cells. For this reason mycobacteria are highly hydrophobic and lead them to form clumps [40].

In 2015, 170 species and 13 subspecies are included on the genus *Mycobacterium* [41]. Based on their clinical impact are classified in:

M. tubereculosis complex: in this group all mycobacteria are able to cause TB in human and other genus are included. Some examples are *M. tuberculosis*, *M. cannettii*, *M. bovis* and also BCG.

Mycobacterium leprae complex: this group includes *M. leprae* and *M. lepraemurium* responsible of lepra disease in human and in rodents, respectively.

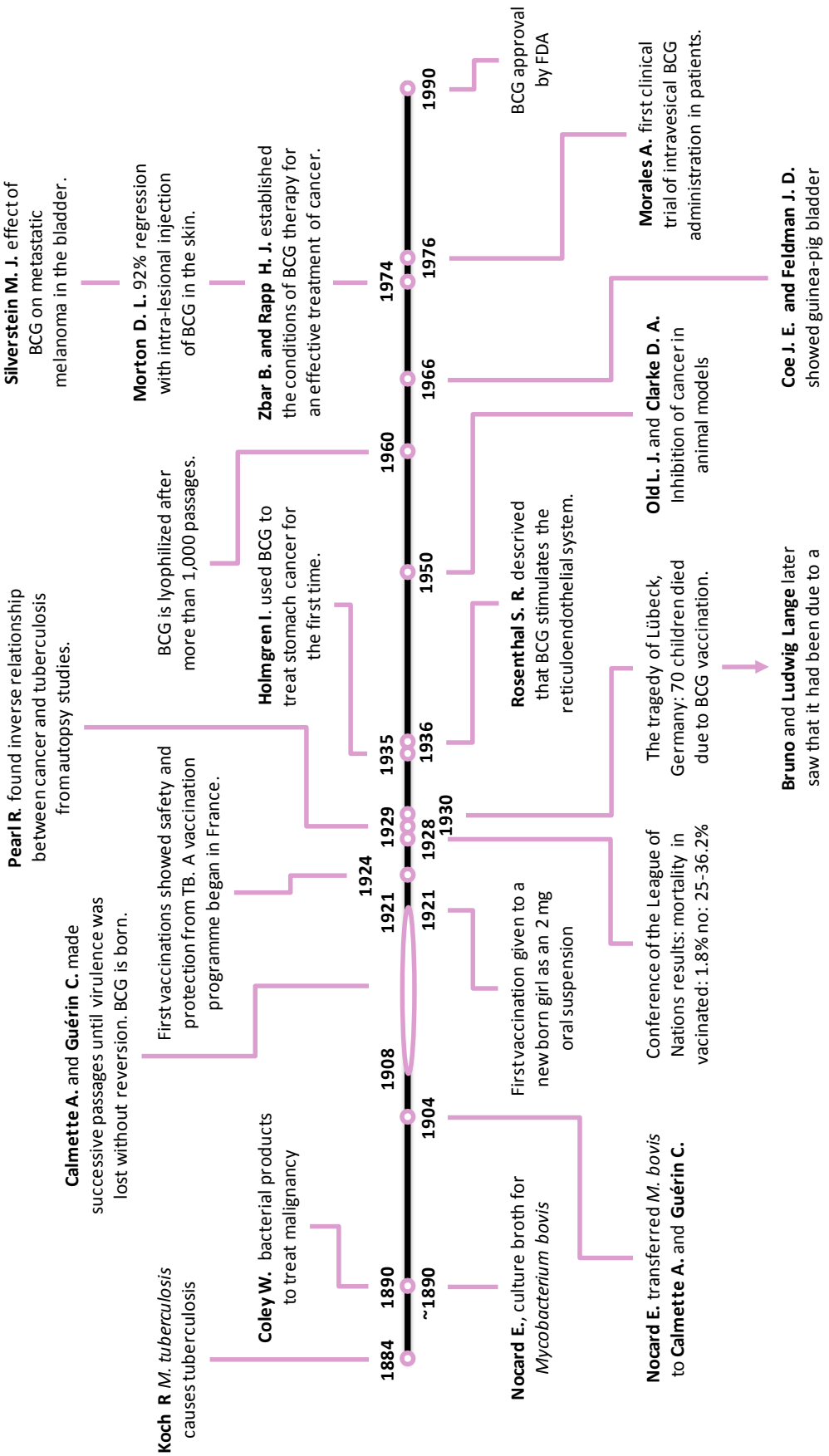
Non-tuberculous, atipic or environmental mycobacteria: this group includes the rest of *Mycobacterium* species. Low or lack of virulence is the main characteristic of non-tuberculous mycobacteria. Their natural habitats are usually soil, fresh water and salt water, among others.

A.2.2. HISTORY: BEYOND THE CURRENT SCOPE

In the previous section, the use of BCG for the treatment of NMIBC, has been briefly described. However, the question of how an attenuated form of the slow-growing *M. bovis*, which is responsible for tuberculous disease in humans and in bovine and other genus, is the most widespread treatment for NMIBC is a matter of history (see Figure A.2-1).

Once *M. tuberculosis* (MTB) was described by Robert Koch as the agent causing tuberculosis (TB) in humans, scientists at the end of the IX century raced to discover a vaccine against the disease [42]. E. Nocard isolated *Mycobacterium bovis* from the milk of a heifer and, in 1904, gave this strain to Dr. Albert Calmette and Dr. Camille Guérin who worked at Pasteur Intitute in Lille. In 1908, Calmette observed that low doses of the extremely virulent *M. bovis* were unable to produce the disease in bovine so he deduced that the presence of bile in the culture media composition attenuated the strain [43]. Thus, from 1908 to 1921, Calmette and Guérin made 231 successive passages of the original virulent strain over bile-potato medium every three weeks. In 1921 at the *Academie des sciences*, they presented a new strain of *M. bovis* with demonstrated avirulence without reversion in a guinea pig model: *M. bovis* Bacillus Calmette-Guérin abbreviated as BCG [42,44].

Figure A.2-1: the history of BCG for the treatment of BC



This same year, the first newborn girl was vaccinated with three 2 mg BCG doses during the first week of her life. Close follow-up of the first newborns who received vaccinations showed safety and protection from TB, so an official vaccination programme began in France and it rapidly spread to the rest of Europe. Mortality due to TB in vaccinated people diminished to 1.8% while in non-vaccinated control it was 25-36.2%. These findings were presented in 1928, at the Conference of the League of Nations in Paris.

However, in 1930, an unfortunate incident happened in Lübeck, Germany. Around 70 of 249 vaccinated children died. It was not until Bruno and Ludwig Lange investigated the case, that it was discovered that some live bacillus of *M. tuberculosis* had ended up in the final preparation of the vaccine lot by mistake [42,43,45]. Sadly, Calmette, who died in 1933, never knew that the deaths had been due to an exceptional contamination [42,45]. Obviously, the tragedy of Lübeck created such distrust in BCG that it would take several decades restore the faith in BCG again [42].

A series of observations by different scientists led to the idea that BCG could play a role in the treatment of malignancies. Years earlier, in the 1890s, in New York, Dr. William B. Coley focused his interest on a cancer patient who was cured of cancer thanks to a fever episode. He hypothesized that if he was able to induce an infection-like symptomatology to a cancer patient, the patient might be cured. He developed a vaccine made of killed *Streptococcus pyogenes* and *Serratia marcescens*. This vaccine was called 'Coley's Toxins'. His first success was with a patient bearing an inoperable sarcoma [46].

In an autopsy study at the Johns Hopkins Hospital in Baltimore, the brilliant scientist Dr. Raymond Pearl revealed significantly lower incidence of cancer in TB caused deceased compared to the control group [42–44].

The first one to employ BCG for the treatment of cancer was I. Holmgren. In 1935, he reported success in 35 stomach cancer patients and by 1960 he had already assayed BCG efficacy in melanoma, leukaemia, colon and lung cancers [27,42]. Later, other approaches were tried, for instance in 1936 S. R. Rosenthal observed that BCG stimulates the macrophage system, known at that time as the reticuloendothelial system [42]. In 1944 EC Armstrong developed the first BC animal model. Moreover, in the 1950s, S. R. Rosenthal described lower leukaemia incidence in people immunized with BCG at the time of birth. In that period, animal studies, performed by L. Old and D. A. Clarke at the Sloan-Kettering Institute in New York, showed that intravenous BCG made mice more resistant to tumour transplantation [44,47].

BCG was not lyophilized until the perfection of the technique in 1960, so BCG needed to be maintained by continuous serial passage [36,48]. This fact led to the formation of many multiple

daughter strains. Six years later, J.E. Coe and J. D. Feldman showed that guinea-pig bearing BC tumours respond to BCG [27,42,44].

1974 was a year of discoveries: first, D. L. Morton found a 92% regression of tumours with intralesional injection of BCG in the skin; second, M. J. Silverstein lead by Morton also found this tumour regression in metastatic melanoma to the bladder; third, B. Zbar and H. J. Rapp established which were the optimal conditions of BCG therapy for an effective treatment of cancer [42–44].

At that moment, clinical trials using BCG for the treatment of lung, prostate, colon and kidney cancers rushed [44]. However, advances in chemotherapy and radiotherapy left aside BCG immunotherapy in all cancer types besides BC [27,42].

In 1976, the Canadian urologist Alvaro Morales published the results of clinical studies evaluating intravesical BCG administration in patients with BC [49]. He established the current induction protocol of BCG for high risk NMIBC patients [27,42]. Finally, it was not until 1990, when local and systemic adverse effects were described, that BCG received Federal Drug Administration (FDA) approval for the treatment of NMIBC [50].

Nowadays, BCG remains the standard treatment for NMIBC.

A.2.3. BLADDER CANCER AND BCG

Before introducing alternative treatments to BCG for the therapy of NMIBC it is necessary to understand why it is important to develop new therapies, which are the known mechanisms of action of BCG and which attempts have been assayed for the replacement of the current therapy for superficial BC.

A.2.3.1. Positive and negative effects

Many factors have varying influence in the outcome of BCG intravesical treatment, for instance: tumour's grade, risk of recurrence and/or progression, BCG dosage and treatment duration, if the patient undergoes only BCG induction treatment or induction plus maintenance, if the patient has been previously treated with chemotherapy after TUR, Purified Protein Derivative (PPD) test status of the patient, age, genetic background, etc. BCG strain needs a special mention because, recently, it has been demonstrated *in vitro* [51] and *in vivo* [48,52] to have huge variability in their efficacy for the treatment of NMIBC,

So it is difficult to achieve consensus between the different clinical trials performed along the past years and along many countries. However, overall, some positive and negative effects of BCG therapy seem to be clear.

A.2.3.1.1. Positive effects

BCG exerts two positive effects in intermediate and high risk NMIBC patients: BCG reduces recurrence and progression compared with TUR alone [53,54]. Many meta-analyses have stated that TUB followed by chemotherapy is not as good as TUR plus BCG induction and maintenance treatment for preventing recurrence of high risk NMIBC tumours. Clinical trials compared in the above cited articles differ in many factors but what it is clear is the long lasting effect of BCG in the reduction recurrence risk and the elongation of the RFI.

Moreover, for intermediate risk BC patients it is not clear the superior prophylactic effect of BCG on recurrence compared to MMC [50]. That is why the EAU guidelines recommend both, BCG treatment or further chemotherapy and allow the physician, together with the patient, to make the decision/choice [9].

As progression occurs in a low percentage of patients, it is more difficult to declare that BCG prevents progression to a higher tumour stage or the development of metastatic cancer compared to chemotherapy. Two systematic reviews [53,55] and an individual patient meta-analysis [50] concluded that BCG prevents tumour progression. However, while Böhle, *et al.* and Sylvester, *et al.* found statistically significant superiority of BCG compared to MMC, if BCG maintenance therapy is provided; Malmstrom, *et al.* did not find statistical differences; that may be because Malmstrom, *et al.* reanalysed the individual patient data of nine studies with different treatment protocols.

A.2.3.1.2. Negative effects

From the above discussion, it can be deduced that BCG maintenance is necessary to have a better prognosis but unfortunately BCG associated toxicity increases with additional doses.

Most patients tolerate and respond well to BCG, but practically all of them experience mild side effects and many of them serious and potentially fatal toxic effects. Once overcome the first year, the patients that are able to continue with the treatment, they usually tolerate well BCG therapy [56,57]. However, it is mainly in the first year of therapy, when patients are advised to stop the treatment. For these cases, the alternative treatment is usually radical cystectomy but the quality of life of the patient is very low (see Section A.1.5.1.3). To avoid removing the bladder, there are some less toxic treatments than BCG but also less efficacious [58].

Problems associated to BCG therapy will be addressed in this section. After the second or third instillation, as a result of the generated immune response, patients suffer adverse effects. The most common are frequency, cystitis and dysuria, and fever [27,42]. This symptomatology is associated with a subsequent low recurrence [59]. Other common side effects are haematuria, other irritative bladder symptoms, granulomatous prostatitis, epididymo-orchitis, flu-like

symptoms and some allergic reactions. More rare complications are uretral obstruction and contracted bladder [56].

Traumatic catheterisation is bound to intravenous absorption of BCG by the patient and may lead to severe reactions or BCG infection. When BCG sepsis occurs, some steroids and antituberculosis drugs are orally or intravenously administered to avoid patient death [42,56]. Maintenance therapy increases the risk of infection.

Based on the severity of the side effects physicians might decide whether to postpone the instillations or definitively stop them [56]. For example, BCG is also contraindicated to patients who are immunosuppressed, suffered BCG sepsis, have gross haematuria or active urinary tract infection [27].

In daily practice BGC failure is defined as 1) patients that have to stop BCG treatment due to toxicity, BCG intolerant patients, 2) BCG resistance which means that further BCG has to be instilled to the patient, 3) tumour recurrence after an effective BCG therapy, called BCG relapse, and 4) the disease persist after induction and the first maintenance course (BCG-refractory BC). Physicians have to be especially careful with high risk T1 and CIS patients that have an increased potential to progress to muscle invasive BC [17,36].

Many clinical trials have tested some options to avoid or to mitigate BCG toxicity. However, these strategies are highly related to lower efficacy of the therapy [60]. In general, the first steps to lower BCG toxicity are to inform properly the patients about the potential adverse effects of the therapy, and to train nurses and doctors to instillate well and to make a correct manage of patient side effects [56,61].

A.2.3.2. Mechanism of action

As seen before, BCG for the treatment of high risk NMIBC has been in use for nearly 40 years. However the mechanism of action of this immunotherapeutic agent is still under investigation. In this section, the existing knowledge regarding this issue is summarized.

A.2.3.2.1. Normal urothelial and BC cells role

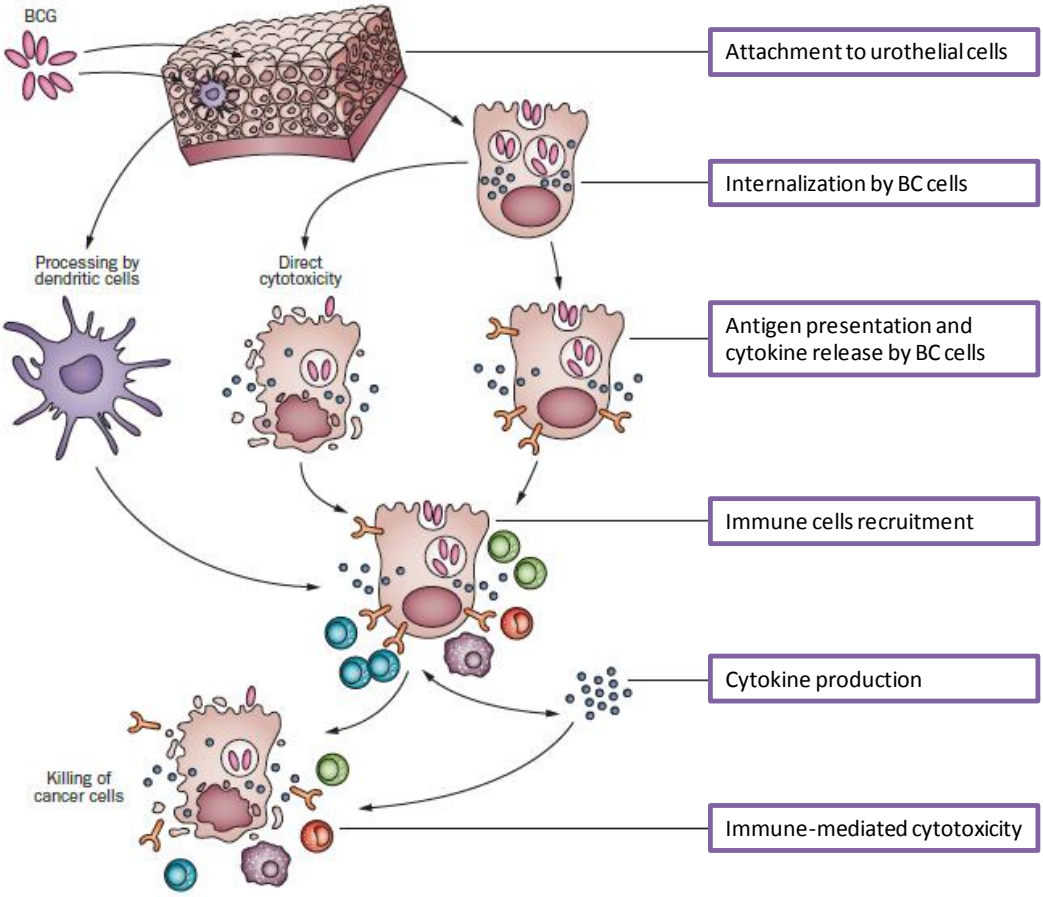
The first step for BCG therapy is the attachment of the mycobacterium to the bladder cell wall. This union is not favoured by the hydrophilic environment on the bladder wall, which as seen in Section A.1 has an impermeability function. First, BCG seems to accumulate near the bladder wall without adhering [62] because both, BCG and the luminal side of the bladder are negatively charged so they repel each other. Tumour cells synthesize less mucin layer than normal urothelium so the access of certain therapeutic agents, including BCG, is facilitated [63]. Second, it is been observed *in vitro* that BCG's Fibronectin Attachment Protein (FAP) attaches to the integrin $\alpha_5\beta_1$ (fibronectine receptor) of the tumour cell through fibronectin [64,65]. This

interaction may be also mediated by the Toll-Like Receptors (TLRs)-2, 4 and 9 which are not only expressed on some immune cells, but they are also expressed on normal urothelium and on BC cells [66]. Third, BCG is only internalized by high-grade BC cells but not by low grade or non-malignant urothelial cells [67,68]. BCG internalization and BCG interaction with TLR drives to the release of several cytokines and other molecules that activate the immune system [69–71].

A.2.3.2.2. Immune system role

As mentioned, BCG mechanism of action for tumour clearance implies the activation of the patient's immune system, both at a local level, in the bladder, and at a systemic level. When BCG interacts with the bladder wall, exerts a direct cytotoxic effect on BC cells and also a complex response is generated. That includes the recruitment and activation of different immune cells subsets that release of cytokines and chemokines that in turn gather other cell subsets and all together induce the death of the BC remaining cells (see Figure A.2-2). This response has to be strong enough to clear the tumour however an overactive response might be a reason to stop the treatment [27]. In this section it will be reviewed the knowledge about the immune response to BCG BC therapy elucidated in *in vitro* studies using human cells or in clinical trials.

Figure A.2-2: immune response generated by BCG during the treatment of BC.



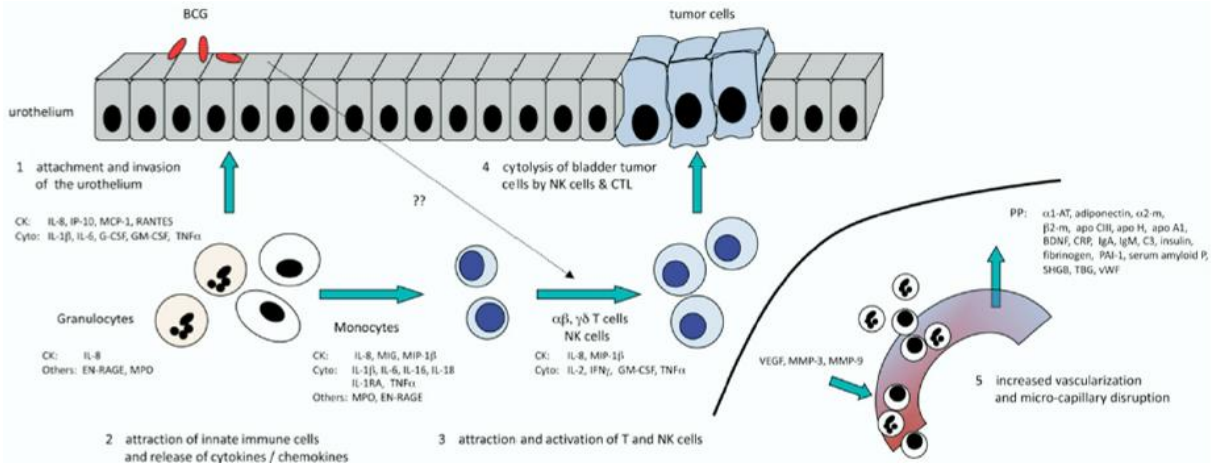
Adapted from the original article of Redelman-Sidi, G [72].

A.2.3.2.2.1. Local response

Few hours after BCG instillation proinflamatory cytokines and chemokines are released and detected in patient's urine including interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-18, interferon-γ-inducible Protein-10 (IP-10), Granulocyte-Colony-Stimulating Factor (G-CSF), Granulocyte Macrophage-Colony-Stimulating Factor GM-CSF, Monocyte Chemoattractant Protein-1 (MCP-1), Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) and Tumour Necrosis Factor-α (TNF-α) [73,72,74]. These molecules trigger an increase in the number of leukocytes present in the patient's urine, these include granulocytes and a less amount of macrophages [72,74–77].

In the following instillations a stronger response is triggered and it is not only a more powerful innate response but also implies acquired immune response [74].

Figure A.2-3: early response after BCG administration in the BC patient's bladder



Adapted from Bisiaux, A. et al. [74].

The role of macrophages

Macrophages are the usual host for mycobacteria which are internalized by fagocytosis [78]. Regarding the role of the BCG-activated macrophages more investigation has to be performed in order to unravel their role *in vivo*. However, many *in vitro* studies have been published. For instance, Pryor *et al.* showed that the cytotoxicity of BCG-activated macrophages was mainly mediated by the released soluble factors [79].

The role of dendritic cells

Similarly to macrophages, several *in vitro* studies have assessed the role of dendritic cells however studies implying patients are still lacking. These immune cells might ingest BCG and present its antigens to T lymphocytes that would trigger the release of cytokines, like IL-12 and

TNF-α, and chemokines able to attract more T lymphocytes, γδ lymphocytes, Natural Killer (NK) cells and neutrophils that are cytotoxic to the T24 BC cell line [27,80,81]. (see Figure A.2-3) [27].

The role of neutrophils

Due to the presence of IL-8, that is believed to be secreted by BC cells [82] neutrophils influx into the bladder, however they are only highly detected after several BCG instillations [66,83]. Neutrophils secrete granule proteins and Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL), as well as, other chemokines that recruit other immune cells, such as monocytes [83,84]. Moreover, these recruited cells would release interferon (IFN) that, in turn, would recruit T cells and macrophages, and induce the expression of TRAIL on the surface of T cells and the release of soluble TRAIL by neutrophils. Overall, this cascade would result in a high concentration of TRAIL in the urine which would specifically induce apoptosis to tumour cells by binding to TNF-family death receptors [83,85].

The role of lymphocytes

Although most of the lymphocytes present in the urine of the patients are Cluster Differentiation 4^+ ($CD4^+$) T cells; $CD8^+$, NK and B cells are also found. It has to be pointed out that only after the third instillation, these populations influx massively in the patient's bladder [74,76]. Despite $CD4^+$ T cells are the predominant lymphocytes, both $CD4^+$ and $CD8^+$ T cells are required for an effective BCG therapy. T cells, mainly, $CD4^+$ are found in the bladder wall for months after BCG instillations [72,86]. Since BCG make that BC cells express higher amounts of MHC class II molecules in their surface [87,88], it makes that more mononuclear cells, in particular, macrophages, T and B cells form granuloma structures [89,90].

The activation of both Th1 and Th2 immune responses is essential for a proper tumour clearance [91]. On the one hand, Th1 response is characterized by the production of cytokines, such as IFN-γ, IL-2 and IL-12 that trigger the development of cellular immune responses. And on the other hand, Th2 response implies the synthesis of cytokines, for instance IL-4, IL-5, IL-6 and IL-10 that favour the humoral immunity (antibody production) [91,92].

The battery of cytokines mentioned at the beginning of this section, cannot be attributed to Th1 or Th2 response but the presence of IFN-γ, IL-2 and IL-12 plus the absence of IL-4 correspond to a Th1 response. It seems that BCG therapy induces a shift from Th2-like to Th1-like response necessary for and effective treatment [72].

The role of Natural Killers (NK)

BCG and the presence of the Th1 cytokines IL-2, IL-12 and IFN-α activate NK cells (BCG-Activated Killer, BAK). BAK cells are called like this because present cytotoxicity for BC cells

mediated by perforin and IFN-γ [93]. However, the Th2 antinflamatory cytokine IL-10 inhibits the activation of NKs [43,94]. BAKs are able to distinguish between BC cells and cells from the normal urothelium because those have lower levels of Major Histocompatibility Complex class I (MHC-I) [94].

A.2.3.2.2.2. Systemic response

BCG therapy not only activates local immune response but it also induces a systemic immune response. BCG instillations make that more than 40% of patients experience conversion to positive tuberculin skin test (Purified Protein Derivative test, PPD test).

C Biot *et al.* observed that previously subcutaneously immunized mice with BCG showed improved survival after BCG therapy compared to naive mice. Moreover, positive PPD patients showed better recurrence-free-survival rates than negative PPD patients [95]. More than 40% patients who are PPD test negative at the beginning of the BC treatment with BCG convert to positive during the instillations and those who convert present a better prognosis of the BC [96]. Furthermore, specific anti-BCG immunoglobulines (IgG and IgA) are found in patients' urine and serum during the therapy [97].

A.2.4. Alternatives to the treatment for NMIBC

As it has been already seen in Section A.2.3.1, BCG is the most effective treatment for NMIBC regarding the prevention recurrence and progression to more invasive stages. However, in the same section the drawbacks of the therapy have been laid out.

Thus, clinicians agree that safer alternatives to live BCG are needed. In fact, as soon as BCG therapy was proposed, many studies attempted to find agents as efficacious as BCG but safer than BCG. A better understanding of the mechanisms of mycobacteria for the treatment of cancer could lead to the elimination of live BCG in BC therapies or, at least, lowering the doses.

The approaches described below are attempts to avoid the use of live BCG.

A.2.4.1. Immunotherapy using macromolecules

Many researchers have tried to use several different macromolecules for the treatment of NMIBC with different degree of success. To cite some: (1) IFN-α is the most successful one, it has been tested in some phase I and II clinical trials and it has a promising effect in avoiding recurrences [98,99] but worse than BCG. (2) Other interleukins, as mentioned in Section A.2.3.2.2, have a crucial role in the BCG therapy, so it has been studied a possible antitumour effect by themselves in clinical trials [100]. (3) Hemocyanin, which is a glycoprotein purified from *Megathura cranulata* (a mollusc), has been tested in phase I and II clinical trials as intravesical therapy [101,102]. (4) Thanks to recombinant DNA technology, a fusion protein was generated from

the transforming growth factor alpha and a 40-kDa modified *Pseudomonas* exotoxin (TP40). This hybrid protein is specifically internalized by tumour cells and kills them [103]. M. Duchek and coworkers tried to replace BCG by a combination of interferon-α2b and epirubicin. They found that BCG is more effective in preventing recurrences than this combination. Moreover, no differences between the two treatments were found regarding progression or toxicity [104].

A.2.4.2. Dose reduction

The most wide spread BCG induction plus maintenance schedule is based on the Southwest Oncology Group regiment (see Figure A.1-5). As this schedule implies many instillations some authors have tried to reduce the dose in each instillation or to reduce the maintenance time.

J Oddens *et al.* and JA Martínez-Piñeiro *et al.* assayed one-third BCG dose versus full dose in order to reduce BCG toxicity along maintenance [60,105]. On the one hand, J Oddens *et al.* did not found differences in toxicity between the doses and they did found that full dose 3 years maintenance reduced recurrences [60]. And on the other hand, JA Martínez-Piñeiro *et al.*, who used a shorter schedule than J Oddens, found a significant decrease in toxicity with the reduced dose. However, they still recommend the full dose for high risk patients and the one-third dose for intermediate risk patients [105]. J Oddens *et al.* also studied the effect on toxicity and on tumour recurrence of one year maintenance versus three years (SWOG protocol). They conclude that the full dose shorter schedule should be followed by intermediate risk patients because they found no benefit in prolonging the treatment for these patients.

Another attempt to reduce toxicity has been published this year by L Martínez-Piñeiro *et al*. They changed the maintenance schedule used by J Oddens *et al.* by a single BGC instillation instead of three during maintenance. They did not observed differences regarding recurrence and progression rates compared to induction therapy alone [106]. However, they did not compare their alternative protocol to SWOG protocol.

R Järvinen, et al. assayed alternating doses of BCG and IFN-α2b in order to reduce the number of BCG instillations. Again they failed in demonstrating superiority in terms of recurrence [107].

Moreover, combination therapy of BCG plus IFN-α-2b has been assayed in many clinical trials, also in an attempt to lower the dose and improve the therapy [108–111].

A.2.4.3. Killed mycobacteria

When talking about killed mycobacteria, one refers to non-dividing forms. As seen in Section A.2.2, in the 1890s Coley observed 'Coley's Toxins' made sarcomas regress. Since then, other killed bacteria have been tested to treat cancer.

There are many ways to obtain bacilli in this form. Kreider *et al.* assayed BGC inactivated by several ways: heat, sonication, gamma-irradiation and using antibiotics, on the 13762A rat mammary adenocarcinoma and the line 10 guinea pig hepatoma. They found that h-k and irradiated BCG had a strong effect in both animal models [112]. In contrast, several authors assayed h-k BCG [113,114] and sonicated BCG [115] but these approaches showed no better efficacy than live BCG. The following subsections make special mention of heat- and irradiation-killed mycobacteria for the treatment of cancer.

A.2.4.3.1. Heat-killed mycobacteria

Heat-killed (h-k) BCG has been assayed in many studies for the treatment of cancer. In a mouse animal model repeated intravesical instillations of h-k BCG resulted in a proper Th2 activation but no Th1 cytokine response was induced [92]. Moreover, they obtained favourable results *in vivo* in an orthotopic mouse BC model in which in the first three instillations animals received intravesical live BCG and in the other three h-k BCG in an attempt of lowering the dose. They observed a proper Th1 activation that may lead to the clearance of tumours [92]. These results reinforce those presented by D. R. Kelley *et al.* that correlated BCG low viability with therapy failure [116]. Thus, these studies conclude that at least at the first instillations, live bacteria are needed to obtain a favourable immune response [92,95].

At the beginning of the 80ies, *Mycobacterium smegmatis* seemed to be a promising agent for the treatment of cancer, based on the studies of E. Ribi, H. Saito and E. Yarkoni published few years earlier [117–119]. Thus, h-k *M. smegmatis* was used as immunotherapy for lung cancer patients in a clinical trial [120], but showed no immunostimulatory effect. However, in an animal model of thymus cancer, *M. smegmatis* showed protective activity [121].

H-k *Mycobacterium vaccae* (SRL172) has been used in several phase I and II clinical trials as a therapy for different cancers [122]: for malignant melanoma [123,124], for prostate cancer [125], for renal cancer [126] and given along chemotherapy for lung adenocarcinoma [127,128]. The antitumour activity of *M. vaccae* has been attributed to its cell wall components [129]. Later, these published works lead to the study of h-k *Mycobacterium obuense* (IMM-101), an environmental mycobacterium phylogenetically related to *M. vaccae*, in clinical trials and it showed no toxicity on patients of melanoma [130].

H-k *Mycobacterium indicus pranii* demonstrated immunotherapeutic potential in a myeloma and thymoma [131] and melanoma [132] mouse models and it has been used in a couple of small clinical trials in India for the treatment of bladder along with radiotherapy [133] and lung cancers [134].

A.2.4.3.2. Irradiation-killed bacteria

To avoid systemic infection, gamma-irradiated BCG has been also used intravenously administered and was found to be effective for the treatment of hepatoma in rats [135]. Moreover, N. Willmott obtained similar results on a rat sarcoma model [136]. Furthermore, as seen above, favourable results were obtained in rat adenocarcinoma and in guinea pig hepatoma [112].

In the case of BC γ-irradiated BCG showed, *in vitro*, similar antitumour effects to live BCG and regarding the triggered immune response, although lower than using live BCG, it induced higher responses to those obtained by using h-k BCG [137,138].

The dose of γ-irradiation determines the possibility to have Killed But Metabolically Active (KBMA) bacteria. Not only γ-irradiation has been described to induce KBMA state [139], KBMA bacteria can be also obtained by other genotoxic methods and result in non-replicating microorganisms that retain some metabolic activity sufficient to induce immunity. Thus, KBMA have the best qualities of live and of death bacteria, they retain immunologenic properties of live bacteria but they are as safe as killed organisms [140,141].

A.2.4.3.3. Other inactivation methods

E. De Boer *et al.* also studied the immune response generated when BCG killed by sonication was used and observed the same results as when using the h-k form, no Th1 response activation [92].

Some clinical trials assayed BCG therapy with antibacterial drugs like oxybutynin in order to reduce BCG toxicity without success [142]. In contrast, ofloxacin showed reduced side effects but long-term efficacy is still unknown [143]. Whether the antitubercular drug isoniazid (INH) is useful to reduce BCG toxicity is controversial [144,145].

A.2.4.4. Cell Wall Skelletons and other extracts

A. R. Zlotta *et al.* observed *in vitro* that not only live BCG was able to induce a proper cytokine milieu for the clearance of BC tumours but also some other BCG subfactions [146].

In the 1970s, T. J. Meyer, E. E. Ribi, I Azuma and B. Zbar developed an extract of BCG called Cell Wall Skeleton (CWS) which suppressed and regressed a strain-2 guinea pig hepatoma [147,148]. At this period, C. Leclerc *et al*. compared *M. smegmatis*'s cord factor (CF) antigen to different cell wall preparations of BCG. They found a better antitumour effect on a leukemia mouse model when the CF antigen was used than when using any of the BCG preparations [149].

Moreover, other extracts of *M. smegmatis* and *Mycobacterium phlei* were used *in vitro* and *in vivo* tumour models, demonstrating their antitumor effect is comparable to live BCG and *M. phlei* in murine fibrosarcoma and in guinea pig hepatoma [118,119].

Despite all these promising results, cell wall extracts were never assayed in BC patients. Years later, however, A. Morales and his collaborators, developed a formulation containing *Mycobacterium phlei* Cell Wall Extract (MCWE) in combination with *M. phlei* DNA and was as proposed an alternative for BC patients in who either BCG or MMC treatment were interrupted due to toxicity problems. MCWE may provide benefit to BCG-failure cases because although it is not as effective as live BCG, it showed to be safer than BCG [150,151].

A.2.4.5. Live mycobacteria

An study showed that not only the live saprophyte *M. smegmatis* expressing mammalian TNF-α but also the wild type strain tested in a BC mouse model showed better long term survival when compared to live BCG [152]. In addition, *M. smegmatis* is able to elicit a favourable immune response in a subcutaneous thymoma mouse model [121]. Furthermore, three different *M. vaccae* strainsin their live form have been studied for their *in vitro* antitumour capacity, but none of them showed better results than BCG [153].

However, it has to be noticed that infections in humans by these mycobacteria have been described [154,155]. For this reason, these mycobacteria have been also assayed in their non-viable form, as mentioned before.

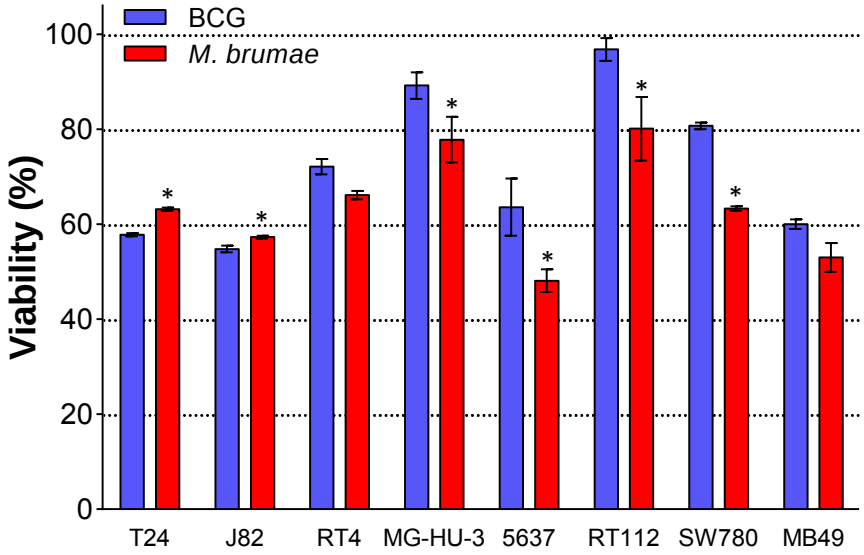
Other genus, like *Lactobacillus*, have been tested *in vivo* in a BC mouse model and either live or h-k and also independently of the route of administration (oral or intravenous) showed an antitumour effect [156]. More recently, intravesical administration of live *Lactobacillus rhamnosus* GG effectively cured tumour bearing mice [157].

A.2.4.5.1. Mycobacterium brumae

An attractive approach to overcome BCG-induced adverse effects is to consider the use of non-pathogenic mycobacteria. As seen in Section A.2.1, the vast majority of *Mycobacterium* species are nonpathogenic and potentially share immunomodulatory antigens with BCG.

M. brumae showed promising results as an alternative antitumour agent *in vitro* [138]. Among a series of 8 mycobacteria tested on 3 BC cell lines (two of them high grade, T24 and J82, and one of them low grade, RT4), *M. brumae* stood out for inhibiting BC cell proliferation at a similar extent to BCG in T24 and J82 cell lines, and for showing an improved effect in RT4 cell line. Thus, based on these results, other series of 7 mycobacteria were tested on 4 different low grade BC cell lines in which *M. brumae* showed a significant differences compared with BCG in terms of inhibiting cell proliferation (see Figure A.2-4).

Figure A.2-4: growth inhibition by *M. brumae* compared to BCG on different BC cell lines



*Percentage of growth inhibition on different human BC cell lines (T24, J82, RT4, MG-HU-3, 5637, RT112, SW780) and one murine BC cell line (MB49) after M. brumae (red bars) and BCG (blue bars) infection. * p < 0.05 versus BCG-infected cells. Figure adapted from the original article of Noguera-Ortega E* [158]

Moreover, *M. brumae* was able to activate human Peripheral Blood Mononuclear Cells (PBMC) *ex vivo* to kill BC cells by both direct contact and using only the soluble factors released by the activated PBMC. *M. brumae* also was able to activate a murine macrophage cell line. Furthermore, unlike BCG, *M. brumae* is unable to persist intracellularly neither in BC cells nor in macrophages which gives a clue of its non-pathogenicity [138].

M. brumae was isolated from soil and water in 1993 by M. Luquin *et al*. [159]. It is considered a rapid grower, non-pathogenic mycobacterium. Although, a clinical isolate of *M. brumae* was described in 2004, it was proved that it has been a case of misdiagnosis [160,161].

Taking in consideration what it has been exposed in this introduction, the characteristics of *M. brumae* and the *in vitro* results described above, *M. brumae* shows to be a promising alternative to BCG for the treatment of NMIBC. To have stronger power to make this affirmation, the antitumour potential of *M. brumae* has to be studied *in vivo*.

However, there was still an issue that needed to be assessed before: the problem of aggregation. Moreover, the interaction between mycobacteria and the target cells could be improved by solving the problem of mycobacteria tendency to form aggregates when resuspended in aqueous solutions. *M. brumae*, like the rest of mycobacteria have a cell wall rich in lipids which make them very hydrophobic [162]. Thus, mycobacteria in aqueous solution (the vehicle used to instil BCG into the bladder of BC patients) tend to form clumps. These clumps

might be hindering the interaction between the mycobacteria and the BC cells, a key step for the initiation of a proper response (see Section A.2.3.2.1).

B. Objectives

The objectives for this thesis are the following:

1. To find a formulation for *M. brumae* able to enhance its antitumour activity

    - to find a stable formulation which maintains mycobacteria viability
    - to find a formulation which avoid mycobacteria clumping
    - to study the *in vitro* antitumor activity of formulated mycobacteria
    - to characterize the physicochemical parameters of the selected mycobacteria formulation

2. To evaluate the efficacy of *M. brumae* as antitumour agent *in vivo*, in an orthotopic murine model of BC

    - to determine the survival rates of tumour-bearing mice treated with formulated and non-formulated mycobacteria
    - to analyse the local and systemic immune response triggered by the intravesical treatment using mycobacteria in tumor-bearing mice

C. Chapter I:
M. brumae and emulsions

C.1. Chapter I outline and introduction

Chapter I talks about the design of a new formulation to resuspend mycobacteria in order to disaggegate mycobacteria clumps and to improve *M. brumae* efficacy against BC cells. The results of this chapter are reflected in manuscript 1 [163] and they have been the basis to publish a patent (Oil-in-water formulations of mycobacterium and uses thereof).

C.1.1. Formulations for intravesical delivery

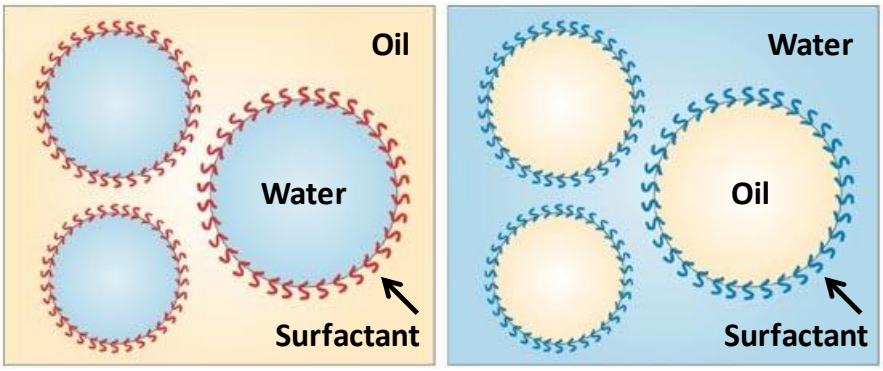
As explained in Section A.1 the bladder has very low permeability in order to prevent leakage of any substance present in the urine into the blood [28]. Among the factors affecting the correct delivery of a drug that has to reach the urothelium there are the hydrophobicity of the drug, the charge, the urine pH and volume, etc. [26]. Many chemical and physical mechanisms to ease the contact between a drug and the urothelium have been described; for instance, applying an electric current, or pretreat the bladder wall with dimetil sulfoxide (DMSO) or hyaluronidase [164]. However, these permeabilization enhancers present undesired side-effects. Thus other strategies to reach the urothelium have been tested. In this section the different drugs carriers for the treatment of BC are addressed.

One of the most broadly studied carriers for intravesical delivery are liposomes because of the ability to be internalized by endocytosis; however, no clinical studies have been performed to apply them on BC. Moreover, solid lipid, protein, polymeric and magnetic nanoparticles, dendrimers and polymeric hydrogels have been studied *in vitro* and/or *in vivo* for intravesical delivery of drugs [28].

Emulsions

An emulsion is a mixture of substances that are immiscible, one is apolar (oil) and the other is polar (water). One of the substances is dispersed (the dispersed phase) in the other (the continuous phase). Between the two phases there are one, two or more surfactants, also called emulsifiers. Whether the oil phase is the continuous phase or the dispersed phase, two kinds of emulsions can be differentiated water-in-oil emulsions and oil-in-water emulsions, respectively (see Figure C.1-1).

Figure C.1-1: depicted water-in-oil and oil-in-water



Adapted from [165].

The first authors who described an emulsion as an adjuvant were Le Moignic and Pinoy. In 1916, they emulsified h-k *Salmonella typhimurium* to immunize mice. However, it was not until Jules Freund and collaborators produced a potent adjuvant using paraffin and h-k mycobacteria that emulsions started to have relevance. Freund's adjuvant has been changing along more than 40 years, the oils has been replaced by others and even the mycobacterium species have.

As mentioned in Section A.2.4, many years ago researchers tried to find safer alternatives to BCG for the treatment of cancer, for instance, mycobacterial cell wall preparations. It was seen that these Cell Wall Extracts (CWE), CWS and many cell wall components were not water soluble which result in aggregation [149,166,167]. An example of success thanks to using an emulsion is the case of Meyer and collaborators who observed no regression in established hepatoma tumours in mice treated with non-emulsified CWS. However, in another set of experiments using an hepatoma guinea pig model, animals were cured when treated with emulsified CWS in mineral oil. In Table C.1-1, are reviewed several examples of success using emulsified mycobacteria or part of their components or other drugs for the treatment of different kinds of cancer or for intravesical delivery.

Table C.1-1: formulations for the treatment of cancer or for intravesical drelivery

Mycobacteria/drug	Emulsion	Oil	Cancer treatment	Reference
BCG cell wall skeleton	O/W	mineral oil	Guinea pig hepatoma (*in vivo*)	[147]
***M. smegmatis* * cell walls**	O/W	mineral oil	Guinea pig hepatoma (*in vivo*)	[168]
BCG cell walls and heat killed *M. smegmatis*	O/W	Drakeol 6VR	Guinea pig hepatoma (*in vivo*)	[169]
***M. smegmatis* Cord factor**	O/W	peanut oil	Murine L1210 leukemia (*in vivo*)	[149]
Killed BCG, *M. phlei,* and *M. smegmatis*	O/W	Squalene	Murine fibrosarcoma (*in vivo*)	[119]
***M. phlei* cell wall extract plus DNA**	O/W	mineral oil	Rat prostate and murine BC (*in vivo*)	[150]
BCG cell wall skelleton	O/W	squalane or mineral oil	Murine lung metastasis from colon carcinoma and melanoma (*in vivo*)	[170]
BCG cell wall skelleton	O/W	Drakeol	Murine melanoma (*in vivo*)	[166]
BCG cell wall skeleton	O/W	Squalane	Murine Lewis lung carcinoma (*in vivo*)	[171]
Mitomycin-C	O/W	soybean oil	Human lung cancer adenocarcinoma and epidermoid carcinoma (*in vitro*)	[172]
Cysplatin	W/O	soybean oil	BC (*in vitro*)	[6]
Plasmid DNA encoding chloramphenicol acetyltransferase or human interferon-α2	W/O	olive oil	Murine skin delivery (*in vivo*)	[173]
BCG Glycolipid A1	-	olive oil	Guinea pig hepatoma (*in vivo*)	[174]

*Review of W/O and O/W formulations of mycobacteria compounds or drugs for treating different cancer types. * it was wrongly classified as M. phlei* [119].

C.1.2. BLADDER CANCER *IN VITRO* MODELS

Any new agent for the treatment of cancer has to be previously tested in an *in vitro* model, in order to prioritize promising agents to be subsequently tested in *in vivo* models [175]. There are many BC cell lines coming either from human carcinomas or from other animals. Moreover, the available cell lines represent different grades of differentiation which give the researcher a wide range of options [175]. To give some examples, RT4 (grade 1) and RT112 (grade 2) were obtained from low-grade papillary urothelial tumours so they are models for low-grade disease as well as 5637, again grade 2, SW780 and MG-HU-3 which are both grade 1. Regarding higher-grade *in vitro* tumour's models there are plenty of cell lines available, for instance human T24 and J82 and murine MB49 [158,176]. The exposed *in vitro* models are useful to study the inhibitory effect of new agents, however, to perform other kind of assays such as, inhibition of migration or invasion capacity, some researchers opt for using three-dimensional models [68,177] or transwell assays [178,179].

C.2. CHAPTER I MATERIALS AND METHODS

Below, there are briefly described the methods used to perform objective 1 and its subojectives (see Section B).

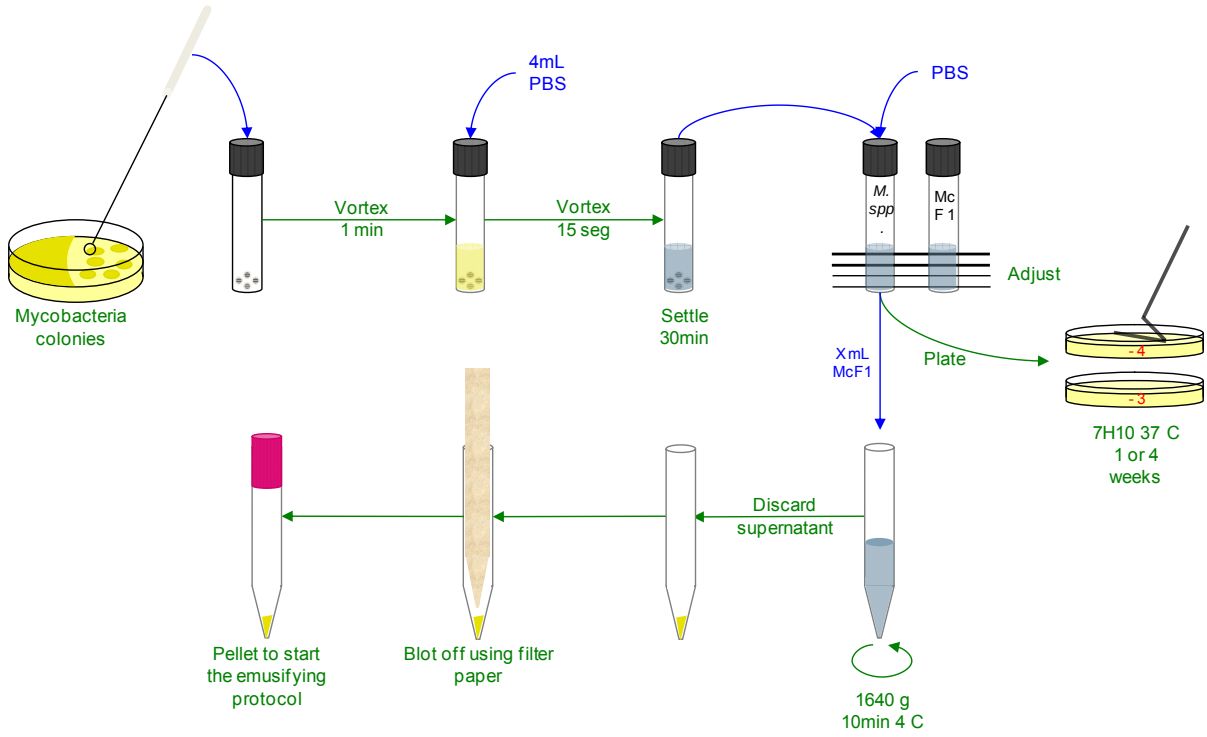
C.2.1. BACTERIA STRAINS AND CULTURE MEDIA

M. bovis BCG Connaught (ATCC 35745) and *M. brumae* (ATCC 51384T) were grown on Middlebrook 7H10 agar medium (Difco Laboratories) supplemented with 10% Oleic-Albumin-Dextrose-Catalase (OADC) enrichment for 4 or 1 weeks, respectively, at 37 C.

C.2.2. *M. BRUMAE* O/W AND W/O EMULSIONS PREPARED

The bacterial colonies were scraped from solid media plates and suspended in phosphate buffer saline (PBS), slightly vortexed with glass beads, and allowed to settle for 30 min. The supernatant was adjusted to 1.0 McFarland standard [180,181]. The cell suspension was pelleted (1640 g, 10 min 4 C), the supernatant was discarded and the pellet was blotted off using filter paper, the amount of mycobacteria cells in this pellet was equivalent to 2 mg or 4 mg of dry weigh (see Figure C.2-1).

Figure C.2-1: preparation of mycobacteria to be emulsified



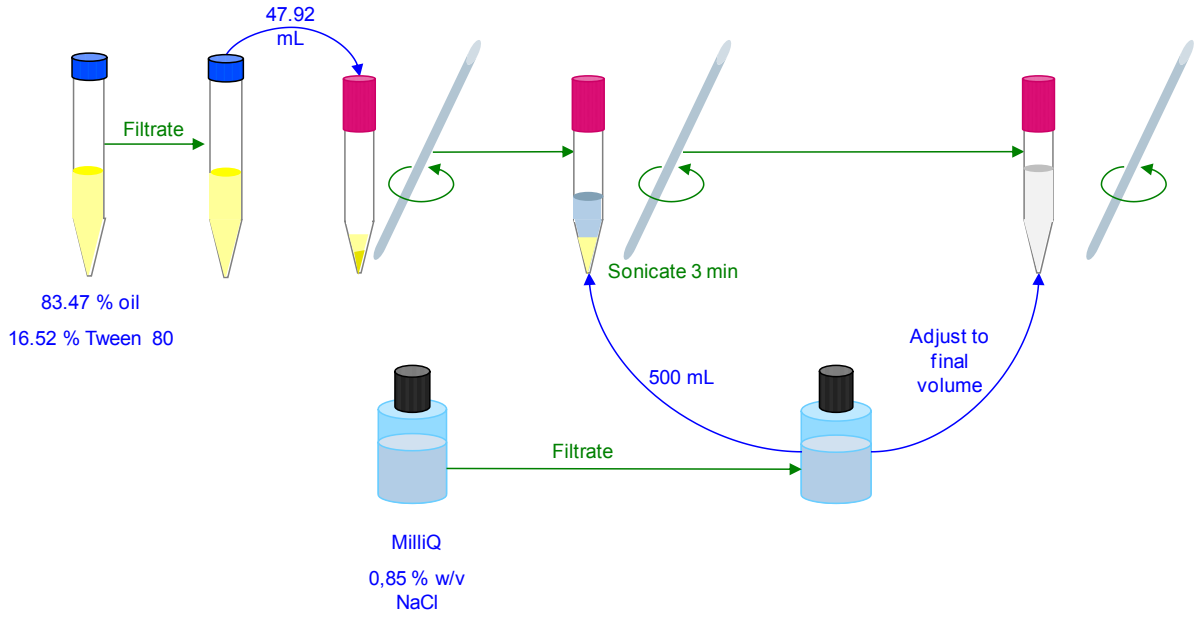
At this point, emulsions of mycobacteria were prepared following previously described protocols with slight modifications. Four different oils: olive oil (OO), soybean oil (SO), squalene (SE) and mineral oil (MO) (all of them from Sigma-Aldrich), two different sonication methods and different proportions between the hydrophobic and the hydrophilic phases were used. Thus, in total, 32 different formulations were studied (see Table C.2-1).

Table C.2-1: different emulsions prepared

Phase	Component	Water-in-oil		Oil-in-water		
		Wu	Hwang	Yarkoni	Morales	This work
Hydrophilic		6	8	99	98	99
	Deionized water	6	8	99	98	99
	Tween 80	-	-	0.2	0.50	-
	“PBS salts”	-	-	-	9.36	-
	NaCl	-	-	0.84	-	0.84
Hydrophobic		94	92	1	2	1
	Oil	46.0	50.0	1	2	1
	Tween 80	18.0	-	-	-	0.2
	Span 80	30.0	30.0	-	-	-
	Brij 98	-	12.0	-	-	-

Percentage of hydrophilic and hydrophobic phases, and percentage of components, in the different emulsions used in the literature and in this work. The experiments were done using the four different oils: olive oil, soybean oil, squalene and mineral oil.

Figure C.2-2: preparation of mycobacteria final emulsion



The first sonication protocol used consists in placing 2 mg of dry-weight of mycobacteria together with the oily phase and then the aqueous phase up to a volume of 2 mL in a tube, proceeding later to sonicate (BANDERLIN electronic, Berlin, Germany) for 5 minutes at 4 C [150].

The second sonication method, which was finally selected, was the rod sonication protocol made in a final volume of 4 mL [119]. To prepare the emulsion using the selected proportions (see Table C.2-1), 4 mg of dry-weight of mycobacteria were placed in a sterile conical glass tube (Duran Group, Wertheim/Main, Germany), and 47.92 µL of a sterile mixture of 16.52% v/v Tween 80 (Sigma) and 83.47% v/v oil were added. Then a sterile glass rod was used to mix them, and 500 µL of 0.85% w/v NaCl (Panreac, Barcelona, Spain) were added and mixed again. The mixture was sonicated for 3 minutes at room temperature (RT). The final volume of the microemulsion was adjusted with the aqueous phase depending on the bacterial concentration needed for each experiment (see Figure C.2-2) [147].

C.2.2.1. Tools choose the most appropriate oil

When the final emulsion protocol was chosen, it was time to decide which one of the four oils was the best option to fulfil the desired properties of the emulsion.

C.2.2.1.1. *M. brumae* affinity to the oily phase

The partition coefficient usually indicates the hydrophobicity of a substance. The technique measures the solubility of a molecule in each of two immiscible phases, in other words, this technique provides information about the affinity that, in this case a drug, has for each phase [6].

Rosenberg *et al*. developed a seminal technique to assess the hydrophobicity of bacteria, Microbial Adhesion To Hydrocarbons (MATH) [182].

By DO determination

To measure the affinity of mycobacteria to each of the oils: OO, SO, SE or MO, 0.150 mL of the oil (hydrophobic phase) or n-hexadecane (VWR) were added to 3 mL of *M. brumae* suspension in PBS (A_{600} 0.44-0.62, referred as A_0). The mixture was vortexed for 1 min and settled for 10 min. Then, the absorbance of the aqueous phase was measured again (A), and the hydrophobicity index was calculated following the formula: hydrophobicity index = $[1 - (A/A_0) \times 100]$ [183,184].

By microscopic observation

Furthermore, 20 µL of the interface were visualized by phase contrast microscope in order to verify the presence of the bacteria in each phase. In another set of experiments, 2% of trypan blue (Sigma) was added to the aqueous phase and mycobacteria were stained with Syto9 (Live Technologies); the interface was then observed by light and fluorescent microscopy DM6000 B and the images were captured by a digital camera DFC480 (both from Leica microsytems).

C.2.2.1.2. *M. brumae* viability in emulsion

M. brumae viability in each microemulsion was determined by using two methods: colony-forming units (CFU) counts and confocal microscopy observation of stained live/dead mycobacteria.

By CFU counting

Serial dilutions of each emulsion were platted on Middlebrook 7H10 agar, and colonies were counted after one week of incubation at 37 C (see Section C.2.1).

By confocal imaging

LIVE/DEAD® BacLightTM Bacterial viability kit (Life Technologies) was used to stain *M. brumae* in each formulation. Stained mycobacteria were observed by using a TCS-SP5 confocal laser scanning microscope (Leica). HCX PL APO lambda blue 63.0x1.40 oil UV objective operating at a zoom of 1.8 was used. To determine both *M. brumae* viability and clump size (next Section) five horizontal (x-z) optical sections (stepsize 1.51 µm) of twenty fields for each condition were captured. Digital images were processed with Metamorf software (Molecular Devices) to calculate the percentage of life and death *M. brumae*.

C.2.2.1.3. *M. brumae* aggregation measurement

Area sizes of the *M. brumae* clumps formed in each emulsion were analyzed from the images taken using confocal microscope as described above, and were automatically quantified using

ImageJ software (National Institutes of Health). Area sizes from 0.7 to 3 μm^2 were considered single bacteria. Larger areas were considered clumps: 3-10 μm^2, small clumps and >10 μm^2, large clumps.

C.2.3. EUKARYOTIC CELL LINES AND CULTURE MEDIA

High-grade human transitional carcinoma cell line T24 was obtained from the Cancer Cell Line Repository (RTICCC-PRBB) in 2007–2011 and was authenticated following short tandem repeat profiling in DSMZ (last time in September 2014). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mixture (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Lonza), 100 U/ml penicillin G (Lab ERN) and 100 µg/ml streptomycin (Lab Reig Jofre) (complete medium), at 37 C in a humidified atmosphere with 5% CO_2, as previously described [158].

High-grade MB49 tumor bladder cells (kindly provided by Dr. Mangsbo and Dr. Tötterman from Rudbeck Laboratory, Uppsala University, Sweden) were maintained in DMEM with stable L-glutamine (Gibco BRL) complete medium at 37 C in an atmosphere containing 5% CO_2, as described previously [158].

T24 and MB49 cell lines were confirmed as negative for mycoplasma contamination by monthly screening using the MycoAlert™ assay (Lonza).

C.2.4. BC CELL LINES INFECTION

Tumour cells were infected with mycobacteria as previously described [51,158,180,185]. T24 and MB49 cells were seeded in 96-well tissue culture plates. Three hours later, *M. brumae* or BCG were used to infect the cells (MOI 10:1) and incubated for 3 hours. In the case of non-emulsified bacteria, they were pelleted, were resuspended in DMEM complete medium without antibiotics and were sonicated in a ultrasonic water bath in order to disaggregate clumps [180,181]. In the case of MO-E and OO-E mycobacteria, the concentration of the suspension was adjusted to this one using the aqueous phase as mentioned before (see Section C.2.2). Extracellular mycobacteria were removed by 3 washes using warm complete DMEM medium. Finally complete DMEM medium was added to each well and the plate was incubated at 37 C in an atmosphere of 5% CO_2 for 72h.

Cytokine analysis

After 72 hours cell culture supernatants were harvested, centrifuged, and stored at -40 C until use. To quantify cytokines in these cell culture supernatants, mouse IL-6 and Keratinocyte Chemoattractant (KC/CXCL1), and human IL-6 and IL-8 enzyme-linked immunosorbent assays

(ELISA) were performed on cell culture supernatants using commercially available kits (BD Biosciences)

Direct growth inhibition

Cell proliferation was measured using MTT colorimetric assay (Sigma). Immediately after removing the culture supernatants, complete DMEM medium containing 10% of MTT was added to the wells and incubated for 3 hours at 37 C in an atmosphere of 5% CO_2. After removing the medium, insoluble blue formazan was dissolved in 100 µL of acidic isopropanol and absorbance was measured at 550 nm in a plate reader (Tecan , Männedorf, Switzerland).

Survival of mycobacteria inside bladder cancer cells

To determine the intracellular viability of mycobacteria, T24 cells were seeded onto 48-well tissue culture plates and infected as explained in above (MOI 10:1). At different time points BC cells were lysed with 400 µL/well of 0.1% Triron X-100 (Sigma). Lysates were plated to count intracellular CFU (see C.2.1), as described previously [180].

C.2.5. FINAL EMULSION CHARACTERIZATION

OO-*M. brumae* emulsion was tested to confirm that the final emulsion was O/W, and to test its potential to adhere to the bladder wall.

C.2.5.1. O/W confirmation

To observe the O/W nature of the mycobacteria emulsion, the droplet test and three different microscopy techniques were performed as previously described [186].

By the droplet test

The emulsion was prepared as previously described (see Section C.2.2). However, this time the aqueous phase of the emulsion was previously stained with 2% trypan blue (Sigma) then, the O/W emulsion was performed. Based on the bibliography [187] a W/O emulsion was designed in order to compare. 47.92 µL of 0.85% w/v NaCl were mixed with the bacteria and then 500 µL, of a sterile mixture of 16.52% v/v Span 80 in OO were added to the mixture and sonicated. A 20 µL aliquot of each emulsion were dropped in the bottom half of a polystyrene Petri dish containing 20 mL of cold distilled water. The behaviour of the drops was recorded with a photo camera (Nikon)[186].

By bright field and fluorescence microscopy

For the light and fluorescent microscopy observation, again 2% of trypan blue was added to the aqueous phase and mycobacteria were stained with Syto9 when performing the emulsion in olive oil. A five µL aliquot of the emulsions were placed in a slide and the cover-slip was sealed

with transparent nail polish. Samples were observed at 1000X using light and fluorescent microscope at 1000X using light and fluorescent microscopy DM6000 B and the images were captured by a digital camera DFC480 (both from Leica microsytems).

By Field Emission Scanning Electron Microscopy (FESEM)

Finally, the ultrastructure of emulsified mycobacteria was observed in a near native stage in a FESEM microscope. Emulsified and non-emulsified *M. brumae* were fixed with 1:1 osmium tetraoxide (4 %) at 4 C for 30 minutes. Then, 5 µl of samples were deposited in silicon wafers (Ted Pella) during 1 minute, excess of sample was blotted with Whatman paper, air dried and observed without coating in a Zeiss Merlin equipped with a secondary electron detector and operating at 0.8kV.

C.2.5.2. Physicochemical properties of the final emulsion

Taking into account the properties of bladder epithelium (see Section A.1), the emulsion needed to fulfil some conditions to be appropriate for intravesical delivery.

By measuring OO-M. brumae adhesion to plastic surfaces

Fifty µL of OO-emulsified or non-emulsified mycobacteria were added into 96-well flat-bottom polystyrene plate in triplicates (Nunc). After incubating for 24 h at RT, five washes with PBS were carried out. Polystyrene adhered mycobacteria were stained with 0.05 ml of crystal violet (1%) for 15min at RT, followed by 5 final washes. Absorbance was measured at A550 using an ELISA plate reader. As negative control, 0.05 ml of PBS were used [184].

By measuring ζ-potential

ζ-potential was determined in OO-emulsified and non-emulsified mycobacteria by laser-scattering method (Zetasizer Nano ZS, Malvern Instruments) [6]. The determination was repeated three times per sample for three batches.

By measuring pH

pH was determined in OO-emulsified and non-emulsified mycobacteria by using pH strips. The determination was repeated three times per sample for three batches.

C.2.6. STATISTICAL ANALYSIS

The statistical significance of the differences between the mycobacteria affinity for the different oils, and between BC cell growth inhibition, was assessed using ANOVA Prism software (GraphPad). Student's t-tests using PAST software was used to compare mycobacterial viability in the different emulsion, intracellular mycobacteria survival, and hydrophobicity of the final emulsion. Kruskall-Wallis (Mann-Whitney, bonferroni corrected p values) test (PAST) were used

to compare aggregates size, cytokine levels produced by infected and non-infected BC cells and ζ-potential.

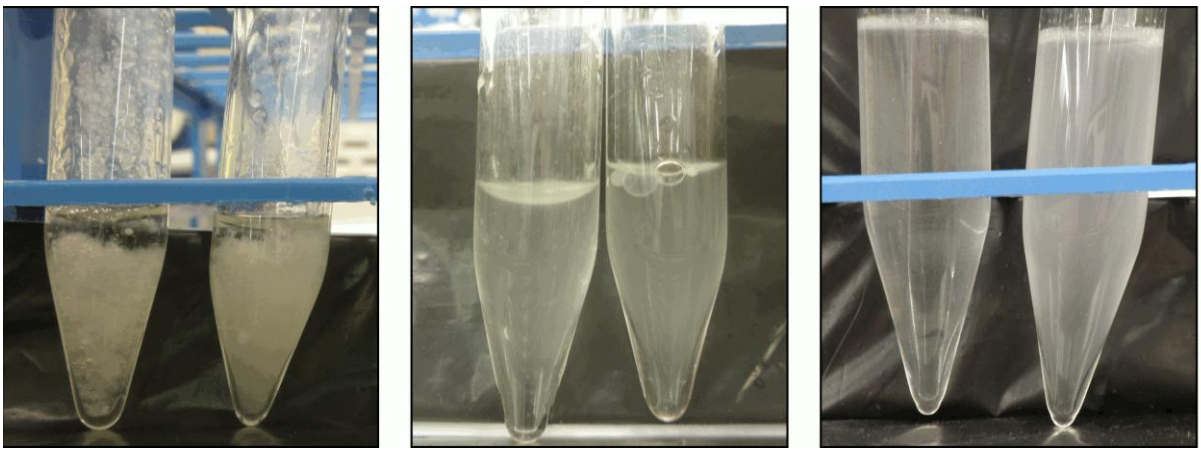
C.3. CHAPTER I RESULTS AND DISCUSSION

In this section there are featured the results and discussion regarding the objectives of chapter I (see Section B).

C.3.1. O/W EMULSION WAS THE MOST HOMOGENEOUS MYCOBACTERIA SUSPENSION

The initial criterion chosen for optimizing the mycobacteria emulsion was based on the homogeneity of the suspension and simplicity of the protocol. By visual inspection, it was observed that some of the emulsions present different phases either because they were unstable or because only a part of the liquid was properly emulsified. Furthermore, some of the surfactants were solid at RT so the oil needed to be warmed which made the protocol more difficult and which could affect *M. brumae* viability. In particular, no homogeneity in the emulsions made neither by using the simple sonication protocol nor when W/O emulsions were made (see Figure C.3-1). So these kinds of emulsions were discarded. In contrast, when O/W emulsions were made by using the rod sonication protocol, homogeneous emulsions were obtained. It was also observed that if the mycobacteria were blended with the oil already mixed with the surfactant, the emulsion was more homogeneous (Fig. 1c).

Figure C.3-1: macroscopic appearance of OO-emulsions



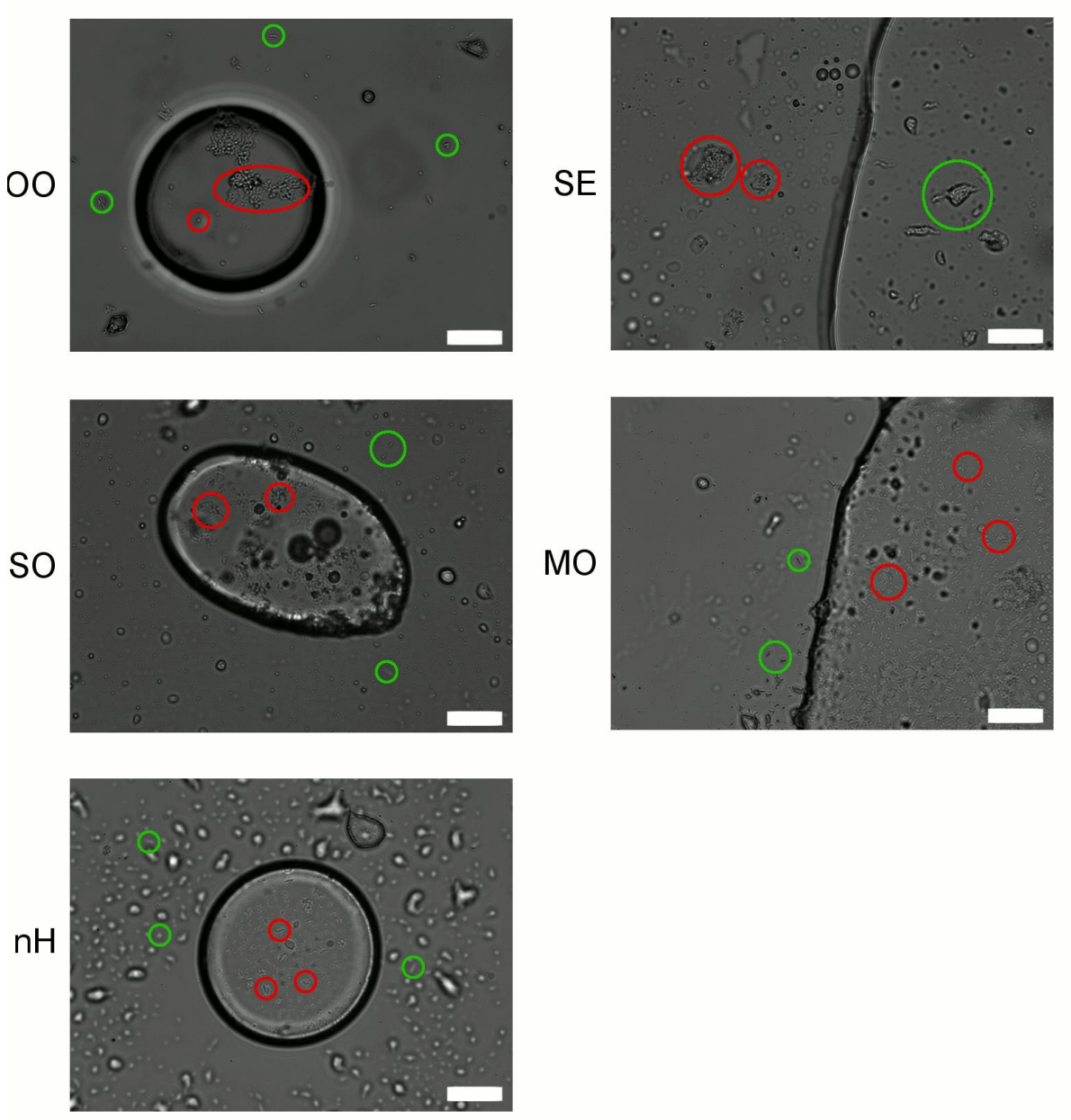
Following the sonication protocol W/O (on the left) and O/W (on the right) emulsions were obtained; on the right a picture of O/W emulsion obtained following the protocol finally chosen. Right tubes correspond to M. brumae preparations, and left tubes are emulsions without mycobacteria.

After selecting the protocol to prepare the emulsions, it was analysed the affinity of mycobacteria for each of the four studied oils [6], and the capacity of each oil to maintain

mycobacteria viability and to avoid mycobacteria clumping. As expected, *M. brumae* was found to be highly hydrophobic, showing a strong affinity for n-hexadecane (see Figure C.3-4) [184,188]. *M. brumae* also exhibited affinity for all four oils; *M. brumae* can be seen inside the hydrophobic phase in all the images in Figure C.3-2 and Figure C.3-3.

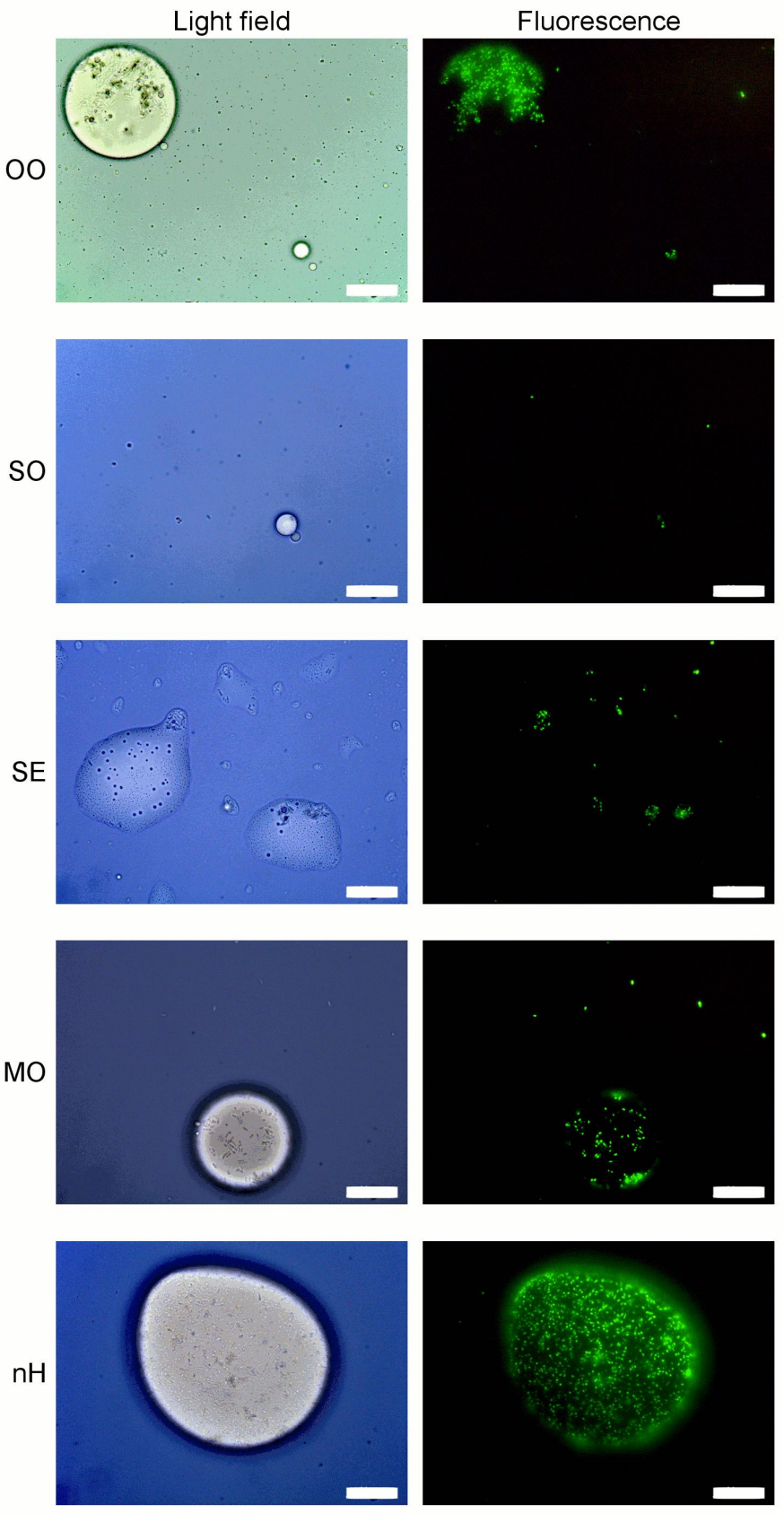
M. brumae showed a high affinity for MO and in decreasing order for SO, OO and SE. As affinity for the different oils could be understood as solubility, maybe there is a correlation between the affinity for the oil and the ability of this oil to better disaggregate clumps (see Section C.3.2).

Figure C.3-2: detail of *M. brumae* in the MATH assay oil-water interface (phase contrast).



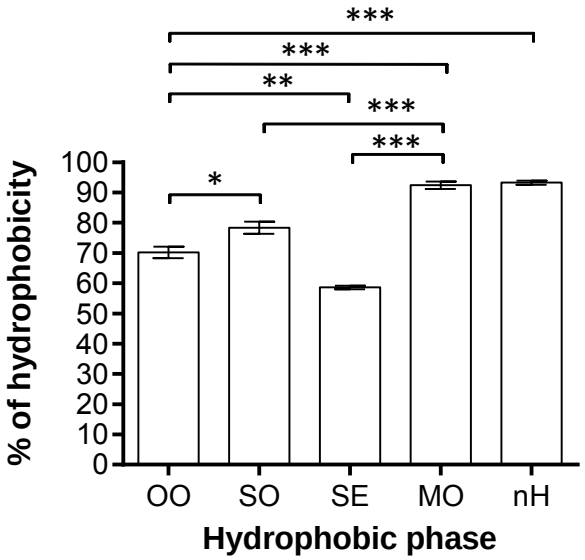
Representative phase contrast images of the oil-water interface of the MATH assay using the different oils and n-Hexadecane (nH). Red and green circles indicate M. brumae in the oil and water phases respectively. Scale bar, 20 µm.

Figure C.3-3: detail of *M. brumae* in the MATH assay oil-water interface (LM-fluorecence).



Representative images of the MATH assay, this time using Trypan blue to stain the aqueous phase (seen in light field images) and Syto9 to stain M. brumae cells (seen in fluorescence images).

Figure C.3-4: affinity of *M. brumae* for the different tested oils.

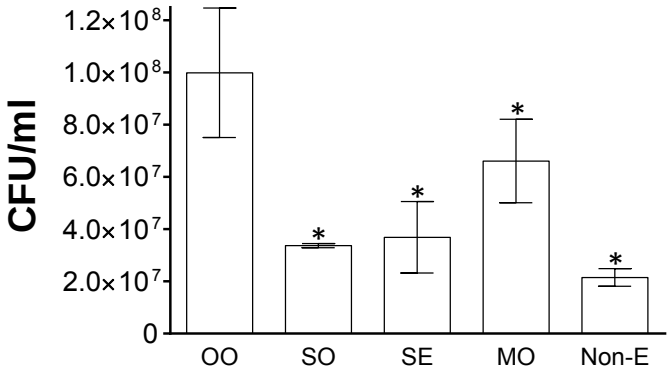


*Hydrophobicity index is represented as mean ± SD of absorbance values from triplicates of one out of at least 3 different experiments. *, p<0.05 ; **, p<0.01; **, p<0.001.*

C.3.2. OO-E MAINTAINED *M. BRUMAE* VIABLE WHILE MO-E BETTER DISAGGREGATED IT

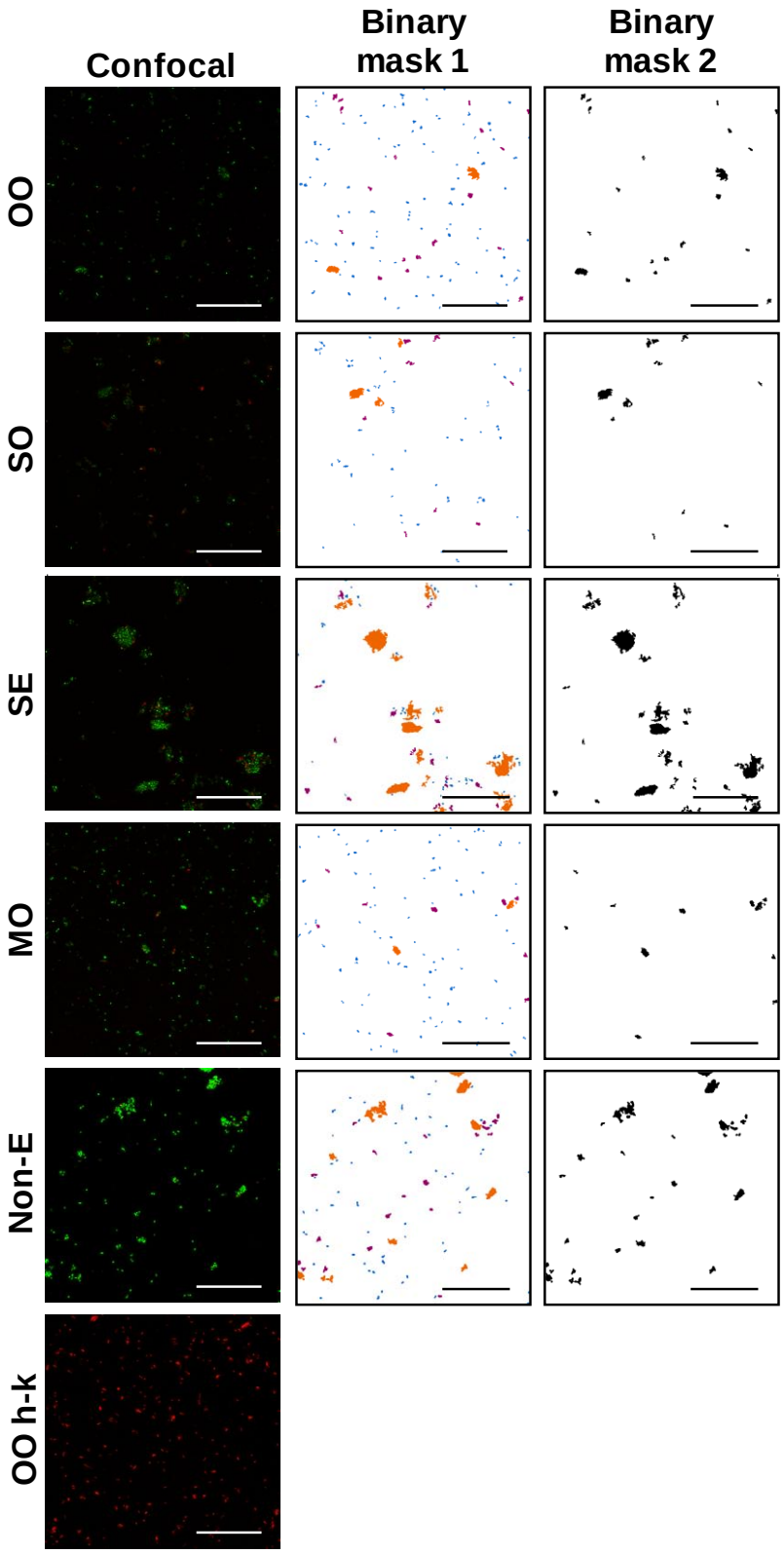
In view that mycobacteria showed a high affinity for all the oils studied, we aimed to unravel how mycobacteria behave when emulsified in each of them. When *M. brumae* viability by CFU counting was studied, it was observed that OO-E better maintained mycobacteria viability than the rest of oils, followed by MO-E (see Figure C.3-5). In contrast, SO emulsion showed to be the one which diminished *M. brumae* viability at higher extend.

Figure C.3-5: CFU counts of *M. brumae* in O/W emulsions made using different oils.



*CFU counts of M. brumae in OO-, SO-, SE-, MO-emulsions or in PBS (Non-E). Values are expressed as the mean ± SD from bacterial culture triplicates of at least three independent experiments. *, p<0.05, with respect to OO-E M. brumae.*

Figure C.3-6: confocal images of *M. brumae* in O/W emulsions made using different oils.

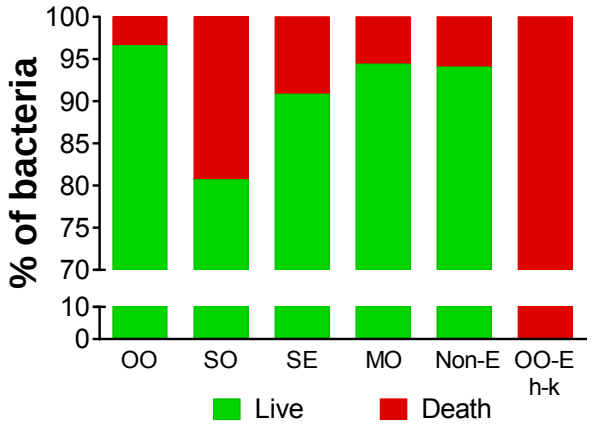


M. brumae in emulsions made by using the different oils observed by confocal microscopy where live bacteria are showed in green (Syto9 staining) and dead bacteria in red (Propidium Iodide staining). In the binary mask 1 there are represented in blue the single mycobacteria cells, in purple the small clumps and in orange the large ones and in the binary mask 2 there are represented only aggregates. Bars indicate 40 µm.

In another set of experiments, emulsions using the four different oils were prepared and *M. brumae* was stained using a viability fluorescent kit. As can be seen in Figure C.3-6, viable cells were stained in green and non-viable cells were stained in red. Non-E *M. brumae* (resuspended in PBS tween 80) was used as positive control and OO-E heat-killed (h-k) *M. brumae* was used as death control. Images of 20 fields per condition were analysed in three different ways to obtain different information. First, by quantifying the intensity of fluorescence, the percentages of live and death bacteria present in each emulsion were obtained (see Figure C.3-7). This approach was used to confirm the CFU counting results explained above. Second, a mask (Mask 1) was applied to the images in order to obtain the area sizes formed by bacteria. Areas from 0.7 μm^2 to 3 μm^2 were considered single cells, from 3 μm^2 to 10 μm^2 were considered small clumps and over 10 μm^2 were considered large clumps. These ranges were established based on the measurement of the area size of 60 single *M. brumae* cells in each emulsion; it was seen that a single cell was never smaller than 0.7 μm^2 or bigger than 3 μm^2. These data was used to calculate the percentage of area that belonged to each of these ranges and elaborate the graph of Figure C.3-8. Third, another mask was applied (Mask 2), this time, area sizes 0.7 μm^2 to 3 μm^2 were not considered; only areas that belonged to aggregates were. The information about clump area sizes are plotted in the graph of Figure C.3-9.

Regarding *M. brumae* viability assessed by the analysis of the confocal images, the same results as using CFU counting were obtained. OO-E *M. brumae* showed the highest percentage of live mycobacteria and SO-E the highest percentage of death mycobacteria.

Figure C.3-7: % of live/death *M. brumae* cells in O/W emulsions made using different oils



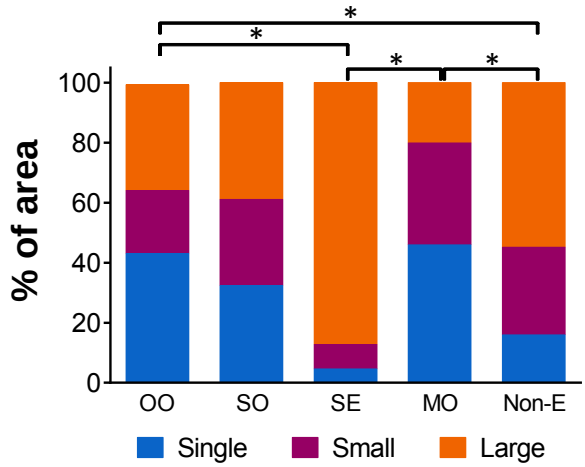
Mean percentage of live (green columns) and death (red columns) bacteria present in the emulsion with respect to the total counts of green and red bacteria, respectively (OO-E h-k, olive-oil emulsion of h-k M. brumae was used as control).

The correlation between the results using the two techniques to assess *M. brumae* viability in the emulsions is good except in the case of SE-E and Non-E. This could be explained by the fact

that these two preparations made the mycobacteria form more clumps (see Figure C.3-8), so CFU are mainly aggregates and no single cells which is translated in biased results in the CFU data particularly in these conditions.

About *M. brumae* clumping, MO was the oil which better disaggregated mycobacteria clumps, obtaining smaller clumps and more isolated cells than when the rest of oils were used (see Figure C.3-8). The aim was that the emulsion diminished the percentage of large aggregates respect the Non-E and by using OO and SO this objective is also fulfilled. Moreover, no significant differences were observed between the percentages of single cells of OO-E or SO-E respect the MO-E. Remarkably, SE-E *M. brumae* only a small percentage of the area is occupied by cells that are forming small clumps or are single (see Figure C.3-6).

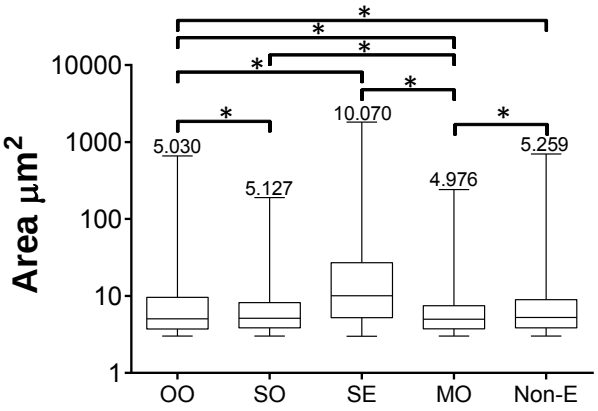
Figure C.3-8: % of single cells, small and large *M. brumae* aggregates in each emulsion



Median of the percentage of single bacteria cells occupied area respect to the total area (in blue) and area occupied by bacteria forming each kind of aggregate (small in purple, and large in orange)., p<0.05, differences in the percentage of single cells.*

In Figure C.3-9, distribution of the area sizes of aggregated *M. brumae* are plotted. In the case of SE-E *M. brumae* it can be observed that not only is the preparation that presented higher amounts of cells forming clumps but also these clumps were the biggest in terms of average (10.070 $\mu m^{2)}$ and in range (from 3 μm^2 to more than 1000 μm^2). As expected, in the case of MO-E *M. brumae* it was observed the contrary case, average size of the clumps was the smallest (4.976 μm^2).

Figure C.3-9: aggregate sizes determination.



*Median size of aggregates (indicated on top of each box) formed by emulsified M. brumae. *, p<0.05.*

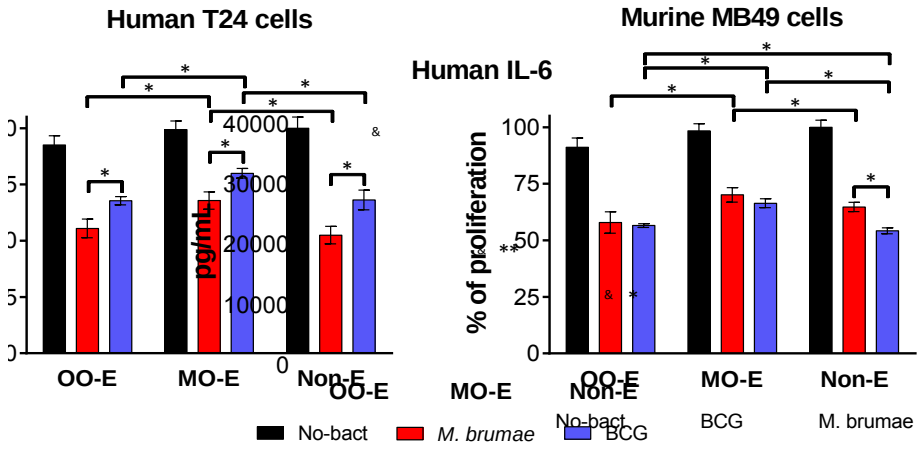
Affinity of *M. brumae* for the different oils do not correlate with aggregation; but it is true that MO-E is the one in which *M. brumae* was less aggregated and the bacterium presented the highest affinity for this oil.

To sum up, OO emulsion better maintained *M. brumae* viability which was confirmed by both techniques and MO followed by OO better disaggregated *M. brumae* clumps. So, only these two emulsions were used for the following *in vitro* experiments.

C.3.3. OO-E *M. BRUMAE* INHIBITS BC CELLS GROWTH MORE THAN MO-E

After selecting only two of the oils (OO and MO), the objective was to demonstrate in vitro the antitumor capacity of emulsified mycobacteria in these oils.

Figure C.3-10: tumour cell growth inhibition exerted by emulsified mycobacteria.

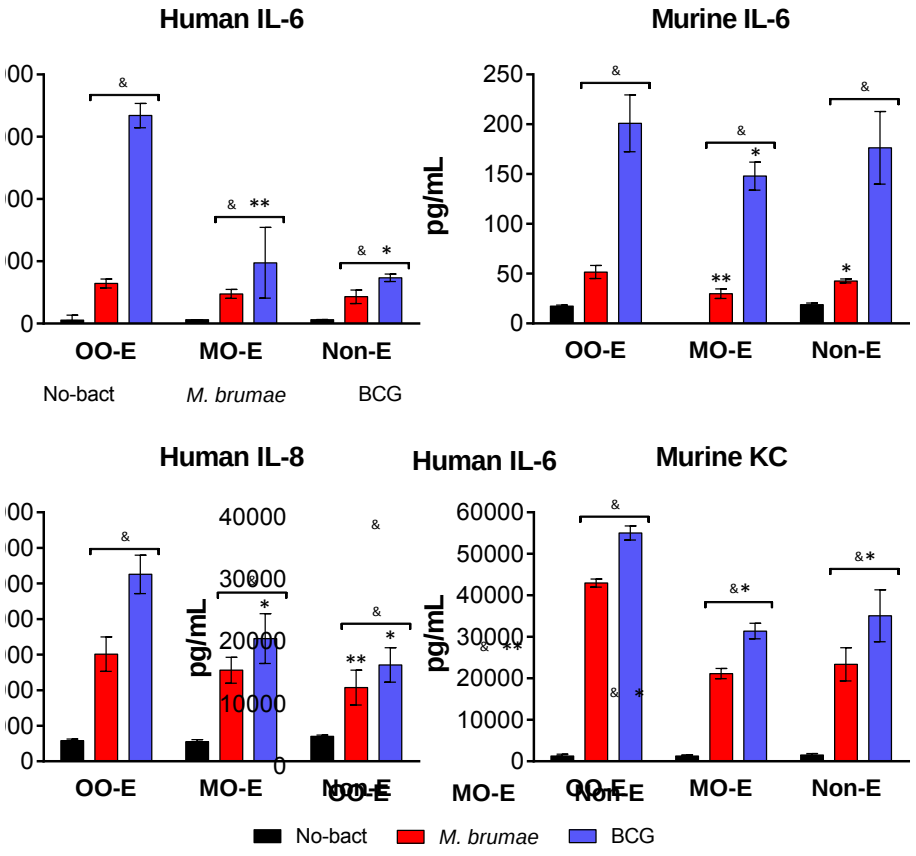


*Tumor growth inhibition on human T24 and murine MB49 BC cells exerted by emulsified or not M. brumae (red columns) or BCG (blue columns) and tumour growth of cells that received emulsions without bacteria or cell culture media (No-bact, black columns). Results from MTT assay are shown as percentage of proliferation related to control cells (Non-E No-bact). Data are expressed as mean ± SD of cell culture triplicates of three independent experiments. * p <0.05; ** p<0.01*

Firstly, the infection protocol [51,158] was optimized to assure that the emulsion was totally removed from the culture after washing the cells 3 hours after infection and there was no affectation of the cells. This was confirmed when it was observed that cultures treated with the emulsion without mycobacteria (OO-E or MO-E No-bact, considered the negative control) showed similar growth rates as the control (Non-E No-bact) (see Figure C.3-10).

As Figure C.3-10 shows, OO-E mycobacteria inhibit proliferation of both BC cell lines similarly to non-emulsified mycobacteria. Only in the case of infecting BC cells with OO-E *M. brumae*, the inhibition of MB49 growth was statistically significantly higher that infecting with non-emulsified mycobacteria. Remarkably, MO-emulsified mycobacteria were less efficacious than the rest of preparations in inhibiting BC cell proliferation ($p<0.05$).

Figure C.3-11: cytokines detected in cell culture supernatants



*IL-6 and IL-8/KC production by cells infected with emulsified or not M. brumae (red columns) or BCG (blue columns) and tumour growth of cells that received emulsions without bacteria or cell culture media (No-bact, black columns). Results are shown as median ± range of triplicate preparations from three independent experiments. *, p <0.05; **, p<0.01 respect to respective OO-emulsified bacteria; &, p<0.05 respect to No-bact.*

Unlike growth inhibition results, the production of cytokines by infected cells differs clearly between infection with emulsified and non-emulsified mycobacteria. Except for the production

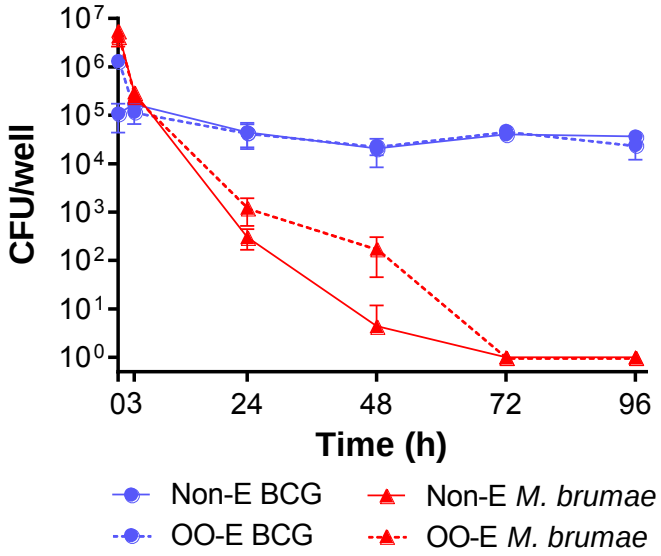
of IL-6 in MB49 cell culture supernatants after BCG infection, OO-E mycobacteria trigger statistically significantly higher levels of cytokines than the rest of conditions ($p<0.05$) (see Figure C.3-11).

Thus, for triggering a significant release of cytokines and inhibit BC cells growth, only OO-E was chosen to continue with the following experiments.

C.3.4. OO-E AND NON-E MYCOBACTERIA SHOWED SIMILAR INTRACELLULAR PERSISTENCE

After observing *in vitro* the antitumor superiority of mycobacteria formulated in OO emulsion, the next step was to demonstrate that the formulation did not affect the non-pathogenicity of *M. brumae*. Figure C.3-12 shows that *M. brumae* (both in the emulsion and in complete DMEM medium) did not persist inside BC cells three days after infection, while BCG remained viable in both vehicles. This behaviour had been already described for BCG and *M. brumae* [158], and the fact of being in emulsion did not change it.

Figure C.3-12: BCG and *M. brumae* survival inside T24 BC cell line.



BCG and M. brumae survival inside T24 BC cell line. The mean ± SD of CFU from cell lysates at different time-points after infection are represented. Values are obtained from three serial dilutions of triplicate culture wells of infected T24 cells. The data are representative of one out of three independent experiments.

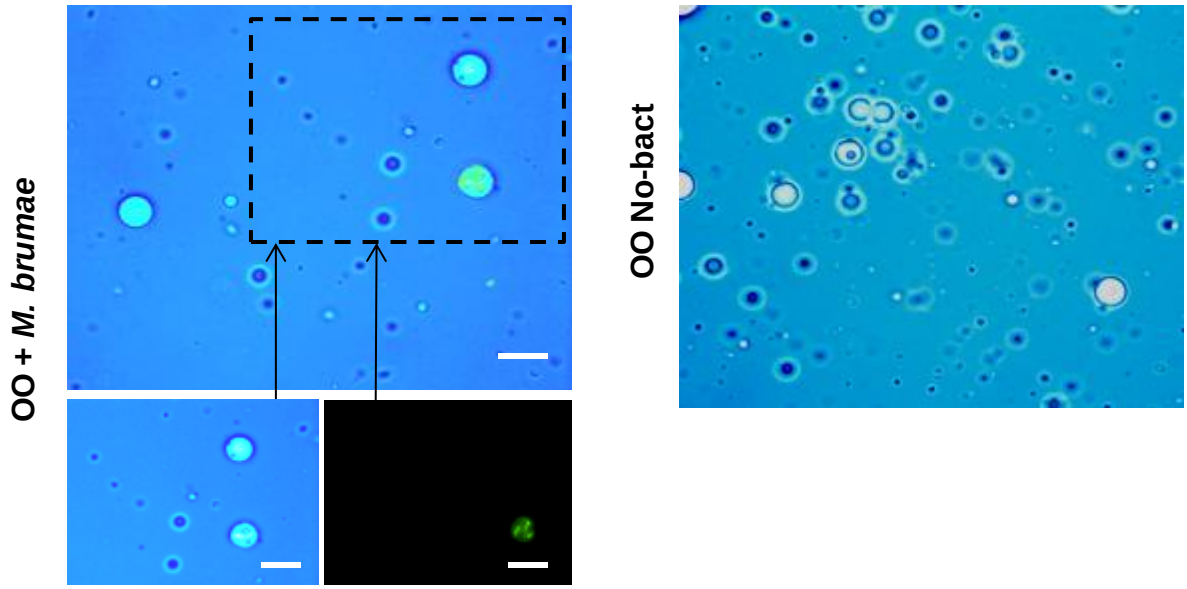
C.3.5. THE O/W NATURE OF THE EMULSION WAS CONFIRMED

The O/W nature of the emulsion needed to be confirmed. There are many parameters that determine if a mixture of water, oil and surfactants will result in a O/W or a W/O emulsion. One of the most determinant is the surfactant or the combination of surfactants. Based on the bibliography, an emulsion like the one selected, which basically is made of water and the

surfactant used is tween 80, should be an O/W emulsion because this water soluble surfactant favours the formation of this kind of emulsions [187]. However, the presence of the mycobacteria could alter the nature of the emulsion.

To this end, there were used different techniques. When the OO-E-*M. brumae* was dropped into cold water, its behaviour corresponded to an O/W emulsion, being completely diluted in after shaking the plate (see audiovisual content in the CD: "Drop test"). To further corroborate the nature of the emulsion it was visualized in a microscope with dual mode; bright field and fluorecent microscopy (see Figure C.3-13). Oil droplets containing mycobacteria were clearly observed comparing the images of light and fluorescent microscopy.

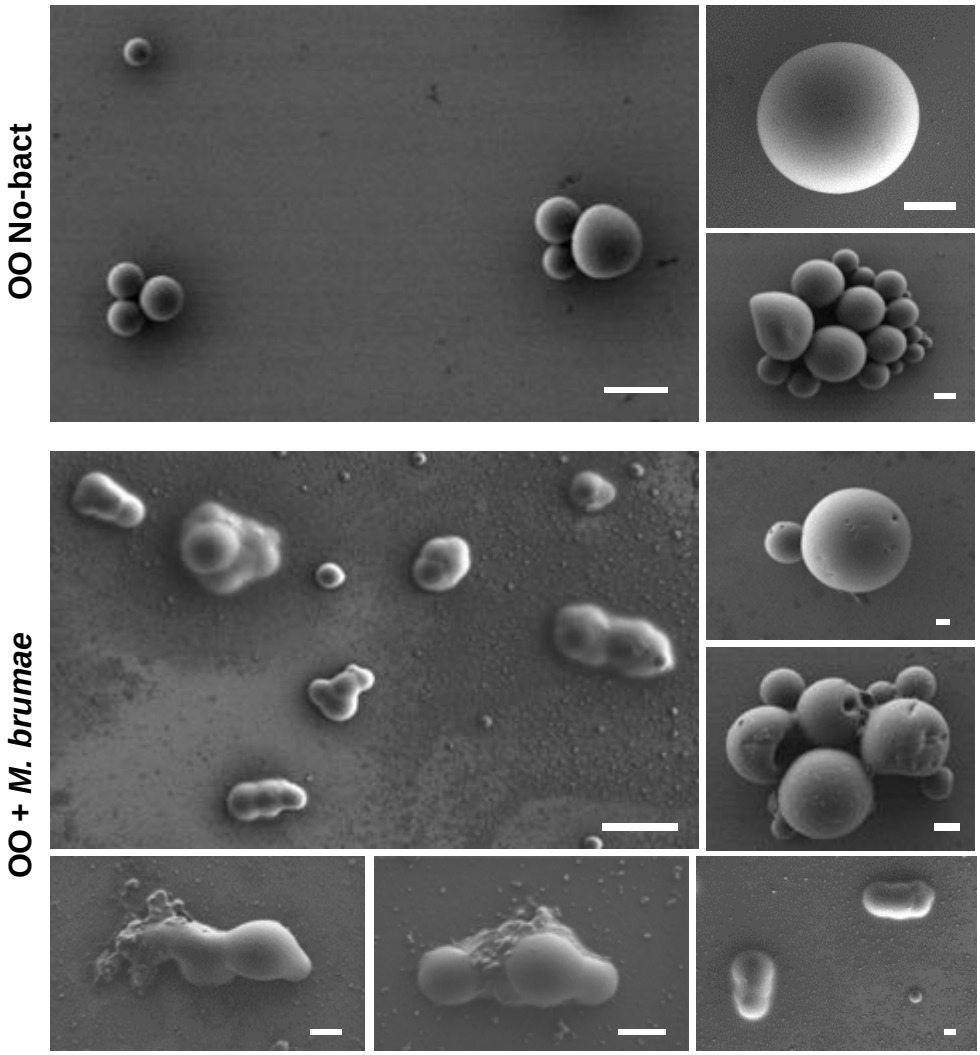
Figure C.3-13: bright field microscopy images of emulsions stained with trypan blue



O/W nature confirmation.Light Microscopy (LM) images of OO-emulsions No-bact Non-E (emulsions without bacteria) or OO-emulsified M. brumae. Bars: 10 µm

Moreover, by FESEM, non-uniform spheres were observed in the OO-E-*M. brumae* emulsion compared to the small uniform spheres observed in empty emulsion preparation (No-bact) (see Figure C.3-14). It can be observed that oil covered, in most of the cases, one or two mycobacterium cells, confirming the results previously obtained by confocal microscopy (see Figure C.3-6).

Figure C.3-14: FESEM images of emulsions fixed with osmiumtetraoxide

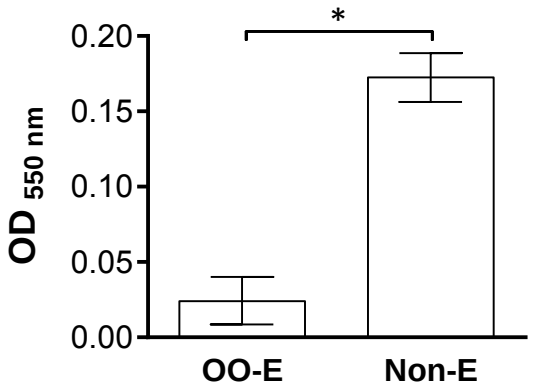


FESEM images of OO-emulsions No-bact Non-E (emulsions without bacteria) or OO-emulsified M. brumae. Bars: 5 µm, in the general views (2 big images) and 1 µm, in images of details.

C.3.6. OO-E APPARENTLY FAVOURS *M. BRUMAE* INTERACTION WITH THE UROTHELIUM

The physicochemical properties of the emulsion containing mycobacteria could be also crucial, *a priori*, for a favourable interaction with bladder epithelium, thus different parameters were analysed. The first result that was observed was that the OO-E *M. brumae* presented reduced hydrophobicity with respect to non-emulsified *M. brumae* as it can be noted since its diminished capacity to adhere to polystyrene plates (see Figure C.3-15).

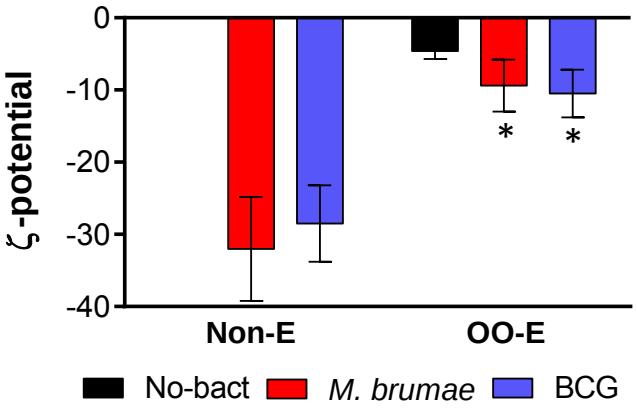
Figure C.3-15: adhesion of *M. brumae* to polystyrene plates.



*Characterization of OO-M. brumae emulsion. Bars represent mean of absorbance values ± SD of triplicate wells from three independent experiments *, p<0.0001.*

Besides, OO-E-mycobacteria showed a significantly neutralized ζ-potential (see Figure C.3-16) with respect to non-emulsified mycobacteria. It has to be considered that the urothelium is hydrophilic and negatively charged [6] and as observed *M. brumae* showed to be hydrophobic (see Figure C.3-4 and Figure C.3-15) and negatively charged (see Figure C.3-16). The fact of emulsifying *M. brumae* improved the values of the two parameters potentially easing the interaction between the bacteria and the bladder epithelium. It has been described that lower pH values favour the interaction between drugs and the urothelium and OO-E mycobacteria showed a pH value around 5 compared to the 7.4 obtained when they resuspended in PBS [6].

Figure C.3-16: ζ-potential of mycobacteria preparations.



*Zeta-potential of mycobacteria preparations. The results are expressed as the mean ± SD of 20 measurements of each triplicate sample from two independent experiments. *, p<0.01 with respect to Non-E.*

D. Chapter II:
M. brumae treatment in the orthotopic mice model

D.1. Chapter II outline and introduction

After screening new therapeutic drugs using *in vitro* models, the selected agents should be studied in more complex systems in order to demonstrate its efficacy before being assayed in clinical trials [189]. The principal objective for chapter II was to evaluate the antitumour activity of *M. brumae in vivo* in the orthotopic murine BC model.

The work performed in this chapter is reflected in several manuscripts. The first results about the antitumour efficacy of *M. brumae in vivo* are published in Manuscript 2 [158]. The part regarding the in vivo efficacy of emulsified versus non-emulsified *M. brumae* appears in Manuscript 1 [163]. In Manuscript 3 [185], there are featured the results about the *in vivo* antitumour effect of γ-irradiated mycobacteria. Finally, the results about the immune response that all these treatments elicit in the mouse model will be the base of a new manuscript.

D.1.1. Bladder cancer mouse models

In vivo models are useful tools to study human diseases and to predict the outcome of new therapies.

The ideal BC animal model should be (1) inexpensive, that is one of the reasons for using rodents; (2) technically reproducible, rapid and easy to perform, regarding the tumour induction method and tumour growth; (3) similar to human natural tumour, in terms of biochemical properties, histology, molecular and genetic characteristics, but the use cells of the same host origin, instead of human cells, is better because an immunocompetent animal can be used without problems of reactivity against the foreigner cells, (4) versatile for testing different therapies, such as chemotherapy or immunotherapy and finally, (5) the tumour should grow intravesically in order to mimic the course of the disease in its environment, like in humans [175,190,191].

In evolutionary terms, larger animals are more similar to human. However, the most commonly used animals as disease models are mice and rats, and BC is not an exception. In some

studies other animals than rodents have been used, for example dogs [192] and pigs [193–195], etc. But these animal models have been used for testing therapies already assayed in rodents, always as a secondary model before clinical trials.

In mice (*Mus musculus*), like in humans, urinary track is composed of two kidneys, two ureters, the bladder and the urethra. The histology of the mouse bladder is also similar to the human bladder although there are some differences in the number of layers of the urothelium (Montine, 2012).

The different types of BC mice models are classified depending on the tumour induction method. These animal models are listed below.

Genetically Engineered Mouse Models (GEMM)

For cancer studies is knock-out mice lacking p53 or RB1 genes (oncogenes' supressors) have been developed, but the animals die in a short time so, inducible transgenic models or with local modifications that only affect the bladder are needed [16]. Thanks to the discovery of uroplakins, a group of integral membrane proteins, nowadays, it is possible to obtain GEMMs that only have the mutation in urothelial cells [176]. Some local gene alterations like CK19-Tag, $Pten^{flox/flox}$ or $p53^{+/-}$ induce BC in mice [175]. GEMMs are powerful tools to study carcinogenesis and to predict therapeutic responses because tumour formation is as it was natural [176]. Despite this, the use of GEMMs is not widespread for testing drug's efficacy because they are very expensive and need time to develop. In the following years, the situation is expected to change, when the scientific community realize of their usefulness and flexibility [175,176].

Chemically induced models

In this type of model the tumour is induced by giving the animals a carcinogen compound, usually mixed in their drinking water. N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) is the most widely used compound because it lacks of systemic toxicity and tumours are exclusively developed inside the bladder [176]. The MB49 tumour cell line was obtained *in vitro* by culturing C57BL/6 mice urothelium with the carcinogen 7,12-dimethylbenzanthracene [197,198]. However, these compounds have to be given during long periods of time for the establishment of the tumour and, consequently, this increases the costs [175,199].

Tumour cell implantation

In this kind of model, BC cells are implanted somewhere in mice body and, subsequently, a tumour is developed. Depending on the localization of tumours, models might be orthotopic, if tumours are inside the bladder, or heterotopic, or also called, ectopic, if tumours are outside the bladder, for example subcutaneous tumours. Subcutaneous implantation of tumours is

technically easier than doing it inside the bladder but intravesical tumours are a better model because they mimic the natural tumour and the results obtained can be easily translated into clinical trials [16,175]. Furthermore, another classification is based on the species' origin of the BC tumour cells. It is known as syngeneic model when the cells used to induce the tumour come from the same species as the animal model, while when the cells come from another species it is called xenogeneic or xenograft model [16]. Nude mice are usually used as xenograft models since they lack of a competent immune system, which avoids the rejection of the implanted cells. Understandably, the cells used come from either human primary cells or human cell lines, for example, T24, RT4 or RT112 BC cells [176].

Syngeneic models, unlike xenograft models, are useful for studies about the interaction of the host immune system and the tumour and the treatment. There are mainly two syngeneic models in use depending on the host mouse strain, C3H and C57BL/6 [197]. In the first syngeneic model the Mouse Bladder Tumour-2 (MBT-2) cell line is inoculated in C3H mice [167,200]. In the second case, the high grade BC cell line MB49 is used in its origin strain C57BL/6. This model is widely used for NMIBC studies [95,200] and was originally optimized by Günther *et al.* [201]. C57BL/6 model is appropriate because it does not develop urinary track diseases or spontaneous tumours (Montine, 2012). Moreover, MB49 induces tumours similar to human BC regarding immunological profile and cell surface markers [197].

D.1.2. RESPONSE TO BCG IN MICE

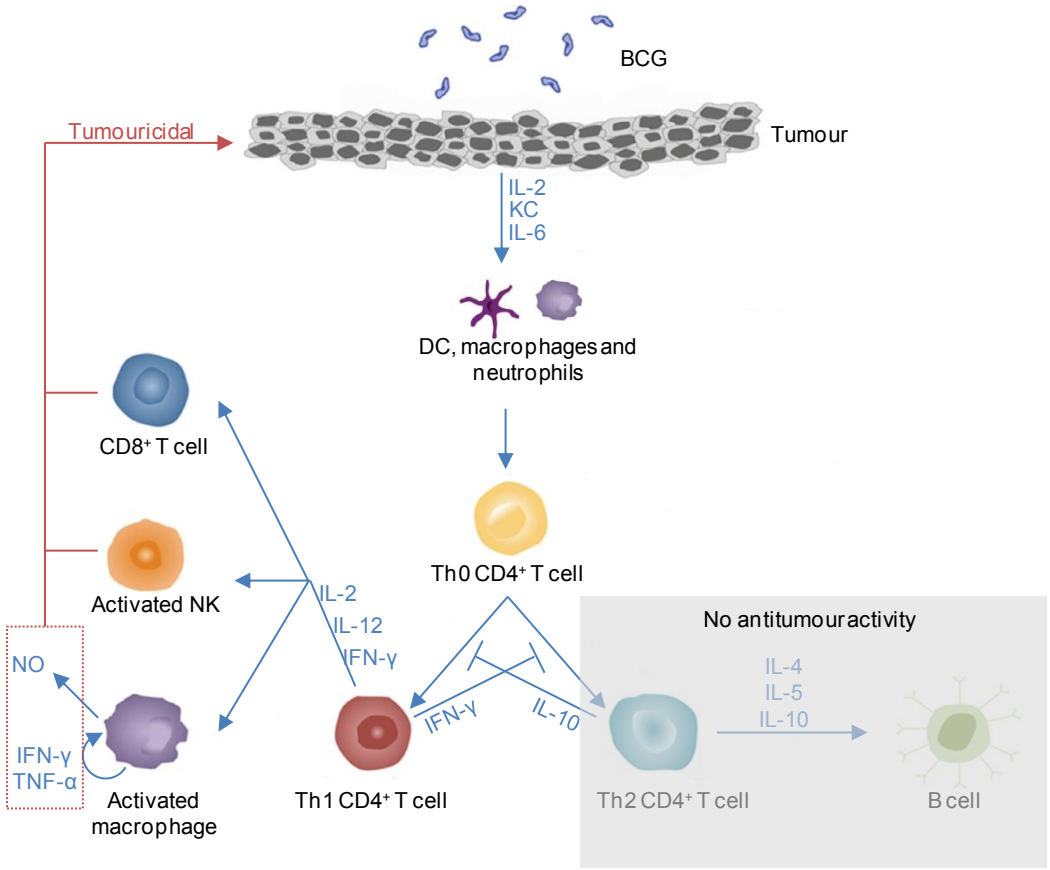
The role of the different cell types of the immune system for intravesical tumour clearance thanks to BCG instillations seems to be similar in mice and in humans. Thus, the use of murine models has allowed to understand the function of macrophages, NK cells, T cells or neutrophils. In Section A.2.3.2.2, it has been addressed what is known about the response to BCG in humans, in this section there are briefly reviewed the situation in mice.

Regarding the role of BCG activated macrophages, different studies have demonstrated that they have a dual tumoricidal role. It was observed that not only BCG had a role on inducing a direct cytotoxic effect by the macrophages on MBT-2 cells but it exerted mainly an indirect effect, because BCG trigger the release of IFN-γ, TNF-α and IL-6. IFN-γ and TNF-α are known to be tumouricidal and to induce the release of NO by the macrophages (see Figure D.1-1)[202,203].

Another subset of immune cells critical for clearing intravesical trumours are NK. NK cells are responsible of eliminating BCG infected BC tumour cells (MB49) in a mouse model [204]. Moreover, when NK are depleted or NK-deficient beige mice are used, BCG therapy is completely ineffective [205]. IL-12 and IFN-α are key factors for the activation of BAKs. These molecules

enhance BAK's cytotoxicity against BC cells while IL-10 not only inhibits their cytotoxicity but also prevents them to influx into the bladder [94,206].

Figure D.1-1: immune response in mice



Adapted from Askeland [207].

Neutrophils are responsible of the migration of T cells into the bladder. Depletion of neutrophils in a BC mouse model results in a reduction of their survival [208]. Both $CD4^+$ and $CD8^+$ T cells are essential for the BCG-mediated antitumour activity. Moreover, animals in which $CD4^+$ subset was depleted were unable to perform a systemic response to BCG and those animals were also unable to clear the tumour [209]. In mice, BCG induces a biased Th1 response indicated by the presence of IFN-γ, IL-2 and IL-12 plus the absence of IL-4, this response is necessary for an effective treatment [72]. J Riemensberger and collaborators, reach the same conclusion, testing BCG therapy outcome in knockout mice for IL-12 and IFN-γ Th1 cytokines and the Th2 cytokine IL-10 [206].

D.2. CHAPTER II MATERIALS AND METHODS

In the following sections there are described the methodologies used to accomplish objective 2 and its subobjectives.

D.2.1. ORTHOTOPIC MURINE MODEL OF BC

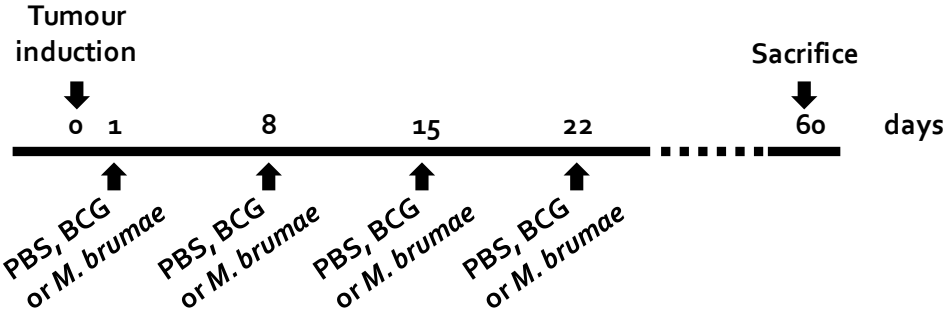
Animal experiments were performed according to procedures approved by the Animal Care Committee at the Universitat Autònoma de Barcelona (UAB) following the international standards. All animals were maintained in quarantine for one week after their arrival at the UAB. Animals were housed six per cage with food and water *ad libitum* in a restricted BSL-2 facility.

C57Bl/6 female mice (6-9 weeks old; Charles River Laboratories, France) were anaesthetized with isoflurane, and chemical lesions were induced on the bladder urothelium by instilling 50 μL of L-poly-lysine (PLL; Sigma) intravesically through a 24-gauge catheter (dwell time 10 min), as previously described [95,210]. Subsequently, 10^5 MB49 tumour bladder cells (MB59 cells were grown as described in Section C.2.3) in 100 μL of culture medium were instilled to induce tumours (dwell time 60 min). One day after tumour induction, mice were randomly divided into groups, and received 100 μL of vehicle, BCG or *M. brumae* cells intravesically (dwell time 60 min), under isoflurane anesthesia. Three subsequent treatment instillations where performed according to the treatment schedules explained below (see Section D.2.1.1). Animals were evaluated on a daily basis for viability and gross haematuria.

D.2.1.1. Treatments

Initially, intravesical treatment using two different doses of *M. brumae* was assayed in the animal model (2-4x10^6 and 2x10^7 CFU).

Figure D.2-1: schedule of tumour induction, treatments and sacrifice



Schedule followed to assay the survival of mice treated with two different doses of M. brumae. At day 0, tumours were induced, 24 h later animals were divided into 4 groups of 6-12 animals and the first treatments were administered. Then, 3 subsequent weekly treatments were intravesically instilled. At day 60 after tumour induccion, the surviving animals were sacrificed.

In these experiments a negative and a positive control were used. The negative control was a group of animals that after tumour induction only received PBS in the subsequent weekly instillations. The positive control was a group of tumour-bearing mice which received BCG (2-4x10^6 CFU) (see Figure D.2-1).

Survival was recorded for 60 days after tumour induction. A death was recorded if animals met the end points approved by the Animal Care Committee at the UAB as a result of tumour burden. Animals were sacrificed by cervical dislocation and, after gross examination, bladders and spleens were carefully collected immediately.

Then, in another set of experiments, in which 6 animals per group were used, 100 μL of the following treatments were administered (see Table D.2-1):

- Healthy animals: in these animals, tumours were not induced. Bladders were administerd PLL, but, instead of MB49, 100 μL of PBS were intravesically instilled and in the subsequent treatments only the vehicle was instilled (PBS or OO-E, No-bact)
- Control with tumour: in these mice tumours were induced but they only received the vehicle in the subsequent instillations (PBS or OO-E, No-bact)
- Live BCG or *M. brumae*: these groups of mice were treated with 2x10^6 CFU of live BCG or 2x10^7 CFU of live *M. brumae* either in emulsion or Non-E (in PBS).
- γ-rradiated BCG or *M. brumae*: these groups were treated with 2x10^6 CFU of live BCG or 2x10^7 CFU of live *M. brumae* either in emulsion or Non-E in the first instillation (24 h after tumour induction). In the following 3 weeks, the same dose of mycobacteria subjected to γ-irradiation (5 kGy) [137,138,185], was intravesically administered in their respective vehicle.

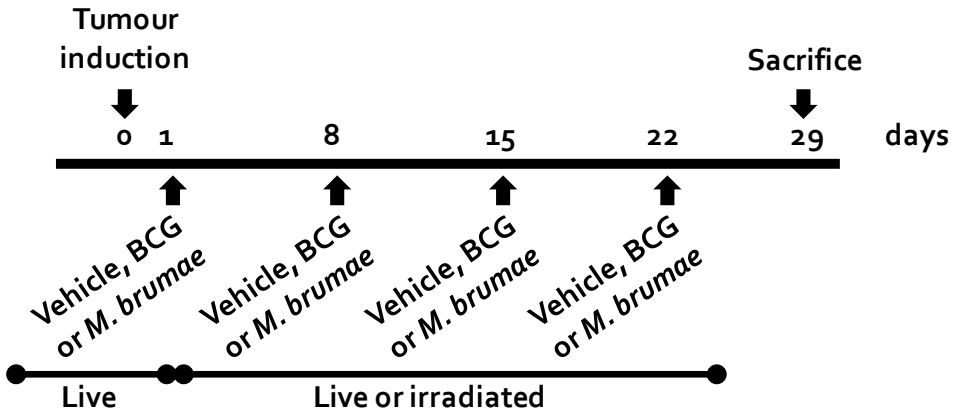
Table D.2-1: treatment groups

Mice group	**Vehicle**	**Mycobacteria**	**Live or irradiated**	**Day 0**		**Day 1**	**Day 8/15/22**
				PLL	**MB49**	**Treatment**	**Treatment**
Healthy (Non-E)	**PBS**	**No**	**-**	Yes	No	PBS	PBS
Healthy (OO-E)	**OO-E**	**No**	**-**	Yes	No	OO-E without mycobacteria	OO-E without mycobacteria
Non-E No-bact	**PBS**	**No**	**-**	Yes	Yes	PBS	PBS
OO-E No-bact	**OO-E**	**No**	**-**	Yes	Yes	OO-E without mycobacteria	OO-E without mycobacteria
Non-E live BCG	**PBS**	**BCG**	**Live**	Yes	Yes	Non-E live BCG	Non-E live BCG
Non-E γ-irradiated BCG	**PBS**	**BCG**	**γ-irradiated**	Yes	Yes	Non-E live BCG	Non-E γ-irr BCG
Non-E live *M. brumae*	**PBS**	***M. brumae***	**Live**	Yes	Yes	Non-E live *M. brumae*	Non-E live *M. brumae*
Non-E γ-irradiated *M. brumae*	**PBS**	***M. brumae***	**γ-irradiated**	Yes	Yes	Non-E live *M. brumae*	Non-E γ-irr *M. brumae*
OO-E live BCG	**OO-E**	**BCG**	**Live**	Yes	Yes	OO-E live BCG	OO-E live BCG
OO-E γ-irradiated BCG	**OO-E**	**BCG**	**γ-irradiated**	Yes	Yes	OO-E live BCG	OO-E γ-irr BCG
OO-E live *M. brumae*	**OO-E**	***M. brumae***	**Live**	Yes	Yes	OO-E live *M. brumae*	OO-E live *M. brumae*
OO-E γ-irradiated *M. brumae*	**OO-E**	***M. brumae***	**γ-irradiated**	Yes	Yes	OO-E live *M. brumae*	OO-E γ-irr *M. brumae*

For this experiment 6 animals per group were used. Mycobacteria treated mice received $2x10^6$ CFU of BCG or $2x10^7$ CFU of M. brumae. Surviving animals were sacrificed at day 60 after tumour induction.

In a posterior experiment, the same treatment groups were used but the group of healthy animals receiving OO-E No-bact was eliminated. Along the experiment urine samples were collected at different time points and, at 29 days after tumour induction, surviving animals were sacrificed by intracadiac puncture to collect the blood under isofluorane anethesia. Again, the bladders and the spleens were carefully taken (See Figure D.2-2).

Figure D.2-2: schedule of tumour induction, treatments and sacrifice



Schedule followed to assess the immune response generated due to the treatment with live or γ-irradiated mycobacteria whether in emulsion or not. At day 0, tumours were induced (except in a group of 6 animals), 24 h later animals without tumour, healthy animals, received PBS in all the instillations. The rest of the animals were treated exactly the same way as explained before in Table D.2-1. At day 29 after tumour induction, all mice were sacrificed.

D.2.2. URINE ANALYSIS

Urine samples were collected in microtubes by applying general pressure at the pelvic area. Collection was performed at day 0, before PLL instillation; at day 1, before treatment instillation; at days 2 and 15, 24 h after the intravesical treatment; and at day 29 before sacrificing the animals. While not processed, urines were kept at 4 C. Subsequently, urines were centrifuged, stabilized and frozen at -40 C. The 10-fold stabilization buffer was 2 M Tris-HCl (pH 7.6), 5% BSA (Millipore), 0.1% sodium azide (Sigma) and 0.1% protease inhibitor (Roche) [211].

For the detection of cytokines and chemokines in the urine, samples were diluted 1:2 in Calibrator Diluent RD6-52 (R&D Biosytems) and analyzed for the presence of mouse GM-CSF, KC, TNF-α, IL-12 p70, MCP-1, IL-1β, MIP-2, Vascular Endothelial Growth Factor (VEGF), IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ, IP-10 and Matrix Metaloproteinase-9 (MMP-9) using a multiplex kit (R&D Biosystems). The assay was performed following the manufacturer's instructions. The plate was read in a Luminex® Magpix® equipment. xPONENT®4.2 software was used for quantitative data acquisition and Milliplex® Analyst 5.1 for multiplex data analysis. The assay was performed following the manufacturer's instructions.

D.2.3. HISTOPATHOLOGIC ANALYSIS OF THE BLADDERS

For histopathologic examination, bladders were fixed in buffered formalin 10%, embedded in paraffin, and routinely processed for staining using Hematoxylin-Eosin (H-E). Four μm sections from the paraffin block containing each bladder were placed on adhesive-coated slides and H-E staining was performed.

D.2.4. IMMUNE CELLS INFILTRATION INTO THE BLADDER

For immunohistochemistry staining, the bladders were processed as explained in the previous section (see Section D.2.3). Formalin-fixed, paraffin-embedded tissue sections (3 μm) were dewaxed with xylene and rehydrated in descending concentrations of ethanol. For antigen retrieval, tissue sections were boiled in bain-marie (96–98 C) for 20 min with 0.01 M citrate buffer (pH 6.0) (CD20 detection) or treated with 0.1% protease (P5147-5G, Sigma-Aldrich) in phosphate-buffered saline (PBS) buffer for 8 minutes at 37 C (CD3 or F4/80 detection). Endogenous peroxidase activity was suppressed with 3% H_2O_2 for 40 minutes. Slides were blocked with goat serum (normal) (X0907, Dako) or rabbit serum (normal) (X0902, Dako) for 1 h at RT.

CD3 immunolabelling was performed using a polyclonal rabbit anti-human CD3 antibody (A0452, Dako) at a dilution of 1:300, over night (ON) at 4 C. The positive control tissue consisted of sections of mice normal lymph node.

CD20 immunoreactivity was assessed using a polyclonal rabbit anti-human CD20/ MS4A1 antibody (PA5-32313, Thermo Scientifc™ Pierce™) at a dilution of 1:200, ON at 4 C. The positive control tissue consisted of sections of mice normal lymph node.

F4/80 immunolabelling was performed with a monoclonal rat anti-macrophage F4/80 antigen antibody (BM4008S, Acris antibodies) at a 1:50 dilution, ON at 4 C. The positive control tissue consisted of sections of mice normal liver.

Immunohistochemistry for F4/80 was carried out using a polyclonal rabbit anti-rat immunoglobulins biotinylated antibody (E0468, DakoCytomation) at a dilution of 1:200, 1h at RT, and the immunoreaction was amplified with the ABC peroxidase staining kit (Thermo Scientifc™) at a dilution of 1:100, 1h at RT; an EnVision+ System-HRP (DAB) Rabbit Kit (K4011, Dako) was used for CD3 and CD20 detection. The chromogen substrate used was 3, 3' diaminobenzidine (K5007, Dako) and counterstaining was performed with haematoxylin (Merck). Negative control tissue sections from the same specimens were identically processed, replacing the specific primary antibody with an isotype-control IgG of the same species and concentration as the primary antibody.

To quantify the different immune cells infiltrated in the bladders by flow cytometry, bladders were minced using a scalpel followed by digestion with RPMI 5% FBS containing 0.5mg/mL collagenase (Sigma-Aldrich, Spain) at 37 C for two successive 30 min cycles with intermittent shaking. The cell suspension obtained was consecutively filtered through a 70-µm disposable plastic strainer (BectonDickinson) and pelleted for staining. Dead cells were stained with a live/dead fixable Aqua Dead Cell Stain kit (Invitrogen). Cells were analyzed by flow cytometry using the following antibodies: CD45-PERCP and NK1.1-PECy7, purchased from Biolegend; CD3-FITC, CD4-PE, CD8-APC, CD19-PE and CD11b-APC, from Immunotools. Samples were acquired in a LSRII flow cytometer (Becton & Dickinson) and the analysis was performed using FlowJo software (Tree Star, Inc). Absolute cell numbers were obtained by using Perfect-Count Microspheres (Cytognos).

D.2.5. CFU COUNTING IN THE SPLEENS

The mouse's spleens were aseptically removed from the animal and were kept in 1 mL of PBS or 1 mL of SensiCell™ RPMI medium 1640 with glucose, sodium pyruvate and stable glutamine (Life Technologies) supplemented with 10% FBS (complete RPMI medium). Spleens were individually disrupted with forceps in 5 mL of PBS or complete RPMI medium and a homogeneous suspension was obtained using a syringe attached to a 23G needle. 100 µL of this suspension were serially diluted and platted in 7H10 for CFU counting and were cultured as previously described in Section C.2.1.

D.2.6. SPLENOCYTES CULTURE

After spleen disruption in 5 mL of complete RPMI medium, the cells were collected in a tube and let settle at 4 C for at least 30 min. Supernatants were collected and the cell concentration was adjusted to $3x10^6$ cells/mL in complete RPMI medium. One hundred microliters of the cell suspension were added to each well of 96-well flat-bottomed microtiter plate (Nunc, Roskilde, Denmark) with or without 1 mg/mL of h-k BCG or *M. brumae* cells, or 5 µg/mL of Concanavalin A (ConA, Sigma) as a positive control. Plates were incubated for 72 hours at 37 C in 5% CO_2 atmosphere.

Cell proliferacion was measured as explained above (Section C.2.4). Pooled supernatants from triplicate wells were collected and stored at -40 C until used. Supernatants were analyzed for the presence of IFN-γ and IL-4 using commercially available ELISA kits (Mabtech AB).

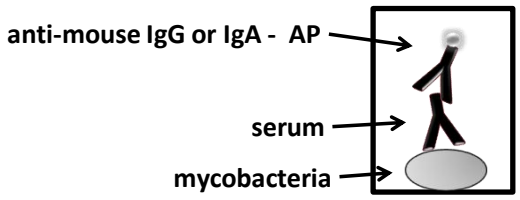
D.2.7. DETECTION OF IGG AND IGA IN SERA

Fresh blood samples were kept at RT for at least 2 hours to allow coagulation. The coagulum was carefully unstuck from the microtube wall with a sterile plastic Kölle inoculation loop. The

tube was then centrifuged at 3500 g for 10 min. The supernatant (serum) was transferred into another tube and frozen at -40 C until used.

Antibodies in sera were analysed by ELISA as described previously with slight modifications (Arko-Mensah, 2009). ELISA plates (high binding; Costar, Corning, NY, USA) were coated with either h-k BCG or *M. brumae* cells (20 µg/mL) in carbonate–bicarbonate buffer (pH 9.6) ON at RT. After washing with Tris-Buffered Saline (TBS), the plate was blocked with 0,5% gelatine from porcine skin (Sigma) in TBS for 2 h. After 3 washes, diluted serum samples were incubated ON at RT. Plates were then washed and incubated for 1 h at RT with alkaline phosphatase-labelled goat antimouse IgG or IgA antibodies (Southern Biotech, Birmingham, AL, USA). The enzyme–substrate reaction was developed using p-nitrophenyl phosphate as substrate (see Figure D.2-3). Optical density was measured in a multiscan reader at 405 nm (Tecan).

Figure D.2-3: detection of antibodies in sera



Schematic representation the IgG and IgA detection in serum samples.

D.2.8. STATISTICAL ANALYSIS

Log-Rank Mantel-Cox tests determined statistical significance of Kaplan–Meier survival curves using GraphPad Prism software. CFU in the spleens were compared using one way ANOVA (PAST). Immunoglobulins in serum were compared using Mann-Whitney test using PAST software. *t-test* was used to compare flow cytometry data (GraphPad Prism) and the proliferation of splenocytes (PAST). Significance was defined as $p < 0.05$.

D.3. CHAPTER II RESULTS AND DISCUSSION

In this section the results and discussion of this chapter are presented responding to the objectives of chapter II (see Section B).

D.3.1. *M. BRUMAE*-TREATMENT PROLONGED SURVIVAL OF TUMOUR-BEARING MICE

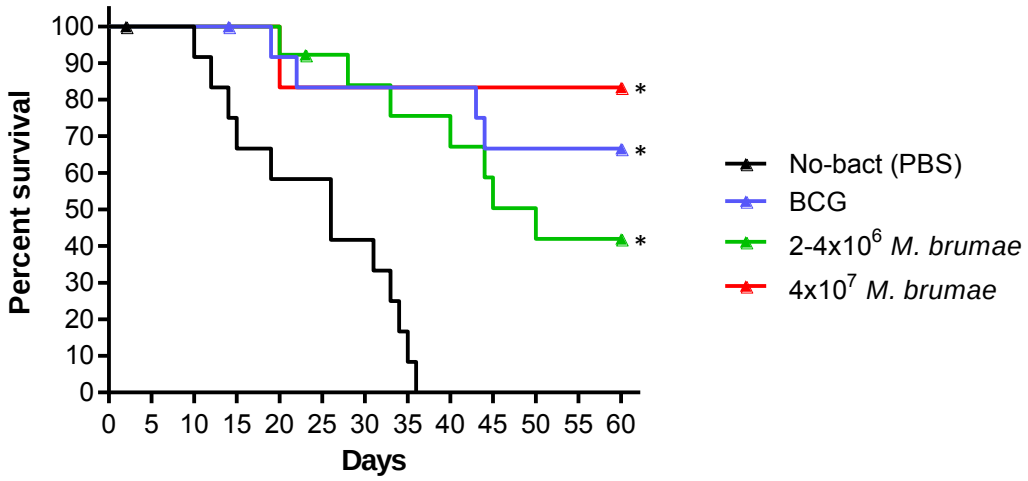
After demonstrating the *in vitro* antitumour activity of *M. brumae*, its *in vivo* antitumour activity using an orthotopic syngeneic mouse model of BC was evaluated following the treatment schedule shown in Figure D.2-1.

Around 8-12 days after tumour induction, all animals presented haematuria, a hallmark of established tumour [95,201]. The animals that only received PBS after tumour induction (No-bact) had to be sacrificed before day forty (see Figure D.3-1). In contrast, at that same time point, more than 80% of the BCG- and *M. brumae*-treated animals were still alive. At day 60, *M. brumae* treatments significantly prolonged survival in tumour-bearing mice ($p<0.01$) as well as BCG did ($p<0.01$) (see Figure D.3-1). No significant differences were observed between BCG- and *M. brumae*-treated groups.

These results obtained in the mice BC model confirm the *in vitro* results seen on chapter I (see Figure C.3-10) and the results presented in Dr. S. Secanella-Fandos thesis which showed that, *M. brumae* was able to inhibit, *in vitro*, MB49 murine BC cell line proliferation (see Figure A.2-4) [138,158].

The second observed result was that *M. brumae* treatment prolonged survival in tumour-bearing mice in a dose-dependent manner (see Figure D.3-1). It has been previously described, both, in a mouse BC model [212] and also in a rat BC model [213], that survival rates and tumour clearance is dose dependent when animals were treated with BCG. Moreover, it had been already observed *in vitro* this dose dependency on the ability of *M. brumae* to inhibit different human BC cell lines [138,158].

Figure D.3-1: kaplan-Meier survival curve of tumour-bearing mice



Kaplan-Meier survival curve of tumour-bearing mice without treatment PBS (No-bact, black line) and after treatment with BCG in PBS (blue), 2-4x10⁶ M. brumae in PBS *(green),4x10⁷ M. brumae* in PBS *(red), * p < 0.01 versus PBS group*

It is worth to mention that in addition to present worse survival rates, histopathology of all bladders showed that animals treated with 2-4x10⁶ CFU of *M. brumae*, present much tumour load than those treated with 2x10⁷ CFU of *M. brumae*. Thus, the higher dose of *M. brumae* used in

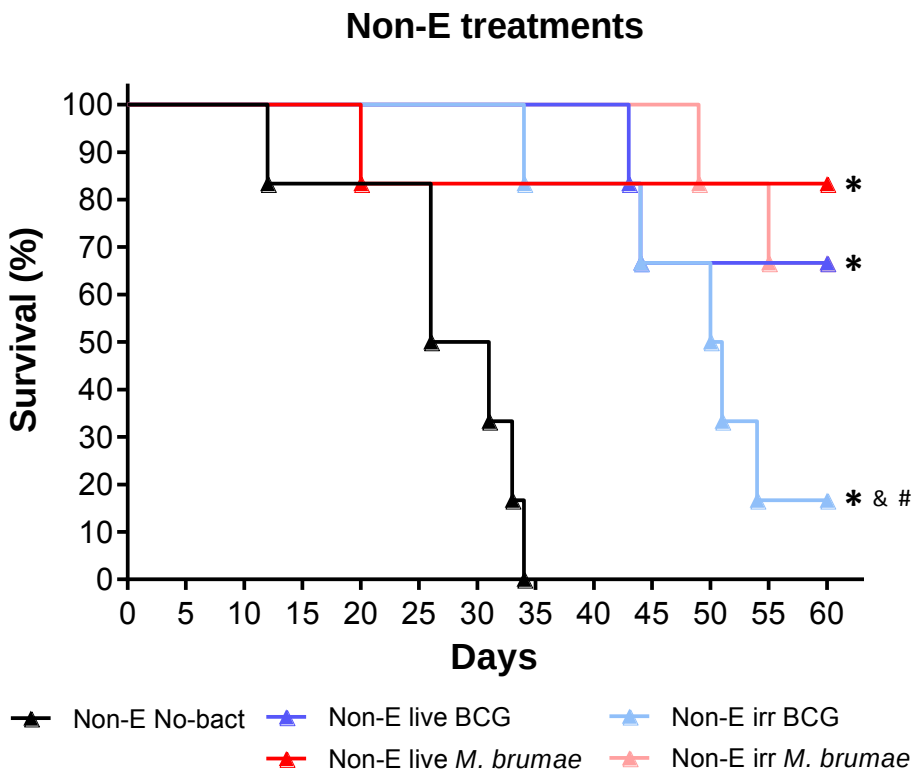
this experiment was chosen as the optimum and in the following sections, all the comparisons are made with animals receing 4x10^7 CFU of *M. brumae* in each instillation.

D.3.2. OO-E *M. BRUMAE*-TREATED MICE SURVIVED UNTIL DAY 60

Once confirmed the antitumour capacity of *M. brumae* in the orthotopic mouse model, and taking into account the previous *in vitro* results, the aim was to explore the therapeutic options that *M. brumae* potentially offered. On the one hand, in chapter I, the results showed that OO-E mycobacteria inhibit BC cell lines growth and triggered a higher production of IL-6 and IL-8 than the Non-E mycobacteria (see Section C.3.3). And on the other hand, γ-irradiated BCG [137,138] and γ-irradiated *M. brumae* [138,185] showed to be able to inhibit human BC cells proliferation, to be able to induce the release of cytokines and chemokines by human BC cells, to be able to activate PBMC to be cytotoxic for T24 cells and to be able to activate murine macrophages.

Thus, γ-irradiated mycobacteria, OO-E mycobacteria and in combination (OO-E γ-irradiated mycobacteria), was instilled into tumour-bearing mice to assess the outcome of these treatments in the animal model.

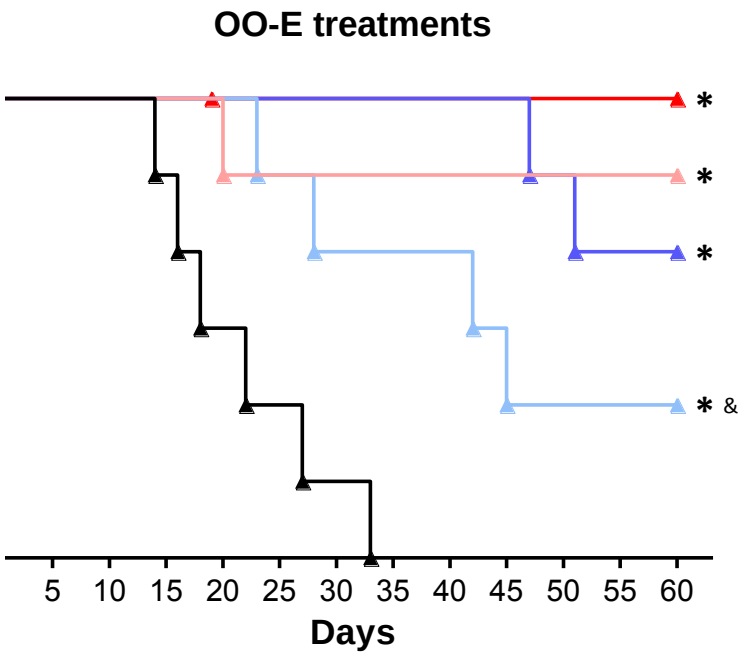
Figure D.3-2: survival of mice treated with Non-E, live or irradiated mycobacteria



*Kaplan-Meier survival curves of tumour-bearing mice that only received PBS (Non-E No-bact, black line), and after treatment with Non-E live M. brumae (dark red), Non-E irradiated M. brumae (light red), Non-E live BCG (dark blue), Non-E irradiated BCG (light blue). * p < 0.01 versus respective control, & p < 0.05 versus live M. brumae, # p < 0.05 versus irradiated M. brumae.*

Regarding the survival rates, tumour-bearing mice control groups (OO-E No-bact and Non-E No-bact) succumbed before day 35 and no differences were found between the OO-E No-bact and the Non-E No-bact groups. It is also worth to mention that animals treated with *M. brumae* obtained in all cases better survival rates that those treated with BCG.

Figure D.3-3: survival of mice treated with OO-E, live or irradiated mycobacteria



*Kaplan-Meier survival curves of tumour-bearing mice without treatment PBS (Non-E No-bact, black line), and after treatment with live M. brumae (dark red), irradiated M. brumae (light red), live BCG (dark blue), irradiated BCG (light blue). * p < 0.01 versus respective control, & p < 0.05 versus live M.brumae,*

The animals of the irradiated groups, received live mycobacteria in the first instillation and only in the three subsequent doses γ-irradiated mycobacteria was administered. This protocol was designed this way because several authors have described that at least the first dose must be of live mycobacteria to induce a proper immune response [92,95]. In Figure D.3-2 and Figure D.3-3, it can be seen that γ-irradiated mycobacteria significantly prolonged mice's survival with respect to their control groups. However, the animals treated with γ-irradiated mycobacteria did not reach the survival rates of those treated with live mycobacteria during the four weeks.

In the particular case of γ-irradiated BCG groups present significantly lower survival rates than *M. brumae* groups. Statistical differences were found when OO-E irradiated BCG was compare to OO-E live *M. brumae* and when Non-E irradiated BCG was compared to Non-E live and irradiated *M. brumae*. In contrast, in the case of *M.brumae* this lose of efficacy is not

observed, although a slight decrease in the survival rates was observed in groups that only received one instillation with live *M. brumae*, this decrease is not as dramatic as in the case of BCG.

Focusing in the effect of the emulsions on the survival rates, in general, groups treated with emulsions containing mycobacteria had a slightly better effect than their respective Non-E groups; remarkably, 100% of OO-E *M. brumae* treated mice survived until the end of the experiment, which has never been achieved with BCG treatment in this animal model (Günther et al., 1999; Noguera-Ortega et al., 2014). The group that showed higher rates among BCG treated groups was also the OO-E live BCG treated one. These results confirm the *in vitro* results showed in chapter I (see Figure C.3-10) in which it was showed that levels inhibition of BC cell proliferation by mycobacteria, emulsified or not, were similar.

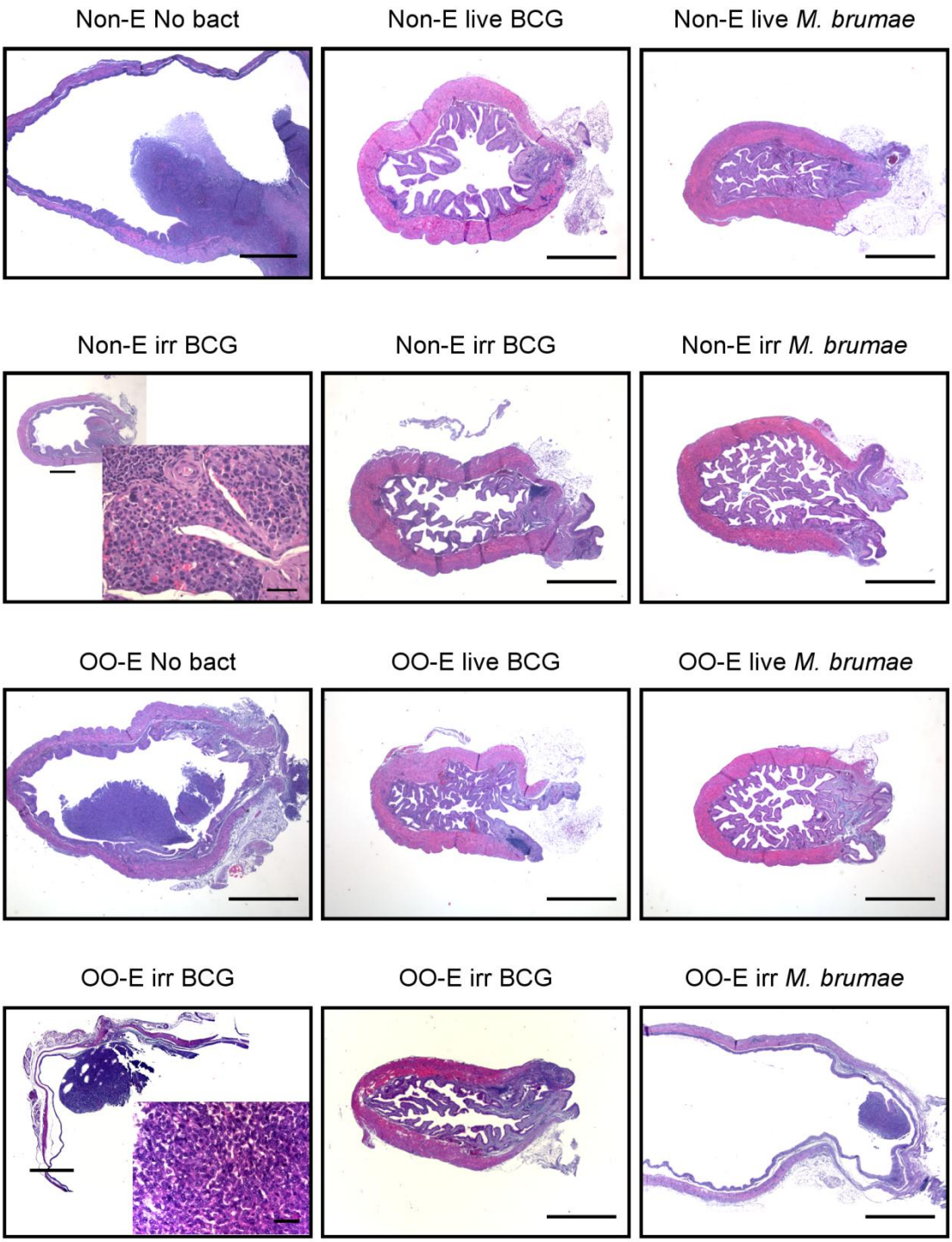
D.3.3. HISTOPATHOLOGY CONFIRMS THAT MYCOBACTERIA REDUCE TUMOUR BURDEN

Histopathological analysis of the bladders of intravesical treated mice was carried out to confirm the presence of tumours. From this analysis, the first result obtained is that the emulsion by itself had no damaging effects on the bladder wall, since microscopically no differences were observed between the Non-E and the OO-E groups; including the healthy animals, the tumour-bearing mice without mycobacteria teatment and the mycobacteria-treated groups.

Histopathological examination of the bladders from the control animals that did not receive mycobacteria (Non-E No-bact and OO-E No-bact) showed a solid mass growing inside the bladder lumen, infiltrating under the *lamina propia* (See Figure D.3-4).

In the case of γ-irradiated-BCG treated mice, remaining diffuse tumour cells or solid tumours were observed in some of the bladders but in the majority of them the tumour was cleared (examples of both cases are showed in Figure D.3-4). In addition, one of the mice which received γ-irradiated-*M. brumae* present a solid tumour in its bladder lumen (see Figure D.3-4). In the rest of bladders of animals which received mycobacteria, no tumours were observed 60 days post-tumour induction. Mild to moderate lymphocytic infiltration into the *lamina propia* or lymphoid aggregates were observed in all mycobacteria-treated tumour-bearing mice (See Figure D.3-4).

Figure D.3-4: histological sections of representative bladders of all groups of treatment



Representative histological images (H-E stain) of bladder sections from the different groups of treatment. In the left column all examples of bladders present tumours. In the second row, example bladders of Non-E irradiated BCG group are showed. The one on the left presents tumour while the one on the middle column does not. In the fourth row, example bladders of OO-E irradiated BCG group are showed the one on the left presents tumour while the one on the middle column does not. The bladder on the right column is the only case of OO-E irr M. brumae treated animal that had tumour at the moment of sacrifice. Scale bar, 1 mm. Magnified images: scale bar, 50 µm. Some of the images were adapted from

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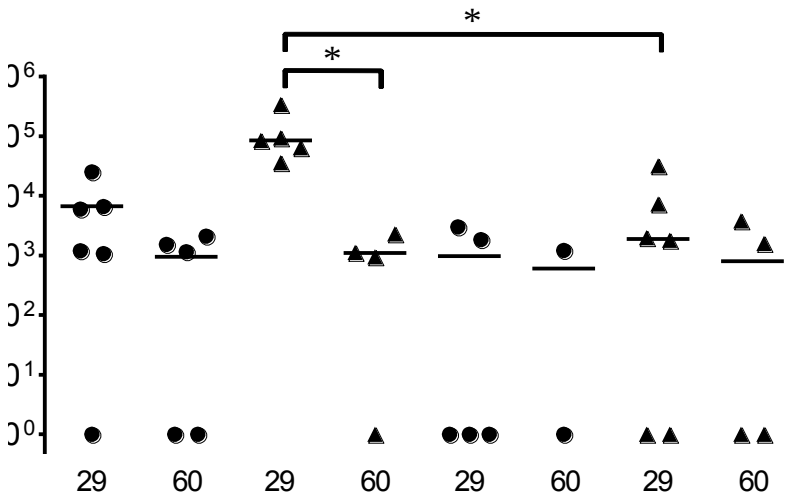
D.3.4. NO VIABLE *M. BRUMAE* WAS RECOVERED FROM MICE'S SPLEENS

In each one of experiments the presence of viable bacteria in spleens was studied. Examination of viable bacteria revealed that BCG-treated mice presented viable bacteria counts (always, NO! Al graphic es veu com hi ha animals que no detectem BCG), while no mycobacteria were detected in *M. brumae*-treated mice's spleens.

The absence of *M. brumae* in the collected spleens could indicate the incapacity of *M. brumae* to persist inside the eukaryotic cells. *M. brumae* is cleared from inside J774 murine macrophage cell line and also from T24 human BC cell line along the time, unlike BCG which persists in both cell lines [138,158]. The same results were showed in Section C.3.4, after infecting T24 cells with emulsified mycobacteria, *M. brumae* did not persist intracellularly while BCG remained viable along the time (see Figure C.3-12). However, only in view of these results another possibility to explain the results could be that *M. brumae* do not reach the bloodstream, thus it is unable to arrive to the spleen.

Figure D.3-5: CFU in the spleen of BCG-treated mice



*CFU of BCG in each individual spleen are represented. The line represents de mean value of the CFU. Animals which received OO-E BCG are represented by dots and the ones receiving Non-E BCG are represented by triangles. * p< 0.05 between the indicated groups.*

Taking into consideration that groups treated with live mycobacteria received four doses while the irradiated-mycobacteria treated animals received only one dose of live mycobacteria (see Figure D.2-2 and Table D.2-1); the initial hypothesis was that irradiated-BCG-treated mice would have less bacterial load in the spleens. However, significant differences on the BCG load where only found between the Non-E treated groups at day 29. Moreover, the results show that

all the groups presented similar levels of BCG, at day 60 after tumour induction, so it can be said that a plateau phase is reached in terms of CFU counts in the spleen(see Figure D.3-5) . Although the presence of BCG in the spleen have not been previously described in the orthotopic murine model of BC, the presence of BCG has been widely analyzed in studies related to tuberculosis vaccination. Mice infected intravenously with BCG reached similar CFU counts in their spleens than those detected in the experiments with the orthotopic BC model and this plateau has been also observed [214,215]. Based on the *in vitro* results mentioned above, probably the fact that CFU counts do not decrease from day 29 to day 60 post-tumour induction is due to intracellular persistence of BCG in the spleens cells.

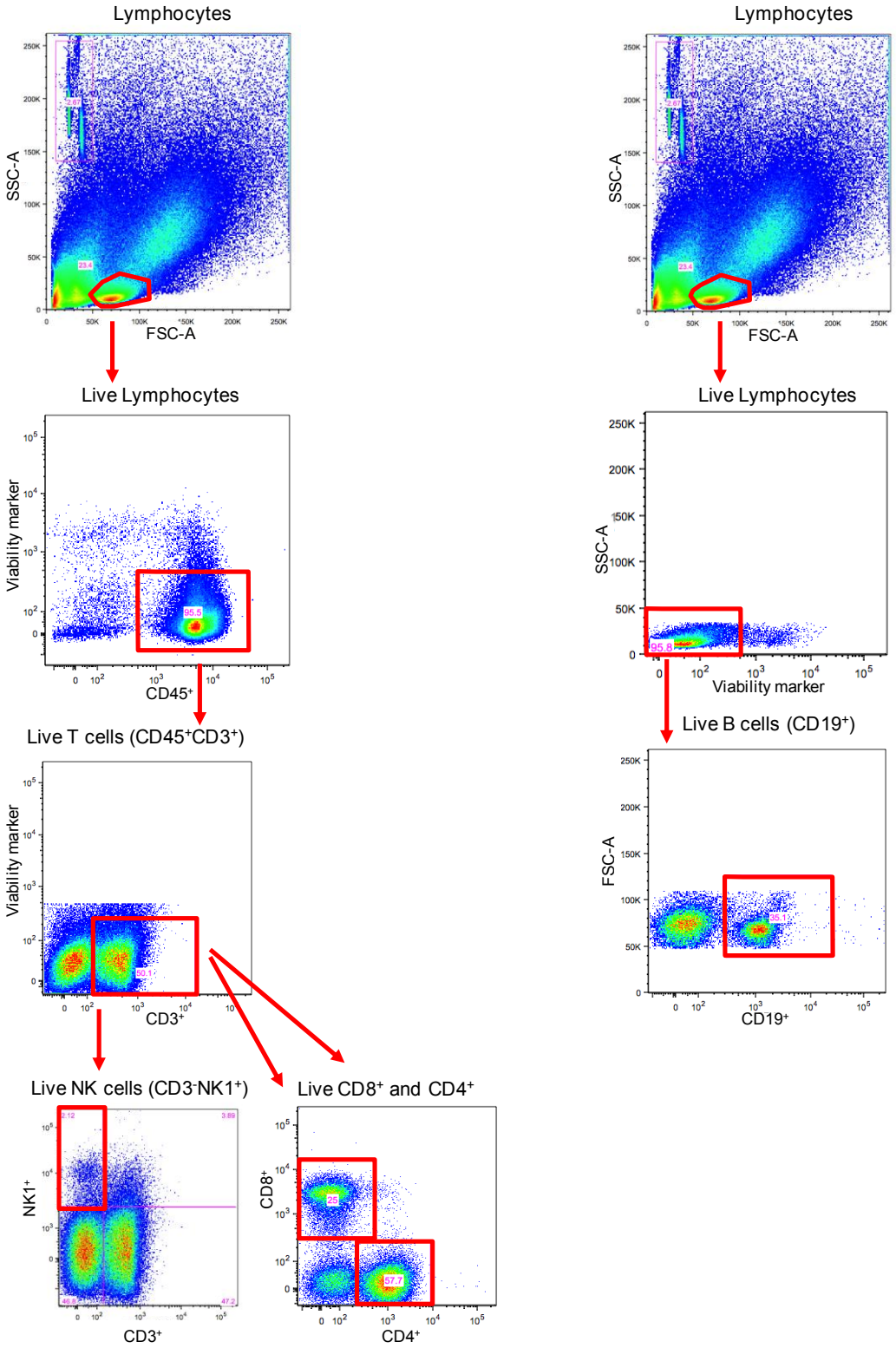
D.3.5. LIVE MYCOBACTERIA TRIGGER AN INFLUX OF IMMUNE CELLS INTO BLADDERS

The influx of immune cells into the bladders in the mouse model has proved to be a powerful tool to unravel the immune response that is locally taking place after BCG treatment [95]. The assessment of the amount and type of immune cells that influx in the bladder during or after the BC treatment could give some clues about the outcome of the therapy in BC patients. However, to date, in humans, any of these parameters has been found to be a strong predictor of the prognosis of the patient [73,36,89,216].

To determine the immune cell infiltration into the bladder, two different techniques were used: flow cytometry, for which four of the six bladders of each group were used, and immunohistochemistry, to localize the different immune cells populations for which the two other bladders were processed for histology.

By flow cytometry, they were analyzed the infiltratiton of lymphocytes, defined as $CD45^+$; B cells, defined as $CD19^+$; NK cells, defined as $CD3^-NK1^+$; and T cells, $CD3^+$, $CD4^+$ and $CD8^+$ (see Figure D.3-6). Overall, all mycobacteria treatments induce a robust infiltration of lymphocytes ($CD45^+$), B and NK cells into the bladder compared to the controls (see Figure D.3-7). Phenotypic assessment showed a high infiltration of T cells ($CD45^+CD3^+$) into the bladder after mycobacteria treatment. This infiltration was composed of both CD4 and CD8 T cells.

Figure D.3-6: flow cytometry gating strategy



Example plot of a bladder of the OO-E live M. brumae group depicting gating strategy followed to identify immune cells into the bladder. In the left column, lymphocytes were gated according to forward and side scatter. Live CD45+ cells were selected. T cells (CD3+) were identified and CD4, CD8 T cells and NK cells were further analyzed. On the right column, live lymphocytes were gated according to forward and side scatter and the use of a viability marker. B cells were analyzed for the expression of the CD19.

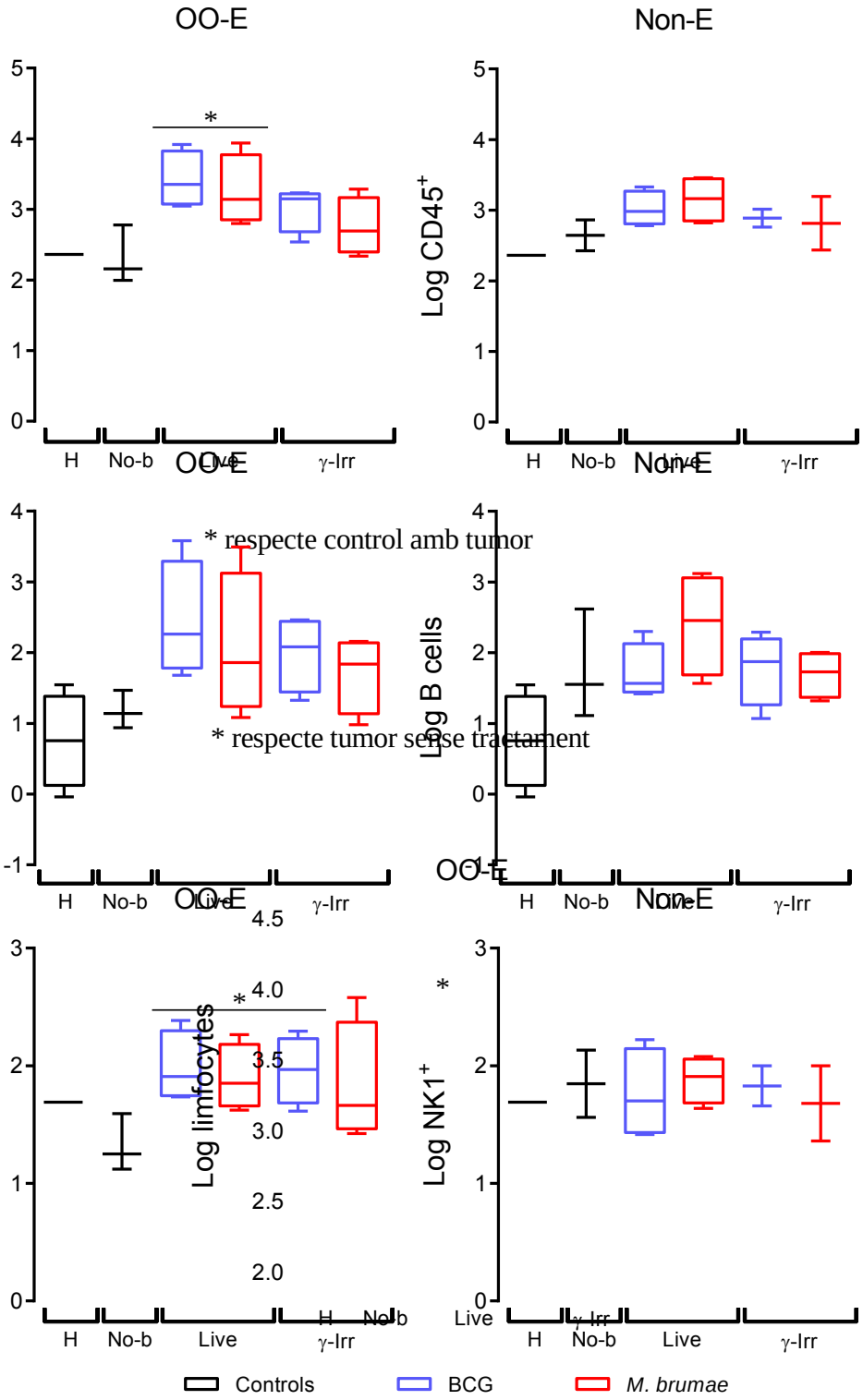
Riemensberger and collaborators unpublished data showed that MB49 by itself induced the infiltration of competent immune cells into the mice bladder masking the infiltration induced by BCG [206]. The results shown in Figure D.3-7, do not agree with this affirmation because in general no significant differences were observed between healthy groups and No-bact groups, in which MB49 was instilled and also some significant differences were found between No-bact groups and mycobacteria treated groups, so the effect of the mycobacteria was not masked. When treatments were compared, it was observed a higher infiltration of immune cells in OO-E treated animals was, in the majority of the cases, higher than in the Non-E treated animals. When live mycobacteria was administered in the four instillations, in general, triggered a higher infiltration of immune cells into the bladder than when irradiated mycobacteria was given from the second dose.

It was observed a higher infiltration of immune cells in the case of the groups receiving the first instillation with live than the controls. These differences could be explained either because the first instillation is with live mycobacteria or because in the subsequent instillations γ-irradiated mycobacteria (and not h-k mycobacteria) are given. It has been observed in these animal models that when h-k BCG is given from the beginning of the course of instillations no differences are observed respect the PBS group [95].

No significantly higher absolute numbers of B cells were found between controls and mycobacteria treated mice. In contrast, these differences were found regarding the absolute number of T cells, CD4 and CD8 when live mycobacteria was administered along the whole treatment, regardless the vehicle used Figure D.3-8.

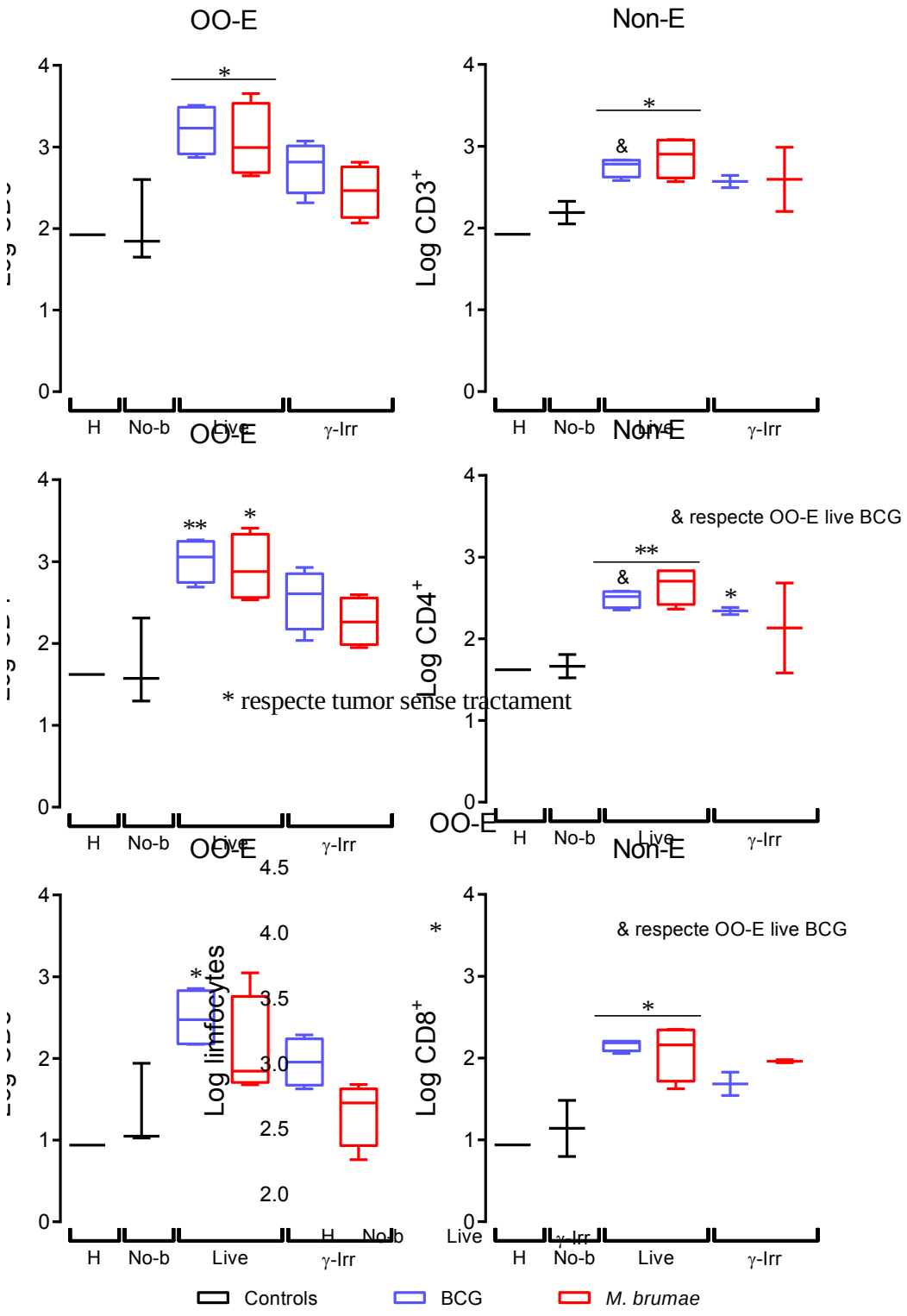
Concerning NK cells, significant differences were only found when OO-E mycobacteria was instilled into the bladders.

Figure D.3-7: log of the absolute number of cells infiltrating into the bladder



*Box plot representing the log of the absolute number of cells infiltrating into the bladder. Black boxes represent the controls (H, healthy animals and No-b, animals that only received vehicle), blue boxes represent BCG treated animals and red boxes M. brumae treated mice. * p< 0.05 respect the No-b.*

Figure D.3-8: log of the absolute number of cells infiltrating into the bladder



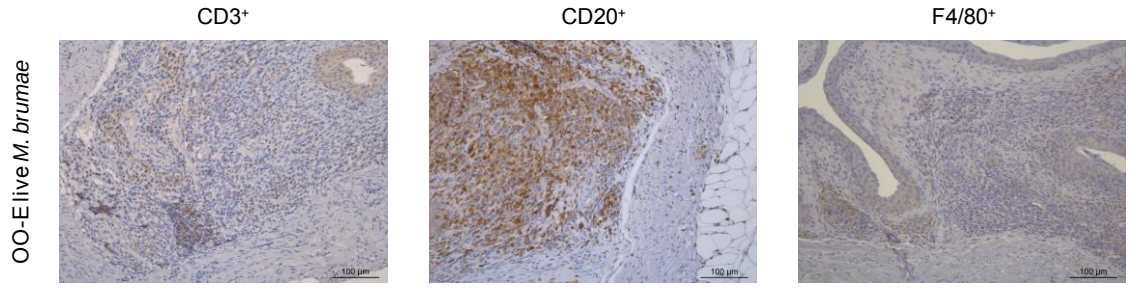
*Box plot representing the log of the absolute number of cells infiltrating into the bladder. Black boxes represent the controls (H, healthy animals and No-b, animals that only received vehicle), blue boxes represent BCG treated animals and red boxes M. brumae treated mice. * p< 0.05 and ** p<0.01 respect the No-b. & p< 0.05 respect OO-E live BCG.*

The results of the immunohistochemistry, although the number of bladders from each animal group was reduced (around two bladders per group), showed that the presence of tumours inside the bladders trigger the infiltration of some $CD3^+$ cells and few number of $CD20^+$ cells (B cells). The treatment of tumour-bearing mice with mycobacteria induce the infiltration of both $CD3^+$ and $CD20^+$ cells, except for the case of animals in which irradiated mycobacteria resuspended in PBS was instilled from the second week. In both cases, animals treated with irradiated-BCG or irradiated-*M. brumae* resuspended in PBS no $CD20^+$ cells are detected into the bladders. However, when irradiated mycobacteria were emulsified the infiltration of $CD20^+$ was similar to the animals instilled with live mycobacteria. This confirms what was already seen with the simpler H-E stain (see Section D.3.3).

Regarding macrophages ($F4/80^+$), immunohistochemistry did not provide much information maybe because a problem with the staining. It was expected much more amount of $F4/80^+$ cells in mycobacteria treated animals.

Regarding the localization of the populations it can be said that $CD3^+$ are generally localized in the peritumoural area in the animals that presented tumour, regardless they were diffuse or solid masses (see Figure D.3-9).

Figure D.3-9: representative images of a bladder stained with the three markers



Bladder of one of the animals of the OO-E live M. brumae group stained with the CD3 marker (right column), CD20 marker (middle column) and F4/80 marker (left column).

D.3.6. IL-6, KC, MIP-2, VEGF AND MMP-9 ARE DETECTED IN URINE SAMPLES

In the analysis of the presence of cytokines, chemokines and other molecules released in the urine, differences among the biomarkers studied were found. However, the results presented in this section are preliminary because it was not possible to collect urine from individual animals in each time point. Thus, samples were pooled. Notwithstanding, the total amount of urine per group and time point was limited and it was not possible to assay different dilutions of urine samples for each biomarker. Among all the molecules tested only IL-6, KC, VEGF, MIP-2 and MMP-9 were detected which means that GM-CSF, TNF-α, IL-12p70, JE, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ and IP-10 were not detected.

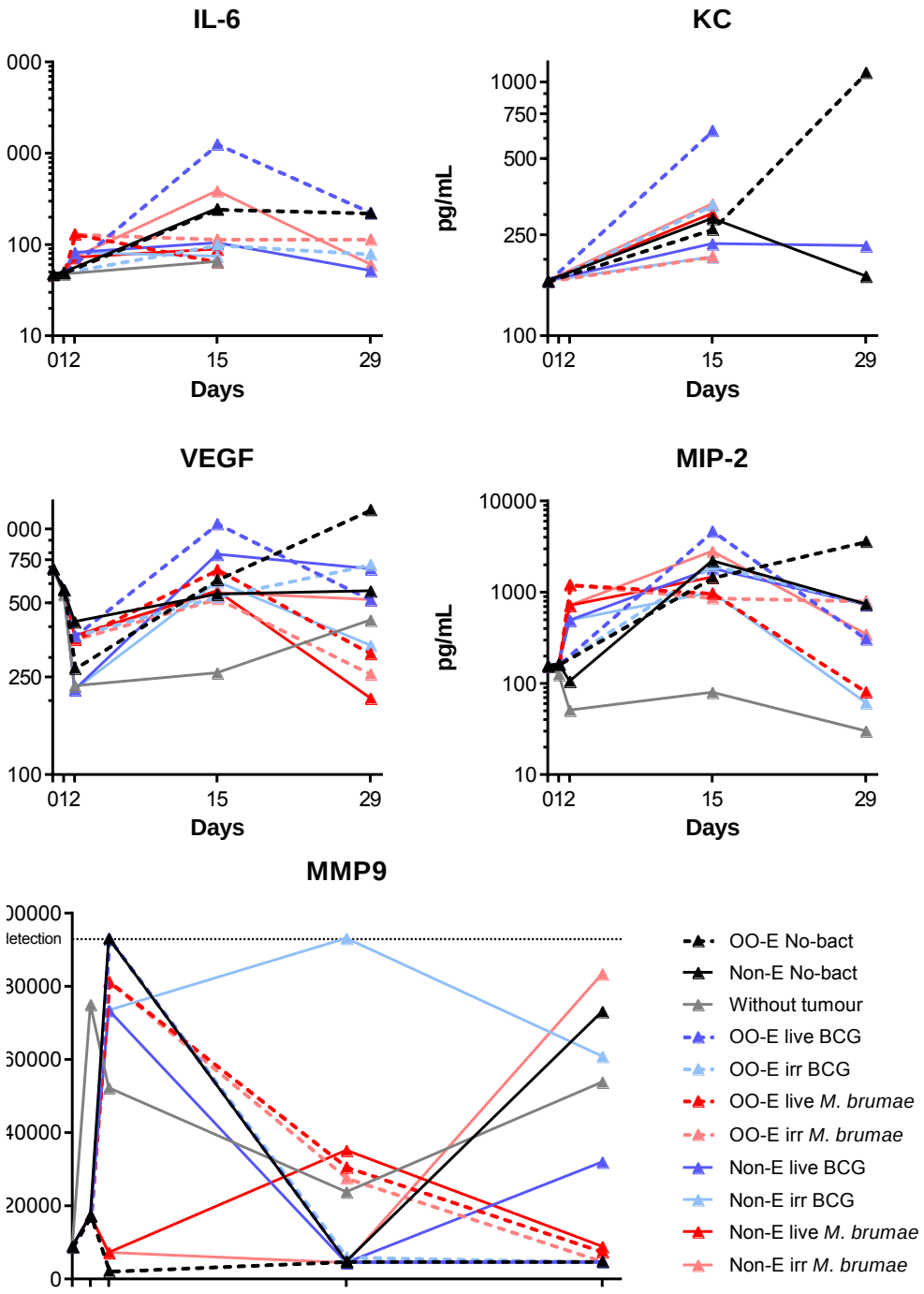
Regarding IL-6 and KC, although many data are missing because the levels were under the limit of detection, it can be said that the presence of these macromolecules correlate with the influx of some immune cells detected by flow cytometry and observed by immunohistochemistry like T cells and macrophages (see Section D.3.5). In bladder cancer patients IL-8 is detected in the urine since the first instillation and this molecule is associated with the a better subsequent local immune response which means a better prognosis. However, in the mouse urines, KC was only detected from day 15 (see Figure D.3-10) [70,82]. Furthermore, although IL-6 induces the Th2 response which might be controlled by the presence of IFN-γ and the absences of IL-4, IL-6 [69] also up-regulates the fibronectine α5β1 receptor which is involved in the interaction of BCG with the urothelium [217,218].

Expression of VEGF is related to the promotion of angiogenesis and it is been associated with BC recurrence [219] and progression [220], in fact, therapies that block this molecule has been assayed for different kinds of cancer in mouse models [221]. Contrary MIP-2 is a chemokine that attracts neutrophils to the inflammation site and is involved in the downregulation of processes of metastasis and angiogenesis [222]. The results show that both molecules present a peak at day 15 in all treated groups regardless of whether they received mycobacteria or not (see Figure D.3-10). The group of healthy animals follows another tendency so the variations in the levels of VEGF and MIP-2 are conditioned by the presence of tumour cells in the bladder. At day 29, the levels of these molecules decrease, indicating lower tumour burden and in contrast, in not treated tumour-bearing mice (No-bact groups), the levels of these molecules do not decrease from day 15 to 29, indicating that tumour burden is not decreasing (see Figure D.3-10). For some groups this affirmation is not valid, however, as said before, it has to be kept in mind that the urines were pooled and data might be biased by a single animal.

The role of MMP-9 in angiogenesis is controversial because it seems to have a double effect, on the one hand, VEGF stimulates MMP-9 release and, on the other hand, some degradation products generated by MMP-9 are antiangiogenic [223,224]. However, MMP-9 has been positively correlated with tumour size in BC patients [225]. In the graph it can be seen that the general tendency of the different groups is to present a peak of MMP-9 at day 2 (48 h after tumour induction) and then, lower or decreasing levels of this molecule were detected in the following weeks (see Figure D.3-10). This could be correlated with the decrease of tumour burden in treated animals. More in detail, both BCG treated groups (OO-E BCG and Non-E BCG) and OO-E *M. brumae* group give a peak of MMP-9 at day 2 but the Non-E *M brumae* treated mice do not. In irradiated BCG in PBS treated animals which is the group with worse survival rates (see Figure D.3-2 and Figure D.3-3) a peak of MMP-9 at day 15 is observed. However, this conclusion is weakened by the fact that the tumour-bearing mice that only received the emulsion present low

levels of MMP-9 along the experiment and also by the fact that the healthy animals present certain level of MMP-9.

Figure D.3-10: macromolecules detected in mice urine



MMP-9, IL-6, KC, VEGF and MIP-2 were detected in mice urine at different time points. Data represent the mean two replicate wells per condition and time point. Tumour-bearing mice without treatment PBS (Non-E No-bact, solid black line) or emulsion (OO-E No-bact, dotted black line) or treated with mycobacteria in OO-E (dotted lines) or Non-E (solid lines), live M. brumae (dark red), irradiated M. brumae (light red), live BCG (dark blue), irradiated BCG (light blue).

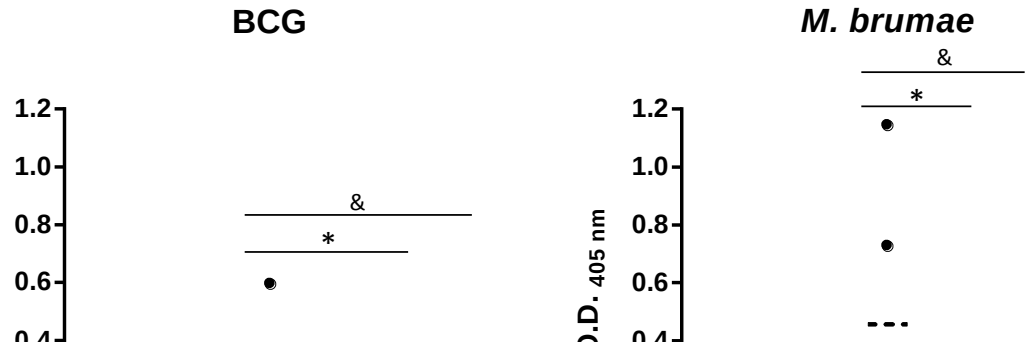
D.3.7. ANTIMYCOBACTERIA ANTIBODIES ARE FOUND IN TREATED ANIMALS SERUM

In order to assess the systemic humoral immune response that took place in the instravesically treated animals, blood samples were collected at day 29 after tumour induction and sera were analysed for the presence of specific anti-BCG or anti-*M.brumae* antibodies (IgG and IgA).

In all treated mice groups, significant levels of specific IgG in sera were found compared to the healthy mice except in the case of group of Non-E γ-irradiated *M. brumae*. When compared to the tumour-bearing mice only treated with the vehicle, these differences were not observed in any of the γ-irradiated-*M. brumae* groups neither in the Non-E γ-irradiated BCG group, (see Figure D.3-11).

Although no significant differences were observed between treated groups, a lower antibody response was observed in *M. brumae*-treated mice respect its homologous treatment with BCG; except in the case of the OO-E live *M. brumae* group compared to the OO-E live BCG that is the other way around, higher IgG levels were found in emulsified-*M. brumae* treated mice than in emulsified-BCG treated mice (see Figure D.3-11).

Figure D.3-11: mycobacteria-specific IgG detected in sera

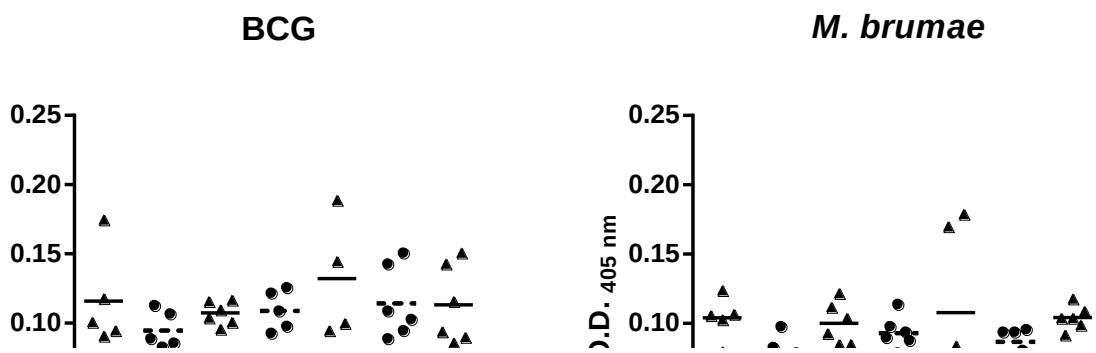


*Data represent the mean of the values of the animals of each group. Black triangles represent Non-E treatments and black dots represent OO-E treatments. In the graph on the left, there are represented the levels of specific IgG anti-BCG found in BCG treated mice sera and on the right, there are represented the levels of specific IgG anti-M. brumae found in M. brumae treated mice sera. * p< 0.05 respect the No-bact. & p< 0.05 respect the healthy animals (H).*

According to data obtained from BC patients, along the treatment with BCG, IgA levels in sera are lower than IgG levels [97]. The data showed that, this relationship between the levels of IgG and IgA in this animal model is also maintained. Moreover, in the case of IgA levels are so low

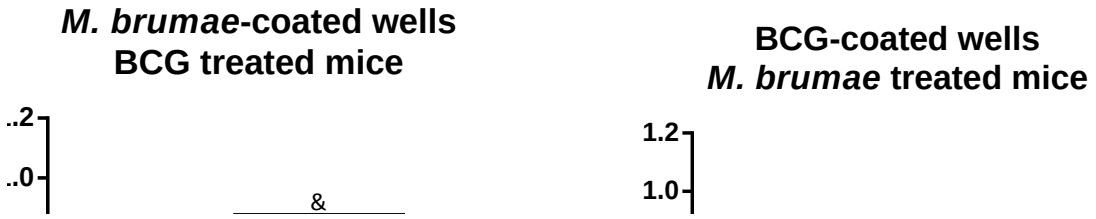
that no significant differences were found between the control groups and the treated groups (see **¡Error! La autoreferencia al marcador no es válida.**).

Figure D.3-12: mycobacteria-specific IgA detected in sera



Data represent the mean of the values of the animals of each group. Black triangles represent Non-E treatments and black dots represent OO-E treatments. In the graph on the left, there are represented the levels of specific IgA anti-BCG found in BCG treated mice sera and on the right, there are represented the levels of specific IgA anti-M. brumae found in M. brumae treated mice sera.

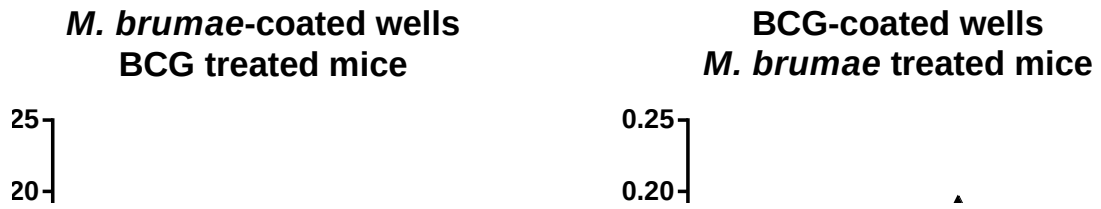
Figure D.3-13: amount of IgG that crosses reacts with BCG or *M. brumae* found in sera



*Data represent the mean of the values of the animals of each group. Black triangles represent Non-E treatments and black dots represent OO-E treatments. In the graph on the left, there are represented the levels of IgG anti-M. brumae found in BCG treated mice sera and on the right, there are represented the levels of specific IgG anti-BCG found in M. brumae treated mice sera. * p< 0.05 respect the No-bact. & p< 0.05 respect the healthy animals (H).*

Regarding cross reaction some inspecific binding was observed, even with the serum of healthy animals (see Figure D.3-13 and Figure D.3-14). In addition, although the differences are not significant, when No-bact groups are compared to the healthy group, it seems that MB49 gives a slight cross reaction with BCG and with *M. brumae* (see Figure D.3-11). Cross reaction between *M. brumae* and BCG is also observed; suggesting that some antigens are shared between *M. brumae* and BCG (see Figure D.3-13).

Figure D.3-14: amount of IgA that crosses reacts with BCG or *M. brumae* found in sera



Data represent the mean of the values of the animals of each group. Black triangles represent Non-E treatments and black dots represent OO-E treatments. In the graph on the left, there are represented the levels of IgA anti-M. brumae found in BCG treated mice sera and on the right, there are represented the levels of specific IgA anti-BCG found in M. brumae treated mice sera.

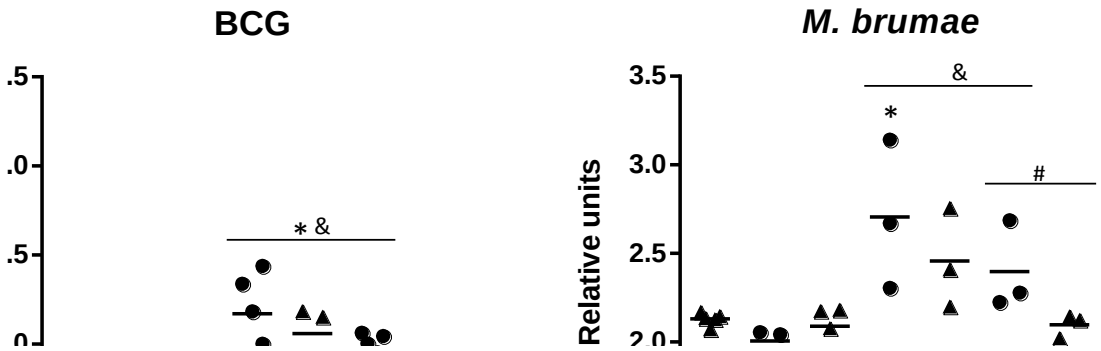
D.3.8. SPLENOCYTES RESTIMULATED WITH MYCOBACTERIA PRODUCE IFN-γ

Spleens were collected at day 29 after tumour induction to analyse the systemic immune response triggered by the intravesical treatments. The splenocytes were cultured in the presence of the same mycobacteria used to treat the animal in the h-k form for 72 h. At this time point, cell proliferation was measured to determine if there was taking place a specific response to the antigens, and also, supernatants were collected to detect the presense IFN-γ and IL-4 cytokines in order to observe if mycobacteria intravesical treatment induce a Th1 or a Th2 response, respectively [226].

Splenocytes proliferation was induced in all groups except in those treated with Non-E γ-irradiated *M. brumae*, compared to healthy animals. This suggests that there was a specific response to the antigens because they were previously been in contact with the mycobacteria.

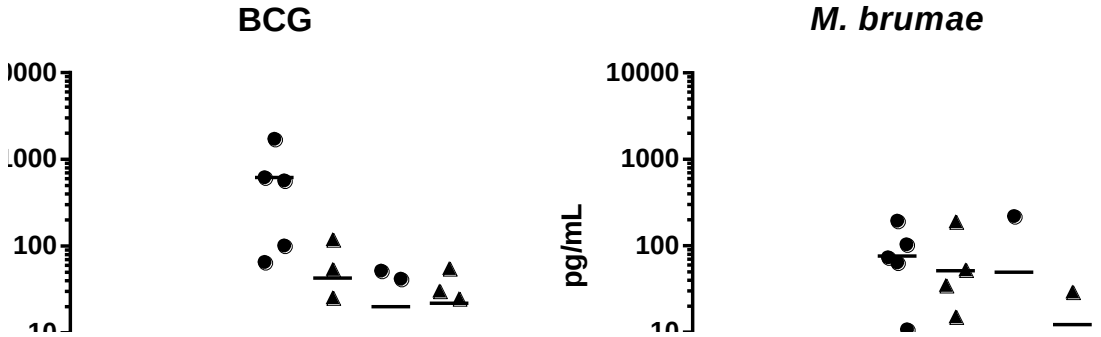
In general, *M. brumae* restimulated splenocytes proliferate more than BCG restimulated ones. But, statistical differences were only found between γ-irradiated *M. brumae* groups and their respective γ-irradiated BCG groups (see Figure D.3-15). No significant differences were observed between any of the other mycobacteria treated groups.

Figure D.3-15: proliferation of the splenocytes restimulated with the same mycobacteria



*Data represent the relative values to the basal proliferation of the splenocytes without being restimulated with mycobacteria. Mean of the values of each group are represented by solid lines. Black triangles represent Non-E treatments and black dots represent OO-E treatments. In the graph on the left there are the animals treated with BCG and on the right, the ones that received M. brumae. * p< 0.05 respect the No-bact. & p< 0.05 respect the healthy animals (H). # p< 0.05 respect the respective treatment with BCG.*

Figure D.3-16: IFN-γ release by the restimulated splenocytes



Data represent the pg/mL of IFN-γ detected in the restimulated splenocytes supernatants. Mean of the values of each group are represented by solid lines. Black triangles represent Non-E treatments and black dots represent OO-E treatments.

When the production of cytokines in splenocyte culture supernatants was analyzed, the first result observed was that ConA triggered the production of IFN-γ in all spleen cell cultures and of IL-4 only in some of them. The levels of IFN-γ (around 5000 pg/mL) were higher than those obtained of IL-4 (around 40 pg/mL). The results of the splenocytes proliferation are consistent with those obtained by the measurement of the produced IFN-γ in their supernatants. Regarding the specific restimulation, in all mycobacteria-treated mice a response was observed, while in any of the control groups an IFN-γ response was induced; all the obtained values were under the limit of detection of the kit (see Figure D.3-16). This fact, make impossible a statistic comparison to the controls and, moreover, no significant differences between the groups were observed (see Figure D.3-16). The fact that splenocytes are specifically restimulated by mycobacteria means that memory T cells are found in the spleen [211].

IL-4 was also measured in the supernatants of the specifically stimulated splenocytes but was not detected in any case. The synthesis of IFN-γ and the absence of IL-4, suggested that the specific splenocytes are Th1 and not Th2 [226].

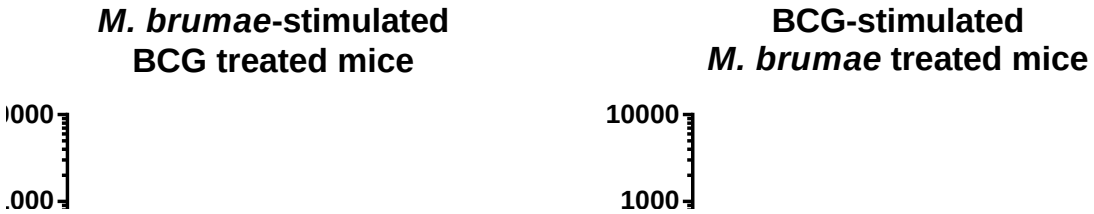
Figure D.3-17: proliferation of the splenocytes restimulated with the other mycobacteria

*Data represent the relative values to the basal proliferation of the splenocytes without being restimulated with mycobacteria. Mean of the values of each group are represented by solid lines. Black triangles represent Non-E treatments and black dots represent OO-E treatments. * p< 0.05 respect the No-bact. & p< 0.05 respect the healthy animals (H).*

Moreover, when splenocytes where cross restimulated, which means that the antigens used to restimulate do not correspond to the treatment that the animal received, it can be observed the same tendency mentioned before: *M. brumae* restimulated splenocytes proliferate more than the BCG restimulated ones (see Figure D.3-17). Again, as was seen for the antibody response,

determinate shared antigens between *M. brumae* and BCG seemed to be present. Interestingly, practically any animal's splenocytes showed induccion of IFN-γ production when cross stimulated (see Figure D.3-18).

Figure D.3-18: IFN-γ release by the cross-stimulated splenocytes



Data represent the pg/mL of IFN-γ detected in the cross-stimulated splenocytes supernatants. BCG treated mice splenocytes were stimulated with H-K-M. brumae and M. brumae treated mice were restimulated with H-K BCG. Mean of the values of each group are represented by solid lines. Black triangles represent Non-E treatments and black dots represent OO-E treatments.

E. General discussion

The clinical practice to treat NMIBC patients consists in a course of repeated instillations of BCG in its live form; this treatment could be elongated from 1 to 3 years. This maintenance schedule avoids recurrence and progression of the disease. Although this last affirmation is controversial, this is the currently accepted treatment schedule [36,106]. NMIBC patients receiving BCG instillations need a close follow up during the therapy and during the following years after finishing the instillations. For this reason, the cost per patient from diagnosis to death is the most expensive of all cancers [19].

BCG therapy presents mild to severe associated side effects. In general, a high percentage of patients abandon BCG therapy due to toxicity [36]. Moreover, due to the need to perform repeated instillations it increases the risk to perform a traumatic catheterization that can easily provoke a systemic infection and cause the death of the patient; even years after finishing the treatment [48,227–230].

A possible alternative to avoid BCG associated toxicity is the use of environmental mycobacteria. In this sense, recently it has been described the antitumour capacity of *M. brumae in vitro*. In view of these results, the general aim of the present thesis was to further investigate the therapeutic potential of this mycobacterium. Thus, the first step was to try to improve the therapy. The highly hydrophobic cell wall of mycobacteria makes them to be prone to clumping. In the present work, it has been demonstrated, that *M. brumae* is also highly hydrophobic since it has high affinity for n-hexadecane (see Figure C.3-4) and also aggregates when suspended in the kind of solutions used to instil BCG in patients, aqueous solutions (see Figure C.3-6) [231]. Aiming to find a proper vehicle which better disaggregate the mycobacteria clumps to improve the interaction with the BC cells and induced an improved immune response different emulsions were designed to emulsify *M. brumae*. Emulsions are oil and water phase systems that have been used not only to overcome the problem of aggregation but also to improve the immune response to different antigens (see Table C.1-1). For instance, Freund's adjuvant is an emulsion that contains or not heat-killed (h-k) mycobacteria that has been used for many years as adjuvant [165,186].

A series of O/W and W/O emulsions were made using two emulsifying methods, different proportions between the hydrophobic and the hydrophilic phases and by using four different oils: Olive Oil (OO), Soybean Oil (SO), Squalene (SE) and Mineral Oil (MO) (see Table C.2-1).

Emulsions were discarded for being heterogeneous or being too difficult to prepare or reproduce (see Figure C.3-1). So, W/O emulsions were discarded, as well as, one of the emulsifying protocols was. The criteria to be chosen were only accomplished by O/W emulsions; particularly, it was observed that more homogeneous suspensions were obtained when *M. brumae* was mixed with the oil containing already the surfactant, instead of having the surfactant in the polar phase, as some authors did [119,150].

Once one of the proportions between oil and water and a protocol to emulsify were chosen, it was time to select one of the oils. One important criterion to choose between the oils was that the emulsified *M. brumae* maintained its viability because it has been observed that BCG viability is important for the efficacy of the therapy; this has been described *in vitro* [137], *in vivo* [52] and also in a clinical trial it was observed that BCG ampoule lots with low viability drove to therapy failure [232].

M. brumae viability was assessed by CFU counting (see Figure C.3-5) and by observing the mycobacteria stained with a live/death kit by confocal microscopy (see Figure C.3-6) and the same result was obtained by using both techniques, OO-E better maintained *M. brumae* viability followed by MO. Noteworthy, regarding the percentages of viable *M. brumae* in the OO or MO emulsions were over 94%, the same as if they were resuspended in PBS (see Figure C.3-7). The fact that the correlation between the two techniques used to assess *M. brumae* viability was not perfect can be attributed to the fact that in certain formulations *M. brumae* is more aggregated which provoked a bias in the CFU counts (see Figure C.3-5 and Figure C.3-7). Indicating that emulsified mycobacteria present the same viability that when resuspended in the vehicle used in the clinical practice and there is the added advantage that mycobacteria cells are less aggregated.

As already mentioned, one of the objectives of using emulsions was better disaggregate *M. brumae* cells. Thus, at the same time, aggregation of *M. brumae* emulsified by using the different oils was calculated based on the same confocal images used to assess *M. brumae* viability. Using MO not only clumps were smaller but also more bacteria were single. Moreover, good results were also obtained when using OO, over 60% of the bacteria were forming small aggregates or were single.

Taking into consideration these results, SO and SE were discarded and only OO and MO were used for the *in vitro* experiments since they were the ones that better maintained *M. brumae* viability and reduced the size of the clumps. Both formulations were assayed for their effect on the human BC cell line T24. Moreover, since the aim was to test the emulsified mycobacteria in the murine animal model it was necessary to test them first in the mouse BC cell line, so MB49 cell line was also included in the experiments.

In the first experiments performed using emulsions on BC cell cultures it was observed that the emulsion by itself had an effect on the capacity of adherence of the eukaryotic cells on the plastic surface, so the protocol was optimized and three hours after infection, washes were performed using complete medium instead of PBS in order to completely eliminate the emulsion from the wells. As seen in Figure C.3-10, emulsions by them self have no inhibitory effect on the BC cells so, this way the effect of the mycobacteria was not masked by the emulsion.

After optimizing the protocol, the results showed that *M. brumae* or BCG emulsified using OO performed a higher antitumour effect that when formulated in MO. Regarding the proinflammtory response, OO and MO emulsions containing mycobacteria triggered the release of higher amounts of IL-6 and IL-8/KC than the Non-E mycobacteria (see Figure C.3-11). The fact that the inhibitory effect of MO-E mycobacteria was not as powerful as the exerted by OO-E does not mean that the initial hypothesis that smaller clumps would ease the interaction between the mycobacteria and the BC cells is wrong, probably it only mean that there is another factor influencing mycobacteria's internalization which prevents the release of proinflamatory cytokines [69]. MO emulsions had been widely used to emulsify mycobacteria cell wall extracts to avoid the problem of aggregation of this kind of adjuvants (see Table C.1-1), however, the results showed in this dissertation suggest that in the case of using whole live bacteria, the use of OO has a higher direct antitumour effect.

In view of the cell culture results, only OO emulsion was selected as the one to be tested in the animal model. But, before moving to the animal model, the emulsion needed to be characterized to claim that it was an O/W emulsion and not the other way around.

The O/W nature of the emulsion was confirmed by three techniques [186]. The drop test confirmed that the behaviour of the emulsion was aqueous which meant that the continuous phase was the water (see audiovisual content in the CD: "Drop test"). The same emulsions used for the drop test were observed by light microscopy in combination with fluorescence microscopy. Light microscopy clearly showed oil droplets contained in a continuous water phase while fluorescence revealed that *M. brumae* was contained in these oil droplets forming small groups or as single cells (see Figure C.3-13). Moreover, the third technique used to assess the O/W nature of the chosen emulsion was the observation of it by FESEM. The observations clearly confirmed what was already seen by confocal, light and fluorescence microscopy: single *M. brumae* cells or in small groups were contained inside the oil phase that was forming droplets (see Figure C.3-14).

Since reaching the target cells is key for any successful therapy, the next step was to unravel whether the emulsion hypothetically favoured the interaction between *M. brumae* cells and the bladder epithelium, by assessing some physicochemical properties of the emulsion. It was

expected that the emulsion favoured this interaction because it was not the first time that emulsions were used for intravesical delivery (see Table C.1-1).

Hydrophobicity, ζ-potential and pH are parameters that have an effect on the interaction of drugs with bladder epithelium. It has been described that if the drug presents pH values around 5, low hydrophobiciy and positive ζ-potential values the interaction with the urothelium is eased because the mucin layer that covers the bladder wall is negatively charged as well as mycobacteria cell wall and also this mucin layer is hydrophilic while the mycobacteria is hydropobic (see Section A.1 and Figure C.3-4) [6,26,28]. Differences in the three parameters were observed when comparing OO-E *M. brumae* and Non-E *M. brumae*. The emulsion seemed to favour the interaction with the urothelium because it present a lower pH and hydrophobicity than Non-E *M. brumae* and, although ζ-potential values were still negative, were closer to the neutrality when *M. brumae* was emulsified.

Therefore, it has been designed an O/W emulsion that is homogeneous, maintains *M. brumae* viability, reduces the size of *M. brumae* clumps and potentially favours the interaction between *M. brumae* and the urothelium.

In view of the promising results obtained *in vitro* with the OO-E mycobacteria, the orthotopic BC murine model was used in order to test the different treatment options already tested *in vitro* and also, the combination of OO-E and/or γ-irradiated mycobacteria, with both BCG and *M. brumae*.

Remarkably, the BC model was well established because all animals experienced haematuria, a hallmark of tumour uptake [197] and tumours were confirmed by histology (see Figure D.3-4). Moreover, the results of survival rates that were obtained using BCG resuspended in PBS were the same as the ones previously obtained by other authors [95,201,210]. All this evidence validates the obtained results.

M. brumae treated mice survived as well as BCG treated animals did. Interestingly, the survival rates of the four groups of *M. brumae* treated mice (live and γ-irradiated, in emulsion or not) were very similar (see Figure D.3-2 and Figure D.3-3) and only when the lower dose was used to treat the animals, they started to progressively die after the second instillation until the end of the experiment (see Figure D.3-1). This dose dependency has been already described *in vivo* for BCG [212,213] and *in vitro* for *M. brumae* [138,158]. Although, in Figure D.3-1 no statistically significant differences are observed, it can be said that the factor that influence the most the survival rates is the dose. This had been already observed *in vitro* on T24 cell line; comparing the

results of BC cell growth inhibition of γ-irradiated *M. brumae* (62.91% of proliferation) compared to its live form (52.54%) [185], and the results of OO-E (55.45 %) and Non-E (52.49 %) *M. brumae* (see Figure C.3-10), only slightly differences are observed, in contrast when lower doses of *M. brumae* were assayed its the inhibitory capacity drastically decreased (90.96% of proliferation) compared to the five-fold (76.26%) and the twenty-five-fold (63.16%) higher doses [158].

Although in the present work it has not been assessed the immune response triggered by the lower dose of *M. brumae* in the animal model, this lose of efficacy could be explained by an unbalance between the local Th1 and Th2 response. As regards the local immune response elicited by BCG related to the prognosis of the NMIBC patients, it has been described that BCG responders release higher amounts of Th1 cytokines like IFN-γ, IL-12 or IL-2 than Th2 cytokines like IL-4, IL-5 o IL-10, in the urine. On the contrary, if the response is balanced to Th2, it drives to therapy failure [206,211]. Furthermore, E. De Boer *et al.* point out that the activation of the Th1 response is necessary for BCG efficacy and they found that Th1 response was decreased when lower doses of BCG are used to treat a BC mouse model. However, Th2 responses were found to be less affected by lowering the dose [92].

M. brumae's non-pathogenicity was confirmed *in vivo*. Previous *in vitro* studies demonstrated its incapacity to persist intracellularly in T24 BC cells and in J774 cells [138,158] and in the present work it was unravelled that the fact of being in emulsion did not change this (see Figure C.3-12). In the animal model *M. brumae* showed that was unable to persist in the mice spleens even one week after the last instillation, either emulsified or not. Since *M. brumae* specifically restimulated splenocytes to proliferate and to secrete IFN-γ, it can be said that *M. brumae* reaches the bloodstream and arrives to the spleen, but, somehow is cleared from there in a short time [211]. However, another hypothesis would be that only some *M. brumae* antigens reach the bloodstream and reach the spleen.

This cellular systemic response that *M. brumae* exerts may be an indicator of good prognosis because some studies described a correlation between becoming PPD positive during the treatment and the clinical response [96,233,234]. However, this correlation is controversial because some other studies were unable to establish it [235,236] or only observed a tendency [59]. In clinical trials it has been proved that the PPD status of the patient before initiating the BCG treatment for BC it is a significant factor to determine the patient recurrence-free survival [95].

Since BCG therapy was first assayed in BC patients, toxicity has associated to the therapy. Since then, with the strong aim to improve the therapy safety, BCG has been assayed h-k [113,114], irradiated [112,135,136] or sonicated mycobacteria [237], or cell wall extracts [147–149,238] obtaining different degrees of success.

Although it has been said that *M. brumae* is unable to persist in mice spleens it was worthy to assay the effect of γ-irradiated *M. brumae* in the BC mouse model for several reasons. First, because it showed better results than h-k in *in vitro* studies [185]. γ-irradiated BCG and *M. brumae* are unable to replicate so are considered not viable, however some metabolic activity is retained so the concept Killed But Metabolically Active (KMBA) can be applied to these γ-irradiated mycobacteria [137,185]. Thus, KBMA bacilli present the advantages of the death mycobacteria (incapacity to replicate) and the advantages of the live mycobacteria because they retain some metabolic activity that is enough to initiate a potent immune response [140,141]. An example of a KBMA bacteria is *Salmonella typhimurium* under UV light [239]. Second, many environmental mycobacteria showed efficacy for the treatment of cancer *in vitro* [158,185] and *in vivo* [112,119,121] and clinical trials [126] but they have been tested in their killed form, because although non-tuberculous they have been isolated in patients. For instance infections in patients have been described to be caused by *M. phlei* [154,240–242] and *M. vaccae* [154,155]. In the case of *M. brumae*, it was described a case of nosocomial infection of a breast cancer patient [160], however, later, it has rectified the infection was not caused by *M. brumae* [161]. Nevertheless, the option of administering death *M. brumae* needs to be considered. Third, because having information about the immune response in the animal model is appreciable in terms of unravelling the mechanisms of action of this mycobacterium in tumour clearance.

At the time to test the efficacy of γ-irradiated mycobacteria in the BC animal model, the animals received a first instillation of live mycobacteria and, only in the three subsequent instillations, γ-irradiated mycobacteria was administered (see Figure D.2-2). This schedule was selected because it has been described in this animal model that if only h-k mycobacteria is instilled, no influx of T cells is observed in the bladders [95]. Moreover, as mentioned in Section A.2.4.3, E. De Boer and collaborators studied, in the orthotopic mouse model, the differences between cytokine gene expression if only live or h-k BGC was administered in a course of 6 instillations and, also, the effect of treating the animals with live BCG in the first three instillations and with h-k in the three subsequent ones. Their results show that when only h-k BCG was administered, Th1 cytokines are not released; this could explain why C. Biot *et al.* are unable to detect T cells in the bladders. On the contrary, when live BCG is instilled and only the three last instillations are with h-k BCG, Th1 cytokine levels are significantly higher than when animals only received h-k BCG and no significant differences were observed compared to the animals that were only instilled with live BCG [92]. Many authors agree that at least the first instillation has to be of live BCG to obtain a proper antitumour response. Repeated instillations

are needed to perform a T cell in the bladder or of the splenocytes. Maybe an antigen only present in the live form is necessary [95,211].

The *in vivo* results featured in the present work showed that this treatment schedule was enough to have a local and a systemic response. However, γ-irradiated BCG treatment was not as successful as γ-irradiated *M. brumae* in maintaining tumour-bearing mice's survival (see Figure D.3-2 and Figure D.3-3). Moreover, the fact of instilling γ-irradiated BCG in emulsion did not improve survival results either (see Figure D.3-3). Higuchi and collaborators, described that BCG activated PBMC (T, γδ T, B, NK and NKT cells) are cytotoxic against T24 cell line and they found NKT and γδ T cells activated by DC to be the main responsible of this cytotoxicity. However, when they activate the DC by h-k BCG they observed no cytotoxicity of the PBMC against T24 [80]. Furthermore, it has been observed that γ-irradiated BCG- and γ-irradiated *M. brumae*-activated PBMC are not as cytotoxic for T24 cells as PBMC activated by live mycobacteria [137,158,185]. Dendritic cells in the spleen process the antigens that arrive to this organ though the bloodstream and process them to be presented to the B cells and $CD4^+$ and $CD8^+$ T cells of the spleen [215,243]. In the results presented in Section D.3.8, it be seen that splenocytes of γ-irradiated BCG treated mice produce less INF-γ than live BCG treated mice, surely because γ-irradiated mycobacteria are not able to activate splenocytes as well as live mycobacteria do.

Regarding the fulfilment of the objective of diminishing the risk of infection by BCG, not significant lower amounts of BCG were recovered from those animals that received one single instillation of live BCG compared to those that received the four instillations. The amounts of viable CFU recovered from the spleens directly correlates with the amounts specific IgG detected in mice sera and with the fact that splenocytes specifically respond to BCG restimulation. This correlations have been previously described for BCG in the tuberculosis infection mouse model [214,215]. Moreover, it is worth mentioning that the date of sacrifice (day 29 after tumour induction) was chosen because it was the proper time point to assess the local immune response that was taking place inside the bladder and time enough to detect a T cell memory response [95,206]. However, to better assess the humoral response, the detection of specific antibodies in sera; it would have been better to choose a later time point, such as 12 weeks after the first mycobacteria treatment. At this time point, it would have been possible to detect greater amounts of antibodies in sera [215].

Despite the fact that all groups treated with live mycobacteria in emulsion give better survival rates than the Non-E in the case of irradiated BCG the fact of being emulsified did not

improve the survival rates of these animals (see Figure D.3-2 and Figure D.3-3). *In vitro* OO-E mycobacteria made MB49 cell line to release higher amounts of IL-6 and KC than the Non-E mycobacteria (see Figure C.3-11). These cytokines are responsible for the influx of macrophages and T cells and in turn these cells induce the activation of T cells and NK cells [72,207]. In the animal model these proinflammatory cytokines has been detected but nothing can be said about whether the fact of being emulsified improves the immune response exerted by γ-irradiated mycobacteria. Moreover, regarding immune cell infiltration into the bladder the data showed that OO-E γ-irradiated mycobacteria were unable to trigger a higher infiltration of immune cells compared to the Non-E γ-irradiated ones (see Figure D.3-2 and Figure D.3-3).

In general in the obtained *in vivo* results, the same tendency is observed in all the parameters studied. OO-E live mycobacteria showed higher survival rates, higher infiltration into the bladder and higher cellular and humoral systemic response than Non-E live mycobacteria. The lower values were obtained when animals received only the first instillation with live mycobacteria and the other three of γ-irradiated mycobacteria.

Despite not having detected Th1 and Th2 related cytokines in treated mice urine, information about lymphocytes subsets infiltrated into the bladder showed high amounts of T cells and B cells and, in addition, memory Th1 splenocytes (INF-γ producers) were found in the animals' spleens. Moreover, specific immonuglobulines were found in mycobacteria treated animals sera. Thus, the results confirm that BCG elicits humoral (Th2) and cellular (Th1) immune responses and that this response is mainly biased to a Th1 response, as well as, *M. brumae* does (see Sections D.3.5, D.3.6, D.3.7 and D.3.8).

The fact that the results obtained using BCG and *M. brumae* are similar is probably due to the observation made when looking at the systemic response; a cross reaction was observed between the two species in both the humoral and the cellular responses. The fact that cross reaction is observed when looking at splenocyte proliferation while these splenocytes do not produce INF-γ indicates that cross reaction induces the proliferation of B cells and/or $CD8^+$T cells, the other memory cells present in the spleen, but Th1 $CD4^+$ cells are not cross stimulated to produce INF-γ.This indicates that BCG and *M. brumae* share some crucial antigens to activate a suitable response for tumour clearance but not some others.

Mice are the tool of choice to investigate in almost every field of the biomedicine, such as physiology, immunology and for testing new therapies. Ectopic cancer models, such as, subcutaneous models, permit a rapid screening of new therapies however, not only in the case of BC, the orthotopic mouse model is the one that more reliably mimics cancer behaviour in the

clinical situation [199,244,245]. in particular, the MB49 orthotopic mouse model is a powerful tool because Chen and collaborators conclude that this cell line mimics the response to BCG that occurs in human BC cell lines, so it is a valid model to make studies *in vitro* and *in vivo* [246]. Regarding the immune response, mice are also the tool of choice of many immunologists and huge advances had been made thanks to this animal model, nevertheless, the differences has to be taken into account [189]. To study immunotherapies, the use of a syngeneic model is mandatory, because xenogeneic for which human BC cells are used to induce the tumour require to use an athymic mouse which is not able to perform the immune response to the BCG [86].

Although the literature is full of cases in which a therapy perfectly worked on mice, failed in human, the results obtained with *M. brumae* have been assayed in parallel with BCG and the results are very similar for both strains, in all the stages of the work, *in vitro*, *ex vivo*, *in vivo* in terms of animal survival rates and in terms of the triggered immune response it can be said that *M. brumae* would be a good candidate to substitute BCG in the clinical practice.

Furthermore, recently, a manufacturing problem occurred and BCG it is being restricted for the treatment of NMIBC patients. Physicians have already noticed these shortages. A survey carried out by the BJU international revealed that the 37% of the survey respondents has no BCG availability in their hospitals and the 45% of the polled ones said there was reduced supplies [247].

Thus, due to these problems, physicians agree that alternatives to BCG need to be found [36,106,210,248].

F. Conclusions

1. An improved O/W emulsion that results in a stable and more homogeneous suspension of *M. brumae* was designed.

2. Olive oil-in-water and mineral oil-in-water emulsions are adequate suspensions to maintain *M. brumae* viability and to disaggregate clumps.

3. The physicochemical properties conferred to *M. brumae* suspension by the fact of being emulsified, pH around 5, lower hydrophobicity and a negative charge closer to the neuter, respect to the non-emulsified suggest that the interaction between mycobacteria and the bladder epithelium would be eased.

4. Mycobacteria contained in olive oil-in-water emulsion inhibit tumour cells growth and trigger a higher release of proinflamatory cytokines response than non-emulsified mycobacteria.

5. Intravesical instillation of live *M. brumae* in tumour-bearing mice, enhanced survival rates at a similar extent to BCG. The response to *M. brumae* treatment is dose-dependent. In fact, the dose was the factor that has a greater impact on *M. brumae* treated mice survival rates, neither the emulsion nor the fact of being administered γ-irradiated had such an impact.

6. Intravesical instillation of γ-irradiated *M. brumae* enhance the survival rates in tumour bearing mice respect the ones that received no bacteria, although not as efficiently as live *M. brumae*. In the case of BCG, intravesical γ-irradiated BCG treatment presents statistically lower survival rates than tumour-bearing mice treated with live *M. brumae*.

7. Intravesical instillation of olive oil-in-water emulsified *M. brumae* present higher survival rates than oil-in-water emulsified BCG treatment.

8. Unlike BCG, after intravesical instillation of *M. brumae*, no bacteria are recovered from spleens, confirming the non-pathogenicity of *M. brumae*.

9. Intravesical instillation of *M. brumae* triggers a local immune response in tumour-bearing mice triggering the infiltration of different subsets of immune cells. This response is higher when four instillations of live mycobacteria are administered compared to γ-irradiated group, in contrast, both treatments induce similar survival rates.

10. The intravesical instillation of both BCG and *M. brumae* induce a systemic immune response in tumour-bearing mice compared to animals that did not received mycobacteria. This response is reflected by an increased production of IFN-γ in mycobacteria-stimulated splenocytes and a significant production of mycobacteria-specific IgG antibodies in sera from mycobacteria treated mice.

11. The intravesical instillation of olive oil-in-water emulsified mycobacteria trigger a higher immune response in tumour-bearing mice that the treatment using non-emulsified mycobacteria.

12. Olive oil-in-water emulsified *M. brumae* shows to be a promising candidate to substitute BCG in the treatment of no muscle invasive bladder cancer.

G. Supplementary material

Due to issues of copyright or for being audiovisual files, some of the supplementary material of this thesis is in the CD placed in the back flap. If this is the case it will be indicated the directory and the name of the file.

G.1. MANUSCRIPTS

This thesis is reflected the following manuscripts. The complete manuscripts are in the folder "Manuscripts" in the CD.

G.1.1. MANUSCRIPT 1

Olive oil-in-water emulsion of mycobacteria improves its antitumor activity against bladder cancer

Estela Noguera-Ortega, Núria Blanco-Cabra, Rosa Rabanal, Alejandro Sánchez-Chardi, Mónica Roldán, Eduard Torrents, Marina Luquin, Esther Julián

To be submitted to Journal of Controlled Release, September 2015

This manuscript contains the majority of the results presented in Chapter I and the other part are in Chapter II.

G.1.2. MANUSCRIPT 2

Non-pathogenic *Mycobacterium brumae* inhibits bladder cancer growth in vitro, *ex vivo* and *in vivo*.

Estela Noguera-Ortega†, Silvia Secanella-Fandos†, Hasier Eraña, Jofre Gasión, Rosa Rabanal, Marina Luquin, Eduard Torrents, Esther Julián

†These authors contributed equally to this work

Accepted in European Urology FOCUS, May 2015

This manuscript contains part of the results presented in Chapter II.

G.1.3. MANUSCRIPT 3

γ-Irradiated mycobacteria enhance survival in bladder tumor-bearing mice although they are less efficacious than live mycobacteria

Estela Noguera-Ortega, Rosa Rabanal, Silvia Secanella-Fandos, Eduard Torrents, Marina Luquin, Esther Julián

In press in The Journal of Urology, September 2015

This manuscript contains part of the results presented in Chapter II.

G.1.4. PATENT

Oil-in-water formulations of mycobacterium and uses thereof

Estela Noguera-Ortega, Marina Luquin, Esther Julián

This text contains part of the results presented in Chapter I.

G.2. OTHER PUBLICATIONS

Killed but Metabolically Active Mycobacterium bovis bacillus Calmette-Guérin Retains the Antitumor Ability of Live bacillus Calmette-Guérin

Silvia Secanella-Fandos, Estela Noguera-Ortega, Francesc Olivares, Marina Luquin and Esther Julián

Published in The Journal of Urology Vol. 191, 1422-1428, May 2014

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