

Investigating the impact of elevated circulating mitochondrial DNA on melanoma metastasis

By

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This thesis is 37,464 in length.

A handwritten signature in black ink, appearing to read 'Mona Saad', with a stylized flourish at the end.

Mona Saad

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Abstract

Melanoma is the most aggressive, deadly form of skin malignancy. Despite only accounting for 5% of skin malignancy cases, it is the leading cause of death in the world of skin cancer. The prognosis of patients with metastatic melanoma is grim, with a 5-years survival rate of 5-19%. Melanoma affects people of working age, having physical, psychological, social and financial implications. Current diagnostic tools are invasive, anxiety inducing and costly. The clinical utility of biomarkers in melanoma remains unclear. This has been identified as one of the National Institute for Health and Care Excellence melanoma future research recommendations. Circulating DNA has been suggested to be a potential biomarker in cancer diagnosis with elevated circulating mitochondrial DNA (mtDNA) being associated with increased risk of cancers such as, breast, non-Hodgkin lymphoma and renal cell carcinoma. In melanoma, a handful of studies show increased mtDNA level is associated with the disease. However, no study has investigated mtDNA in the context of metastatic melanoma. In this thesis I investigate patient serum levels of cell-free mtDNA in metastatic melanoma and its potential role as a novel non-invasive biomarker. I also investigate the role of elevated mtDNA in promoting melanoma disease progression specifically investigating the liver as a known melanoma metastatic site.

My results indicated that circulating cell free mtDNA is elevated in metastatic melanoma in both human and melanoma engrafted mice. In our small sample size, the circulating cell free mtDNA level distinguished between the patient group who remained in remission compared to the group who developed a relapse post tumour resection. This highlights the potential role mtDNA may play as non-invasive biomarker in melanoma. Mechanistically, in mice, I show that melanoma engraftment mediated a downregulation of Stimulator of Interferon Genes (STING) in the liver. STING is known to play a role in innate immunity. The liver macrophages in melanoma engrafted mice exhibited a pro-tumour M2 like phenotype. To our knowledge this is the first data that has demonstrated that melanoma engraftment in mice resulted in the downregulation of STING in liver macrophages. Overall, the data presented provides an insight into the functional role that mtDNA plays in promoting an immunosuppressive microenvironment in melanoma facilitating disease progression.

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List of abbreviations

APC- Antigen-presenting cell

BMDM- Bone marrow derived macrophages

BT- Breslow thickness

CT- Computed tomography

cGAMP- 2'3' cyclic GMP-AMP

cGAS- Cyclic GMP-AMP synthase

CTCs- Circulating tumour cells

ctDNA- Circulating tumour DNA

cfDNA- Cell free DNA

CTL- Cytotoxic T lymphocyte

CTLA-4- Cytotoxic T lymphocyte-associated antigen-4

DC- Dendritic cell

DDR- DNA damage response

ECM- Extracellular matrix

ER- Endoplasmic reticulum

FBS- Foetal bovine serum

IFN- Interferon

IRF- Interferon regulator factors

ICI- Immune checkpoint inhibitors

IL- Interleukin

ICAM- Intercellular adhesion molecule

IV- Intravenous

IP- Intraperitoneal

KC- Kupfer cells

MM- Malignant Melanoma

MRI- Magnetic resonance imaging

MDA- Melanoma differentiation antigens

MDSCs- Myeloid-derived suppressor cells

MHC- Major histocompatibility complex class

DMXAA- 5,6-dimethylxanthenone-4-acetic acid (STING agonist)

MACS- Magnetic activated cell sorting

mtDAMP- Mitochondrial damage-associated molecular pattern

mtDNA- Mitochondrial DNA

NF- κ B- Nuclear factor kappa-B

NK- Natural killer

PET- Positron emission tomography

PB- Peripheral blood

PRR- Pattern recognition receptor

PD-1- Programmed cell death protein 1

PD-L1- Programmed cell death-Ligand 1

PBS- Phosphate-buffered saline

qPCR- Real time quantitative polymerase chain reaction

RPM- Rotations per minute

STING- Stimulator of interferon genes

SASP- Senescence-associated secretory phenotype

SC- Subcutaneous

SLNB- Sentinel lymph node biopsy

TAM- Tumor associated macrophages

TAA- Tumor associated antigens

TSA- Tumour specific antigens

TLR- Toll-like receptor

TLR9- Toll-like receptor 9

TRAF6- Tumour necrosis factor receptor associated factor 6

TBK1- TANK-binding kinase 1

TNF- Tumour necrosis factor

TME- Tumour microenvironment

VCAM- Vascular cell adhesion molecule

VEGF- Vascular endothelial growth factor

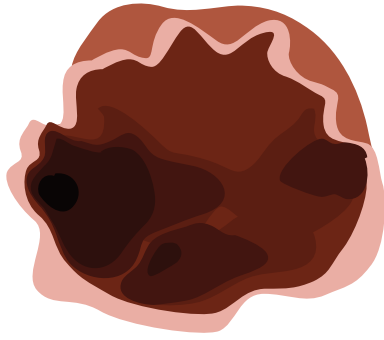
WLE- Wide local excision

1 Introduction

1.1 Melanoma

Melanoma is the most aggressive, deadly form of skin cancer (1). Despite only accounting for 5% of skin cancer cases, it is the leading cause of death in the world of skin cancer (1). The incidence of melanoma is among the fastest growing cancers world-wide, accounting for 55,000 deaths annually (1, 2). Melanoma is on the rise, and it is envisaged that the number of deaths worldwide are predicted to increase to 175% by 2040 (1, 2). Thus, further research is required to understand the complexity of this disease. The prognosis of patients with metastatic melanoma is grim, with a 5-year survival rate of 5-19% (2). Furthermore, melanoma affects much younger patients compared to other major cancers (3). Melanoma affects people of working age, having physical, psychological, social and financial implications (3). Better outcomes can be realised through new therapies or improved diagnosis, developed from a better understanding of the biology of the disease.

Melanoma arises from melanocytes cells situated in the basal layer of the epidermis (Figure. 1.1) (4, 5). Melanocytes are responsible for producing melanin, the pigment absorbing ultraviolet (UV) radiation (6, 7). It is the keratinocytes that initiate the process of melanin production (8). In response to UV radiation, keratinocytes release α -melanocyte stimulating hormone (α -MSH) (7). This binds to melanocortin 1 receptor (MC1R) located on the melanocytes resulting in melanin production (6, 7). This is then transferred to neighbouring keratinocytes, accumulating melanin in keratinocytes (4). It is this melanin pigment in keratinocytes in the top layer of skin that protects the living cells resident deeper in the skin (8).



1-1. **Melanoma**

A pigmented skin lesion with asymmetry, irregular borders and a non-uniform colour

Melanocytes produce two types of melanin, eumelanin and pheomelanin, producing a black/brown pigment and a red/yellow pigment respectively (9). It is the ratio of these two pigments that determines the skin colour as opposed to the number of resident melanocytes (9). Eumelanin the darker pigmentation provides greater protection against UV light (9). It is well known that people with lighter skin, blond/red hair and light eyes tend to have a higher risk of developing melanoma (9). The MC1R partly determines the skin, hair and eye colour (10). Variation in this MC1R gene determines the function of MC1R (10). Fully functional MC1R produces eumelanin, while reduced MC1R function results in the production of pheomelanin, which accumulate mutations mediated by UV exposure (10). Skin cancer arises as a result of the accumulation of such mutations (9, 10).

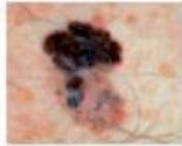
History of Melanoma

The first cases of melanoma documented was in 1651 where patients with 'black tumours' resulted in their death (11, 12). Melanoma was initially thought to be a fungal growth (12, 13). Surgical resection was the mainstay of treatment with a Scottish surgeon, John Hunter believed to have performed the first melanoma mass resection from a patient's mandible in 1787 (11, 13).

The genetics and hereditary components of melanoma was first noted in the 1820s where a patient with 'fungoid disease' was noted to have a father who passed of a similar disease (14). This observation was made almost 50 years before the understanding of Mendelian inheritance. It was also noted that patients with a family history of 'hereditary melanoma' appeared to have numerous moles (15, 16).

1.1.1 Melanoma: diagnosis and management

History and clinical examination form the initial steps in melanoma diagnosis. The 'ABCDE' system is used when clinically assessing a lesion (Figure 1.2) (17, 18).

Asymmetry	Border	Color	Diameter (>6mm)	Evolution or Elevation
				

1-2 The ABCDE system utilised when clinically examining a lesion

Asymmetry of the lesion, irregular borders, non-uniform colour and enlarging lesions are all red flags. The presence of one or more of these features makes the lesion more likely to be a melanoma, warranting urgent confirmation of the diagnosis (18).

A tissue biopsy for histological detail analysis is required to confirm a suspected diagnosis of melanoma (18). A histopathological analysis provides information on defining features of the tumour such as the type of melanoma, depth of invasion, presence of perineural invasion and ulceration, all of which have implications on the management and prognosis (17, 18). The depth of invasion of melanoma to the underlying layers is a crucial part of the histological report as it determines the risk of aggression, invasion and metastasis (17). This is known as the Breslow thickness (BT) which is the measurement in millimetres (mm) of the depth of melanoma from the skin down to the deepest layer of where the tumour resides (17, 18). BT is utilised to determine the prognosis and management of melanoma (19). It dictates the skin margins with which the tumour should be resected with (Table 1.1) (19).

Table 1.1 Breslow thickness and associated prognosis.

Breslow thickness with associated 5-year prognosis (19)			
Breslow thickness (BT)	5-year survival	Recommended resection	surgical margin
<1mm	95-100%	1cm	
1-2mm	80-96%	1-2cm	
2.1-4mm	60-75%	2cm	
>4mm	37-50%	2cm	

Over the years as data about the treatment and survival of melanoma patients mounted, a staging system known as the American Joint Committee on Cancer (AJCC) TNM (Tumour, node, metastasis) was developed (20). This dictates the management of melanoma patients today. The AJCC 8th edition was released in 2016 (Figure 1.3) (20, 21).

Tumour Size

- T1a** <0.8 mm without ulceration
- T1b** <0.8mm with ulceration or 0.8-1 mm without ulceration
- T2a** >1-2mm without ulceration
- T2b** >1-2mm with ulceration
- T3a** >2-4cm without ulceration
- T3b** >2-4cm with ulceration
- T4a** >4 cm without ulceration
- T4b** >4 cm with ulceration

Nodes

N0 No regional nodes

- N1a** 1 clinically occult node (detected by SLNBx)
- N1b** 1 clinically detected node
- N1c** In-transit, microsatellite or satellite with no regional node disease
- N2a** 2 or 3 clinically occult node (detected by SLNBx)
- N2b** 2 or 3 nodes, 1 clinically detected node
- N2c** 1 node and in-transit, microsatellite or satellite
- N3a** 4 or more clinically occult node (detected by SLNBx)
- N3b** 4 ore more, 1 clinically detected, or matted nodes
- N3c** 2 or more or any matted nodes + in-transit, microsatellite or satellite

Metastasis

- M0** No distant metastasis
- M1a** Skin, soft tissue including muscles, and/or nonregional lymph node
- M1b** Lung with or without M1a sites of disease
- M1c** Non-CNS visceral sites with or without M1a or M1b sites of disease
- M1d** CNS with or without M1a, M1b, or M1c sites of disease

There is a sub-classification if LDH is elevated. example M1a(1)

1-3 Melanoma classification (22).

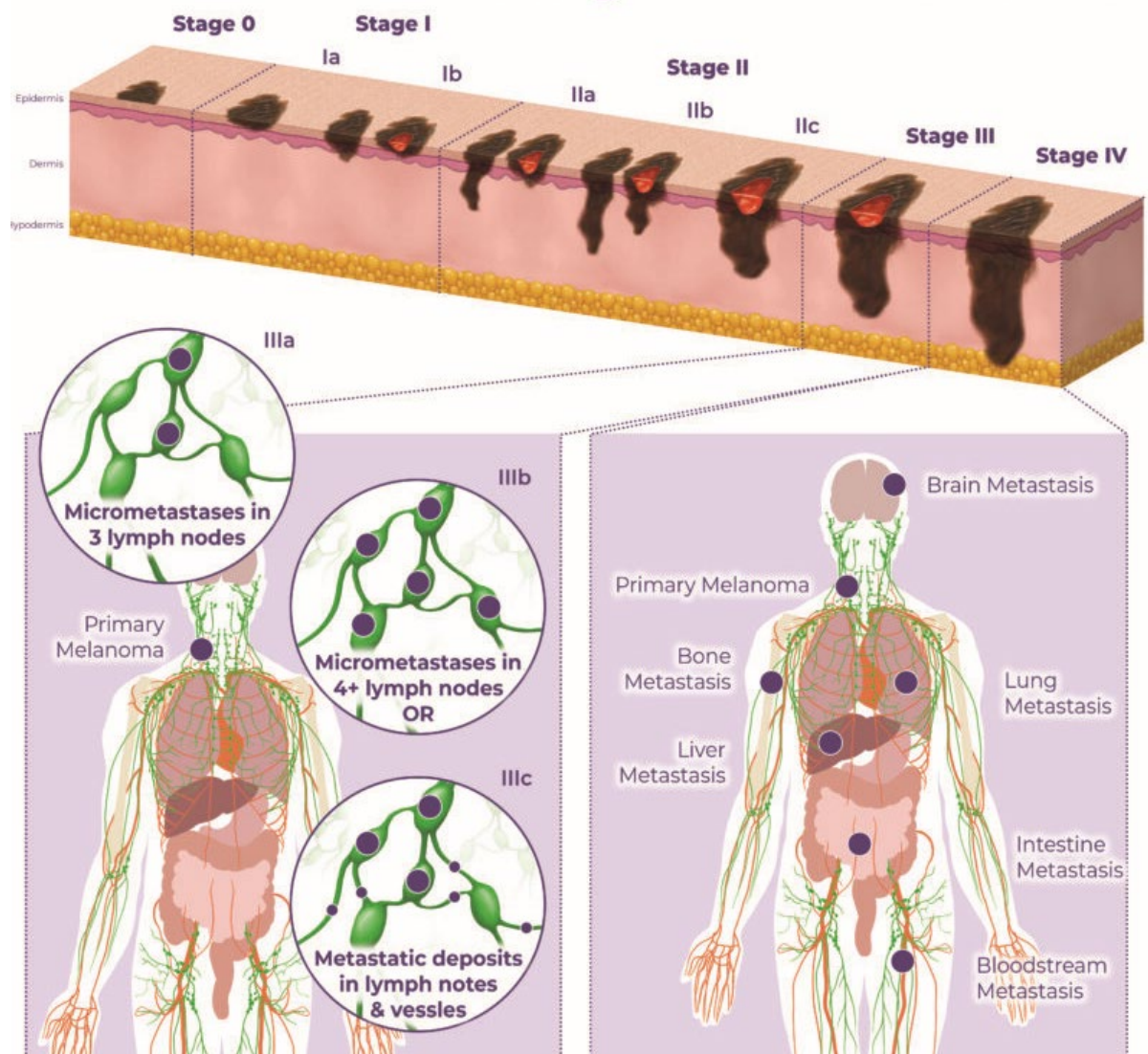
Melanoma is classified and staged using the internationally used TNM system from the American Joint Committee on Cancer (AJCC) 8th Edition (20, 22).

The clinical melanoma stages include stage 1-IV, which depends on thickness of the tumour, infiltrating capacity of the tumour, lymph node involvement and the presence of distant metastases (Table 1.2 & Figure 1.4) (20, 23, 24). Melanoma is staged I or II if there are no lymph node or distant metastasis involvement (20). Stage III signifies the presence of lymph node metastasis, and stage IV includes patients with distant metastasis (20).

Table 1.2 Melanoma staging

Melanoma staging: AJCC clinical and pathological staging assignment (20)		
Stage	TNM classification	5-year survival
Stage 0 (Melanoma in situ)	Melanoma in situ, No=0, M0	>98%
Stage I (A, B, C)	T 0.8-2mm +/- ulceration	92-97%
Stage II (A, B, C, D)	T >1.0-4.0mm +/- ulceration	53-81%
Stage III (local-regional lymph node involvement)	Any T, ≥ 1 N, M0	40-73%
Stage IV (distant metastasis)	Any T, any N, M1	15-20%

Stages of Melanoma



1-4 Illustration of melanoma staging 0-IV (25).

Stage 0: Melanoma in-situ, Stage I-II: depends on the histological features of the tumour, Stage III: local regional lymph node involvement detected either clinically or by sentinel lymph node biopsy (SLNB), Stage IV: signifies metastatic melanoma with metastasis to distant regions (20, 21, 25).

There is currently no consensus on the ‘ideal imaging’ for melanoma (26). Imaging outside of stage IV is not routinely recommended in melanoma, unlike other solid tumours (26). For stage IV, staging computed tomography (CT) of the thorax, abdomen and pelvis as well as magnetic resonance imaging (MRI) brain is carried out (26, 27). CT with or without positron emission tomography (PET) and an MRI are considered in stage III patients and in stage II if the melanoma has high risk features (27).

Melanoma subtypes

Cutaneous or skin melanoma is the commonest type of melanoma accounting for 94% of cases (28). Other types include uveal and mucosal melanoma accounting for roughly 5% and 1% of melanoma subtypes respectively (29-31).

Based on the histology and growth pattern, cutaneous melanoma is given its subtype name (Table 1.3) (32). This includes the most common type superficial spreading melanoma, the aggressive nodular melanoma, lentigo maligna melanoma, and acral lentiginous arising on palms, soles and nails (32). The acral lentiginous type is often detected late on due to its site (32). Other melanoma subtypes include the non-pigmented amelanotic melanoma, and desmoplastic melanoma which has a high local recurrence rate (32).

Subtype	Frequency	Characteristic
Superficial spreading	70%	Arises from existing nevus
Nodular	5%	Absence of radial growth, robust vertical invasion
Lentigo Maligna	4-15%	Typically exhibits slow progression
Acral lentiginous	5%	Higher incidence in patients with darker skin pigmentation, typically occurs on palms, soles, and subungual spaces
Amelanotic	4%	Absent pigmentation and rare
Desmoplastic	<4%	Seen in older patients

Table 1.3 Cutaneous melanoma subtypes.

Cutaneous melanoma subtypes are based on site and histological features and have different prognosis (32)

1.1.2 Treatment: surgical and systemic therapy

Surgical resection

Surgical resection of the tumour including surrounding healthy skin known as wide local excision (WLE) is performed for localised disease, the margins resected depend on the BT (Table 1.1) (19, 33). With this WLE, sentinel lymph node biopsy (SLNB) is indicated in tumours of a BT>1mm and should be performed in the same setting (33, 34). Sentinel lymph node is the first node that receives lymphatic drainage from the area that drains the tumour (35). SLNB is the most important staging and prognostic tool in melanoma (35, 36). It identifies patients for adjuvant systemic therapy and guides the intensity of follow-up (35, 37).

Unlike surgical resection of localised primary melanoma, resection of metastatic melanoma aims to provide local regional control of the disease and not a cure (38). Metastatic melanoma patients (stage IV) require adjuvant therapy post resection (38). Adjuvant therapy can include targeted therapy and immunotherapy, which have surpassed chemotherapy that is rarely used for advanced melanoma these days (38).

Targeted therapies

Several targeted therapies have been developed for known somatic molecular mutations (39). The most common targeted therapy used is the *BRAF* inhibitor such as Vemurafenib. *BRAF* is a gene that encodes the protein B-RAF, which is known to play a role in the signalling pathways regulating cellular proliferation (39, 40). 50% of melanoma contain activating *BRAF* mutations (39). The targeted therapy is utilised in melanoma with *BRAF* mutation in advanced melanoma (40). These agents are highly effective; however, treatment resistance appears to develop in many patients over a relatively short period of time (41, 42).

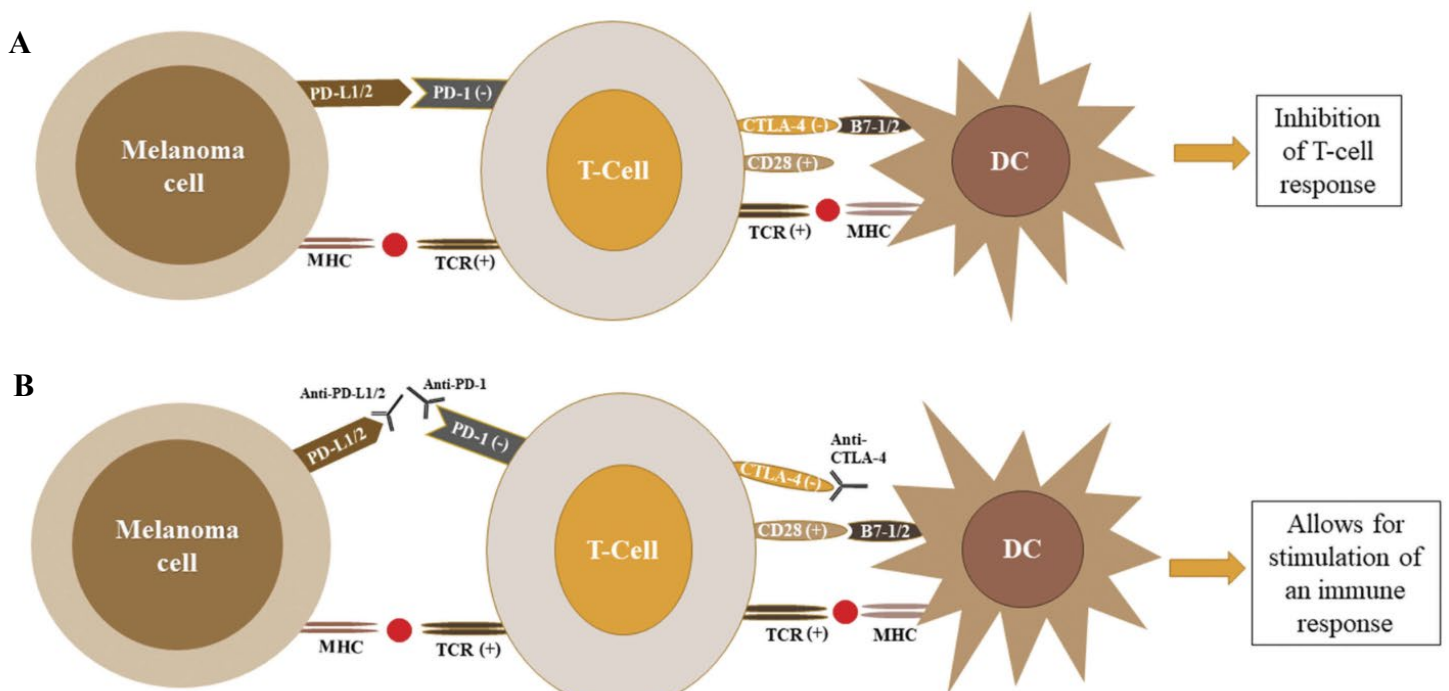
Immunotherapy

The development of immunotherapy over the past 30+ years has transformed the management and prognosis of advanced and metastatic melanoma (43-46). Immunotherapy utilises the body's immune system and its ability to recognise self-

healthy cells against 'non-self' cells (45). The major histocompatibility complex (MHC) class system allows for this information to be communicated with the immune system (47). All cells have MHC class I on their cell surface, which display either 'self-proteins' or 'non-self-proteins' depending on the cell. The displayed non-self-proteins trigger the immune system to mount a response against this non-self-cell (48). Although cancer cells are in essence self-cells, they are mutated cells displaying proteins on the MHC that trigger the immune system (48). The produced and displayed proteins by tumour cells are known as tumour associated antigens (TAA), tumour specific antigens (TSA) and melanoma differentiation antigens (MDA) in melanoma cells (48). These antigens when displayed on the MHC class I proteins trigger the immune system (49). This occurs due to their recognition by the T-cell receptors on cytotoxic CD8⁺ T-lymphocytes (49). These T-cells begin to infiltrate the tumour. Such event is evident in histological slices where tumour infiltrating lymphocytes (TIL) are observed (49). The number of these TIL present can be a prognostic marker, where the absences of TIL can indicate that the tumour has evaded the immune systems response (50, 51). This is often associated with a worse prognosis (50, 51).

Tumours can develop several mechanisms that allow them to escape the immune systems response (52-54). Melanoma which has the highest mutation rate of all cancers is particularly good at achieving this (52, 53, 55). Some of the mechanisms believed to allow melanoma cells to evade the immune system include down regulation of MHC class I proteins, TAA and MDA avoiding attack by the cytotoxic T lymphocytes (52, 53, 56). Additionally, melanoma cells can produce immune suppressive cytokines and inhibitory molecules such as prostaglandin E₂ and β -transformation growth factor (56). Furthermore, melanoma is known to utilise the innate immune check point system to its survival advantage (52, 57). T-cells contain the programmed cell death protein 1 (PD-1) on its surface which recognises the ligand (PDL1) on the surface of somatic cells (58). This interaction allows the immune system to recognise 'self', preventing an immune system overdrive, which would otherwise attack self, resulting in autoimmune pathology (59). Melanoma cells manipulate this and appear to exhibit an upregulation of PDL1 (49, 59). This results in an 'off state' of the immune system, facilitating tumour evasion of the normal immune response (Figure 1.5A) (60). To overcome this, immune checkpoint inhibitors, were developed and came

into clinical use in 2011 (38, 61). This revolutionised the way high risk and metastatic melanoma is now managed, greatly enhancing prognosis (46, 62, 63). Immune check point inhibitors are antibodies against PD1/PD-L 1 blocking the two from binding, thereby allowing the immune system to mount a response against tumour cells (Figure 1.5B) (60).



1-5 T-cell interaction with melanoma cells (60).

(A) MHC class I complex on melanoma cells is detected by the TCR on T-cells resulting in the activation of T-cells. T-cells contain PD1 molecule on its surface that interact with the PDL1/2 on the melanoma cell surface, this binding leads to the deactivation of T-cells. This mechanism is normally in place to keep the immune system in check, preventing immune response against 'self'. However, in melanoma this allows the tumour to evade the immune response.

(B) In immunotherapy the antibody (Anti-PDL1/2, anti-PD1) blocks this interaction, allowing for the immune system to continue to mount a response against the tumour cells.

Abbreviations: DC, dendritic cell; CTLA4, cytotoxic T lymphocyte antigen 4; PD1, programmed death 1; TCR, T-cell receptor; MHC, major histocompatibility complex; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2.

Treatment with Pembrolizumab, an anti-PD1 antibody agent, in patients with metastatic melanoma is said to have a 12-month overall survival rate of 73% compared to 43% in patients treated with the chemotherapy agent, dacarbazine (64).

However, the side effect profile of these checkpoint inhibitors can be extensive given that self-non-pathological somatic cells are also attacked in the process (65). The side effect can include colitis, inflammatory conditions of the skin and endocrine organs (65-67). For some patients the side effects are so debilitating that treatment is terminated and alternatives sought (66, 68, 69). In addition to the side effects, some patients either do not respond to these agents or some go on to develop resistance to the therapy (70-72). To date the reasons for this are not fully understood (70, 73). This has led to the debate about the need to develop a tool such as a biomarker to be able to predict which subset of patients are likely to respond to treatment (65, 74, 75). Furthermore, the need to develop other targeted therapy and agents to further enhance the management of advanced metastatic melanoma is essential.

1.2 Biomarkers in Melanoma

Current diagnostic and prognostic tools used in melanoma are invasive and costly (76). A tissue biopsy requiring the use of anaesthesia is the standard diagnostic test currently (18). Additional investigative tools are also utilised including sentinel lymph node biopsy, and imaging in the form of CT, MRI and PET scans depending on the features of the tumour and patient status (19, 76-78). Sentinel lymph node biopsy is considered the most important staging tool for melanoma (79). Sentinel lymph node biopsy requires patients to undergo an invasive surgical procedure under general anaesthetic to acquire the prognostic information (79). Additional risks of the invasive procedures currently utilised include pain, infection, bleeding, scarring, wound breakdown, and delayed healing (77, 78). Radiological screening is not the answer as it has a low 'pick-up' rate for metastases (26). A non-invasive screening biomarker to identify subclinical disease progression is required for melanoma, particularly now that effective systemic therapy is available for these

patients (80-82). A specific and sensitive biomarker is required for melanoma detection, predicting patient outcomes, and response to treatment (82-85).

The clinical utility of biomarkers for various tumours such as prostate cancer (prostate specific antigen- PSA), and some solid tumours have had a notable impact on the management of such cancers in terms of screening, detection of treatment response, and recurrence (86, 87). The clinical utility of biomarkers in melanoma however remains unclear (88, 89). This has been identified as one of the National Institute for Health and Care Excellence (NICE) melanoma future research recommendations (90). Biomarkers in a form of a non-invasive simple blood test obtained in the outpatient department setting, have a role to play in place of the currently used invasive, costly, and anxiety inducing investigations such as biopsy and imaging (84).

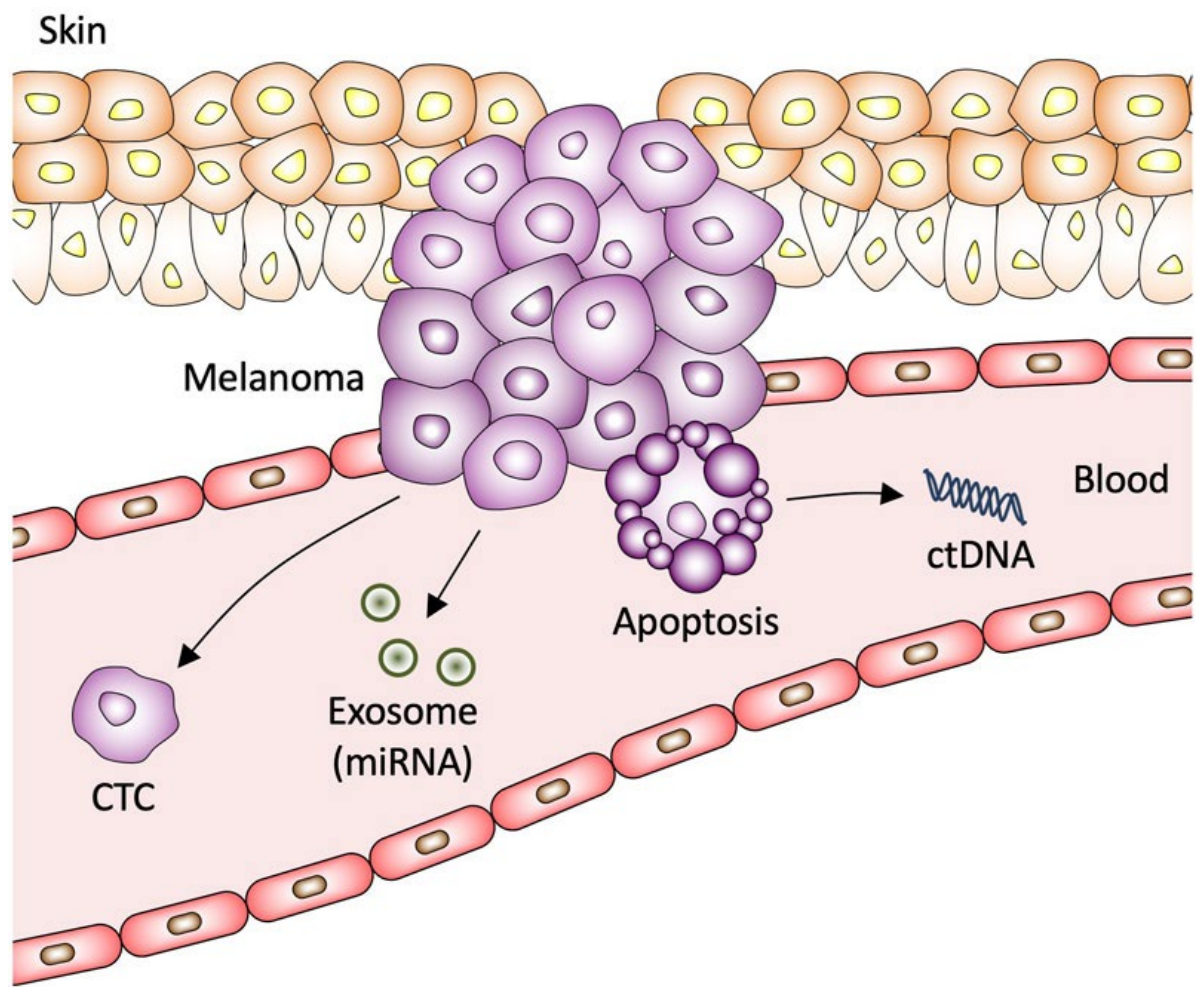
Biomarkers are molecules that can be detected in blood, urine, saliva, and tissue yielding information on disease progression, response to treatment and recurrence (82, 84, 91, 92). Melanoma biomarkers can broadly be divided into diagnostic, prognostic and predictive biomarkers (84). A diagnostic biomarker detecting melanoma in the early stages ideally, whilst a prognostic biomarker would yield information on the patient outcomes and survival independent of the treatment (82, 83). A predictive biomarker on the other hand would indicate the patient's likelihood of benefit from a selected treatment such as immunotherapy or targeted therapy (93, 94). This would assist in selecting the patients most likely to benefit from such treatment, avoiding giving treatment to patients who may not benefit, given the extensive side effects and potential toxicity (65, 66, 68-70). A predictive biomarker would allow for personalised patient specific treatment to be provided (93, 95). Predictive targeted therapy biomarkers such as the BRAF V600, which is an oncogene and if mutated (V600E) as is the case in more than 50% of cases, would guide targeted therapy selection in such patients (96). Whilst a prognostic biomarker would guide patient triage for optimal therapy to be administered (97).

Liquid biopsy, the term given to circulating molecules found in biological liquid samples, are non-invasive and can yield valuable information in real time (98). Over the past two decades research on liquid biopsy as an adjuvant to traditional biopsies in cancer has generated more than 9 000 publications and 3 000 clinical

trials (99). Currently there is no liquid biopsy in clinical practice use for melanoma (98, 99).

Circulating liquid biomarkers are non-invasive, overcome tissue biopsy sampling bias, and allow for patient response to therapy to be tracked and monitored over time (98, 99). Additionally, the information a tissue biopsy provides is limited by the tumour features that were present at the time of sample collection (100). Currently histological features are used as a prognostic and a predictive biomarker, including the tumours thickness, presence of ulceration, and the mitotic rate (101, 102). The histological features are part of the melanoma staging system, 8th edition American Joint Committee on Cancer (AJCC) (20, 21). In addition to lactate dehydrogenase (LDH), the only circulating biomarker currently in use for melanoma in practice as a prognostic tool (88, 103). A high LDH level is associated with poor patient outcomes, and survival in advanced metastatic melanoma (104-106). While a reduction in LDH from baseline is associated with immunotherapy response on the first follow up CT scan (107, 108). Other circulating proteins such as S100B, and melanoma inhibiting activity (MIA) were discovered and thought to have a prognostic value, but none have materialised into clinical practice (84, 109-111). Over the years several molecules have been identified as potential liquid biomarkers for the diagnosis, predictive or prognostic indicators in melanoma (112).

Of the emerging technologies, molecular profiling seems to have resulted in the bulk of melanoma liquid biomarkers discoveries (113-116). This is partly because the signalling and genetic alteration mediating the formation, and progression of melanoma can be identified through molecular profiling techniques (112). Liquid biopsy biomarkers identified through molecular profiling include circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), and cell free DNA (cfDNA) (Figure 1.6) (98, 112, 117).



1-6 Liquid biopsy in melanoma (98).

Liquid biopsy in melanoma discovered and currently investigated include the circulating biomarkers circulating tumour cells (CTC) and circulating tumour DNA (ctDNA) (98).

Circulating tumour cells can be detected in the circulation at a rate of one CTC per million leukocytes (118). Thus, CTCs is a very small proportion of tumour cells in the circulation (118). CTCS reflects tumour burden and is associated with poor patient outcomes (119, 120). CTCs are currently investigated for its diagnostic and prognostic value in advanced cancer namely metastatic breast, colon, lungs and prostate (121, 122). Its use and role in early-stage cancer is limited due to the challenges in its detection at early-stage disease (117). In melanoma, circulating melanoma cells (CMC) have been detected utilising qPCR of melanocyte specific genes (123). Additionally, enrichment of melanocyte surface markers has also been possible owing to the development in enrichment and cell isolation technologies (123). CMCs have been found to have a prognostic value in advanced but not early-stage melanoma, but its detection sensitivity remains low (124-126). This has hindered the use of CMCs in clinical practice. ctDNA is fragmented DNA shed into the circulation by the cancerous cells (127-131). It consists of 130-170 bp and thought to be present because of tumour cell apoptosis, cellular necrosis, or macrophage phagocytosis of the cancerous cell (131). ctDNA has a short half-life of 16 minutes to 13 hours (132-135). In early-stage melanoma ctDNA was undetectable (136). However, in advanced melanoma longitudinal assessment of ctDNA levels including ctDNA *BRAF* mutations, levels were found to be predictive of treatment response in patients receiving immunotherapy (137-139). Additionally, patients with a so called favourable ctDNA profile where the ctDNA is undetectable at baseline for example was associated with a superior progression free and overall survival (140-142). This contrasted with patients who had an unfavourable profile of detectable ctDNA at baseline and throughout treatment, whom had a far inferior overall survival rate (137, 143). ctDNA levels were also found to be associated with tumour burden and overall survival in patients with uveal melanoma (144).

ctDNA quantification is carried out by several techniques including qPCR, digital droplet PCR and next generation sequencing (145). Sampling consistency and variations in the blood collection and processing of samples, seem to have halted ctDNA from making it into clinical use (146, 147). As well as having a short half-life, there is also an increase in ctDNA in blood collection tubes that is time dependent (146). Numerous factors come into play including collection time from serum separation, transportation and storage (148). All these factors can impact white blood cell lysis increasing cfDNA, and ctDNA fraction is low compared to the

total background of cfDNA (146). Unlike ctDNA, cfDNA is DNA found circulating freely in peripheral blood of both healthy controls as well as patients with cancer (149, 150). The detected levels vary where a concentration of 2ng/ml (range 1-6 ng/ml) is detected in healthy controls compared to an average concentration of 8 ng/ml (range 1-42 ng/ml) found in lung cancer patients (151, 152). The source of cfDNA is thought to be from cellular phagocytosis, necrosis and apoptosis, although the exact mechanism is not fully understood (153). Whilst ctDNA are of cancer cell origin, its release contributes to the total amount of cfDNA, thereby increasing the total level of cfDNA detected in the presence of cancer (150). cfDNA has been found to have prognostic value and act as a marker of tumour burden in patients with metastatic melanoma (142, 154). Like ctDNA detection, currently there is a lack of a uniform standard practice in extracting and detecting cfDNA (153). Several studies have utilised a wide range of commercially available cfDNA extraction and purification kits including spin columns or magnetic beads, as well as a variety of blood sample collection tubes (155, 156). However, currently there is no standardised optimal practice when it comes to extracting and measuring cfDNA (156, 157).

1.2.1 Mitochondrial DNA

Much of the current research on melanoma biomarkers has in the main focused on nuclear DNA (158, 159). To date this has not materialised or translated into clinical practice (160). In addition to nuclear DNA, mitochondrial DNA (mtDNA) can also be detected (161). Compared to nuclear DNA, mitochondrial DNA has significantly higher copy numbers from 1000 to 10, 000 per cell, simpler structure, and shorter length (161). This makes mtDNA easier to sample and process during molecular analysis (161, 162). Thus, mtDNA could potentially be a preferable choice for a non-invasive biomarker (162, 163). Mitochondrial DNA could potentially be utilised in the outpatient department skin clinics to correlate levels of mtDNA with clinical severity, recurrence, progression, and response to treatment (161, 162, 164, 165). There have been very few studies looking at mtDNA in melanoma (166, 167). Epidemiological studies have illustrated that there is a significant association between mtDNA copy number and an increase in risk of cancers such as breast, non-Hodgkin lymphoma, renal cell carcinoma,

lung and pancreatic cancer (168-176). However, there has only been a handful of studies looking at mtDNA copy number in melanoma (166, 167, 177). One of which had a large sample size of 500 melanoma patients and 500 healthy controls (167). In this study 500 patients with melanoma and 500 healthy controls had their mtDNA copy number measured in peripheral blood, utilising qPCR (167). They found that the mean mtDNA copy number was significantly higher in melanoma patients compared to healthy controls. Additionally, the study found that increased mtDNA copy number was associated with an increased risk of melanoma by 1.45-fold. This study however only looked at mtDNA copy number in patients with early-stage melanoma. Furthermore, it was a retrospective study, hence no tracking of mtDNA level and monitoring of disease response to treatment or progression were investigated.

1.2.2 Mitochondria and mitochondrial DNA

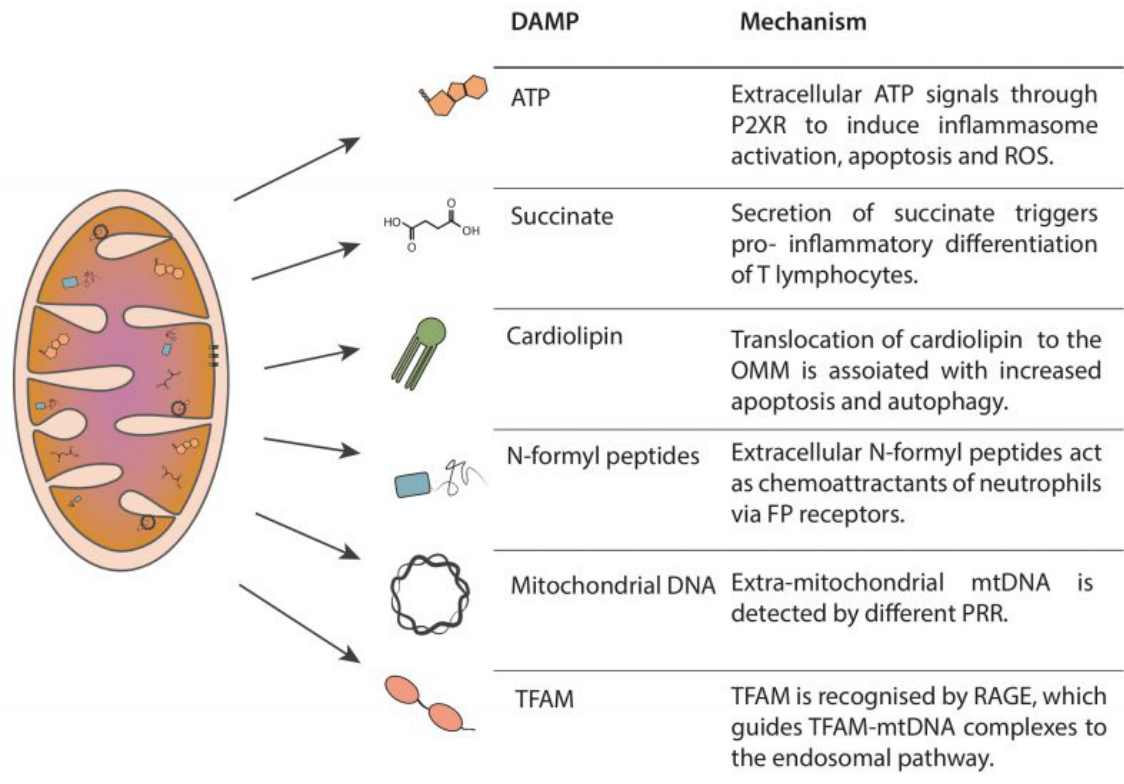
Mitochondria is a double membrane organelle referred to as the 'powerhouse' of cells (178). It supplies the necessary energy in the form of a molecule known as adenosine-5'-triphosphate (ATP) via oxidative phosphorylation and glycolysis in the electron transport chain (178). The mitochondria maintain its own genome, known as mitochondrial DNA (178). mtDNA unlike the linear nuclear DNA, is circular in structure and is much smaller containing 16, 569 base pairs as opposed the 3.3 billion base pairs forming the nuclear DNA (178). Each cell can contain hundreds of copies of mtDNA compared to only one nuclear DNA per cell (178). This genome consists of 37 genes, coding 13 proteins, 22 transfer RNA (tRNA), and 2 ribosomal RNA (rRNA) (178). The 13 proteins such as ATP synthase form the enzyme complexes required for the oxidative phosphorylation generating the energy, ATP (178). Interestingly mtDNA is maternally inherited and can be used to trace back the maternal family tree (178). The clinical relevance of this inheritance pattern is that mitochondria linked diseases are maternally inherited (178).

There are different theories surrounding the origin of mitochondria (179). The endosymbiotic theory is one theory that is commonly accepted (179). It postulates that mitochondria originated from a single cell organism that originally engulfed a 'mitochondria like bacteria' (179). As a result of this, mitochondria retained some

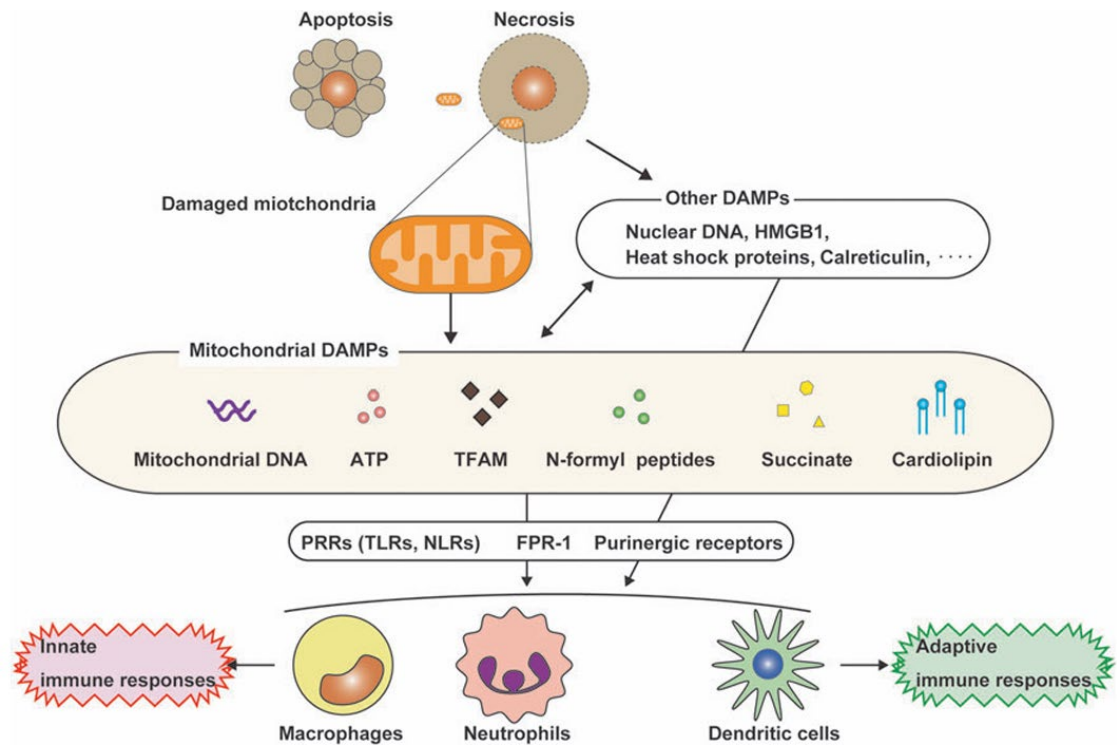
bacterial features such as triggering an innate immune response when its substances are encountered by the immune system (179, 180).

Mitochondria also play a role in regulating apoptotic pathways when triggered by cellular damage and stress (179, 180). In response to such cellular triggers, the mitochondria produce damage-associated molecular patterns (DAMPs) including proteins, lipids, mtDNA, adenosine triphosphate (ATP), mitochondria RNA (mtRNA) (Figure 1.7A) (180-182). DAMPS including mtDNA are released into the cytosol if the integrity of the mitochondria's membrane is comprised and impaired due to trauma and cellular stressors (182, 183). The cGAS-STING (cyclic GMP-AMP synthase stimulator of interferon genes) pathway senses the mtDNA, now present in the cytosol, causing the release of interferons (184, 185). This results in the activation of several inflammatory pathways such as Interferon regulatory factor 3 or 7, which play a role in innate immune responses (184, 185). The activation of such inflammatory pathways results in the release of pro-inflammatory cytokines initiating an innate immune response (Figure 1.7B) (186, 187).

A



B



1-7 DAMPS and the immune response.

(A) Mitochondrial-derived Damage Associated Molecular Patterns (DAMPS).

When mitochondrial function and structural integrity is compromised through trauma, stress, infection or pathology, mitochondrial damage-associated molecular patterns (DAMPs) such as mtDNA, ATP are released into the cytosol (182).

(B) mtDAMPS detection triggers and an immune response.

mtDNA and mtDAMPS are recognised by receptors such as Toll like receptor (TLR) which in turn stimulate immune cells including macrophages and dendritic cells to mount an inflammatory and immune response against the offending stimuli. (186)

In the early phase of this innate immune response, the offending cells are attacked and ejected by the immune system (188). However, if the trigger persists this can result in chronic inflammation (188). It is now believed that in a tumour microenvironment, the initial immune anti-tumour response can eventually lead to a diminished anti-tumour immune response contributing to immunotherapy resistance in cancer patients (188).

1.2.3 mtDNA: innate immune activator

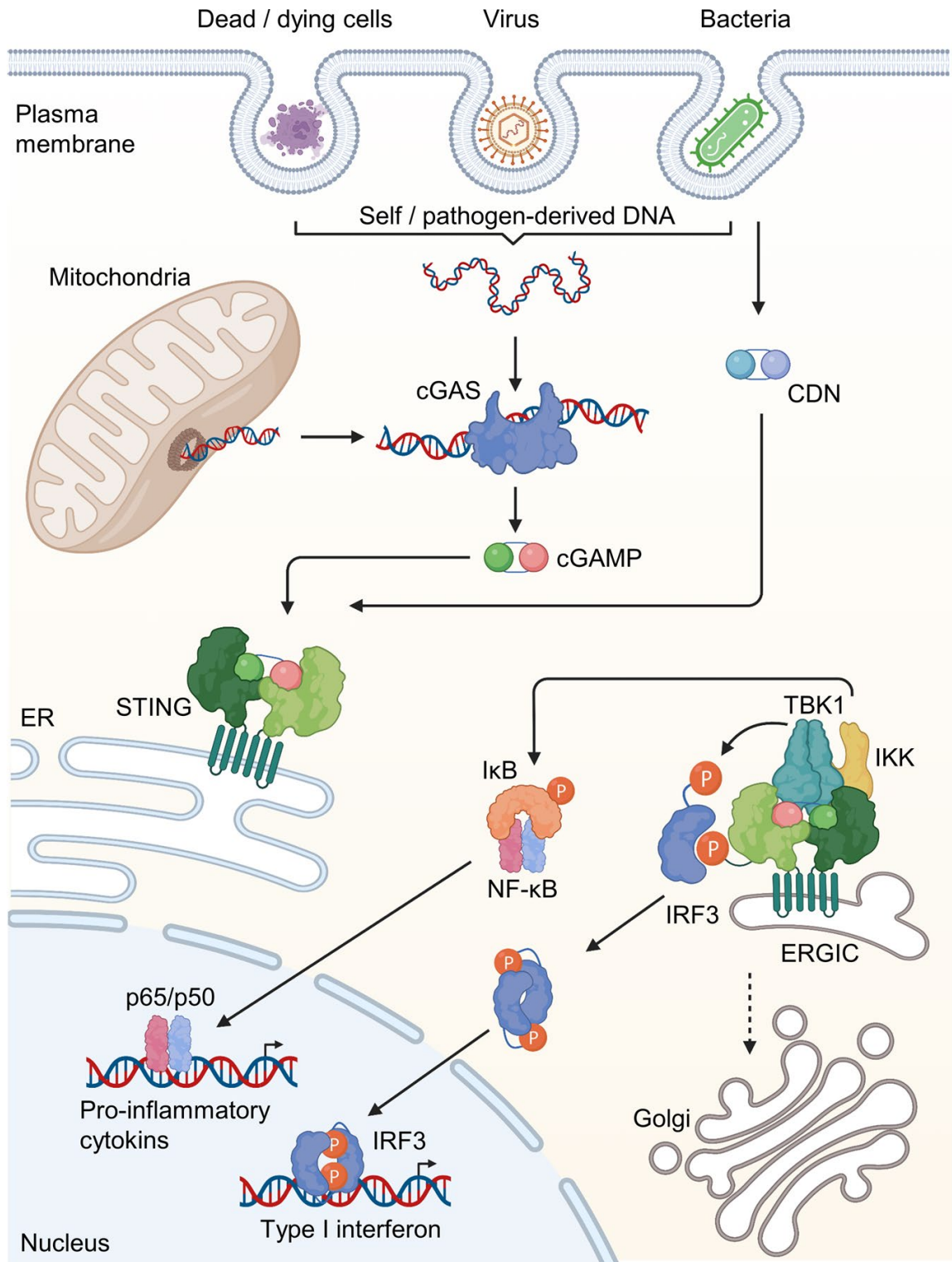
Cell-free mtDNA contains islands of unmethylated cytosine-guanine dinucleotide (CpG) motifs which are recognised by pattern recognition receptors and are potent activators of the innate immune system (189, 190). The CpG islands in the mitochondrial DNA are recognised by the cyclic GMP-AMP synthase (cGAS), and pattern recognition receptors (PRRs) such as Toll-like Receptor 9 (TLR 9) (191). Its recognition triggers the innate immune signalling pathways (182, 190, 191). mtDAMPs in general have come into focus as mediators of inflammation, with mitochondrial DNA (mtDNA) being the most documented (191).

1.2.4 mtDNA and the cGAS-STING signalling pathway

The cGAS activated by sensing mtDNA results in synthesis of 2'3' cyclic GMP-AMP (cGAMP) (192). The cGAMP then leads to the Stimulator of interferon genes (STING) activation from its resident site of the endoplasmic reticulum (192). The STING activation results in the release of IRFs (192). This enhances the action of the immune effector cells such as T-cells and antigen presenting cells (192). It is understood that this activation of the innate immune system in the acute phase results in the anti-tumour/trauma effect (192). However, ongoing activation of this cGAS-Sting pathway has been shown to exhibit an immune suppressive response. Here the immunosuppressive immune cells such as myeloid-derived suppressor cells (MDSCs) infiltrate the tumour resulting in immune suppression and treatment resistance (192, 193).

mtDNA: potent TLR inducer

Toll-like receptors (TLRs) are known to have an established role in detecting pathogen-associated molecular patterns (PAMPs), mounting an innate inflammatory response (Figure 1.8) (192, 194). mtDAMPS released during trauma or cell death are detected by TLRs resulting in the activation of NF- κ B and IRF3 signalling pathways, resulting in the release of pro-inflammatory mediators in the tumour microenvironment and interferons respectively (192-194). mtDNA driven activation of TLR9 has been implicated in several diseases including chronic liver inflammatory disease and non-small cell lung cancer (193, 195-197).



1-8 Innate immune system recognition of mtDAMPs and its components (192).

Toll-like receptors detecting mitochondrial DAMPs results in the activation of several signalling pathways. The activation of transcription factors IRF3 and 7 induces the expression of type I interferon genes. While the translocation of NF-κB results in the upregulation of genes encoding proinflammatory cytokines such

as IL-1, IL-6 and TNF- α . The TLR- NF- κ B pathway can also induce the inflammasome gene expression, which ultimately leads to secretion of IL-1 β and IL-18. All of this results in chronic inflammation in the tumour microenvironment facilitating tumour survival (192, 195).

1.2.5 mtDAMPs and cancer

mtDAMPs appear to play two distinct roles in tumour modulation (198). Acute activation of the innate immune signalling pathways leads to the generation of cytokines, which target tumour cells (198). However chronic stimulation of such pathways is thought to have the opposite effect (198, 199). Here the immunosuppressive cells gather in the tumour microenvironment, facilitating the survival, proliferation and potential metastasis of tumour (198, 199). Additionally, it is believed that chronic stimulation of these innate immune pathways, can induce resistance in cancer cells (199). This is observed in interferon chronic stimulation resulting in over expression of the immune check point programme cell Death-ligand-1(PD-L1), which would normally act to keep the immune system in check (200). However, in a cancer context this allows cancerous cells to avoid attack and apoptosis mediated by T-cells (200, 201). Up regulation of PD-L1 was observed in melanoma where increased levels of TNF- α in the tumour resulted from the activation of NF- κ B transcription factor complex (202). Furthermore, *in vivo* TLR4 was found to activate the signal transducer and activator of transcription 3 (STAT 3) in melanoma cells (202, 203). This resulted in an immunosuppressive tumour microenvironment promoting tumour proliferation, survival and epithelia-mesenchymal transition (EMT) (202, 203).

Stimulation by mtDAMPs molecules of the innate immune system results in the activation of several signalling pathways including NF- κ B, STAT3 and IRF3/7, TLRs, cGAS/STING, and MAPKs (198, 204, 205). This subsequently results in the release of cytokines (e.g. IL-6 and IL-10), chemokines and interferons, which attract immunosuppressive cells such as the pro-tumour M2 macrophages, tumour activated neutrophils, regulatory T cells, MDSCs, and dendritic cells (205). The net effect of this is polarisation to a suppressive phenotype allowing cancerous cells to survive, proliferate and metastasise to distant sites (206). Additionally recent work highlighted the phenomenon of melanoma cells obtaining energy through mitochondrial transfer from its surrounding mesenchymal stromal cells resulting in a survival advantage for emerging cancer cells (207).

Whilst stimulation of the innate immune signalling pathways drives inflammatory and immune responses to the offending agent, they can also mediate a pro-tumour effect (208). Some of the stated signalling pathways such as cGAS-STING have been utilised as part of immune therapy, and yet in some cancers such

pathways can be associated with poor prognosis (209, 210). There is scope for further research to investigate and understand this potential contrasting impact of mtDAMPS between the pro-tumour and anti-tumour effect, which could be utilised to understand contributors to poor prognosis and act as targeted therapy in the future (211).

mtDNA and mtDAMPS are not only implicated in cancer pathology but also a wide range of non-cancer diseases such as trauma, sepsis, inflammatory diseases and neurodegenerative conditions (183, 212, 213). The level of circulating mtDNA detected tends to vary depending on the disease in question (214).

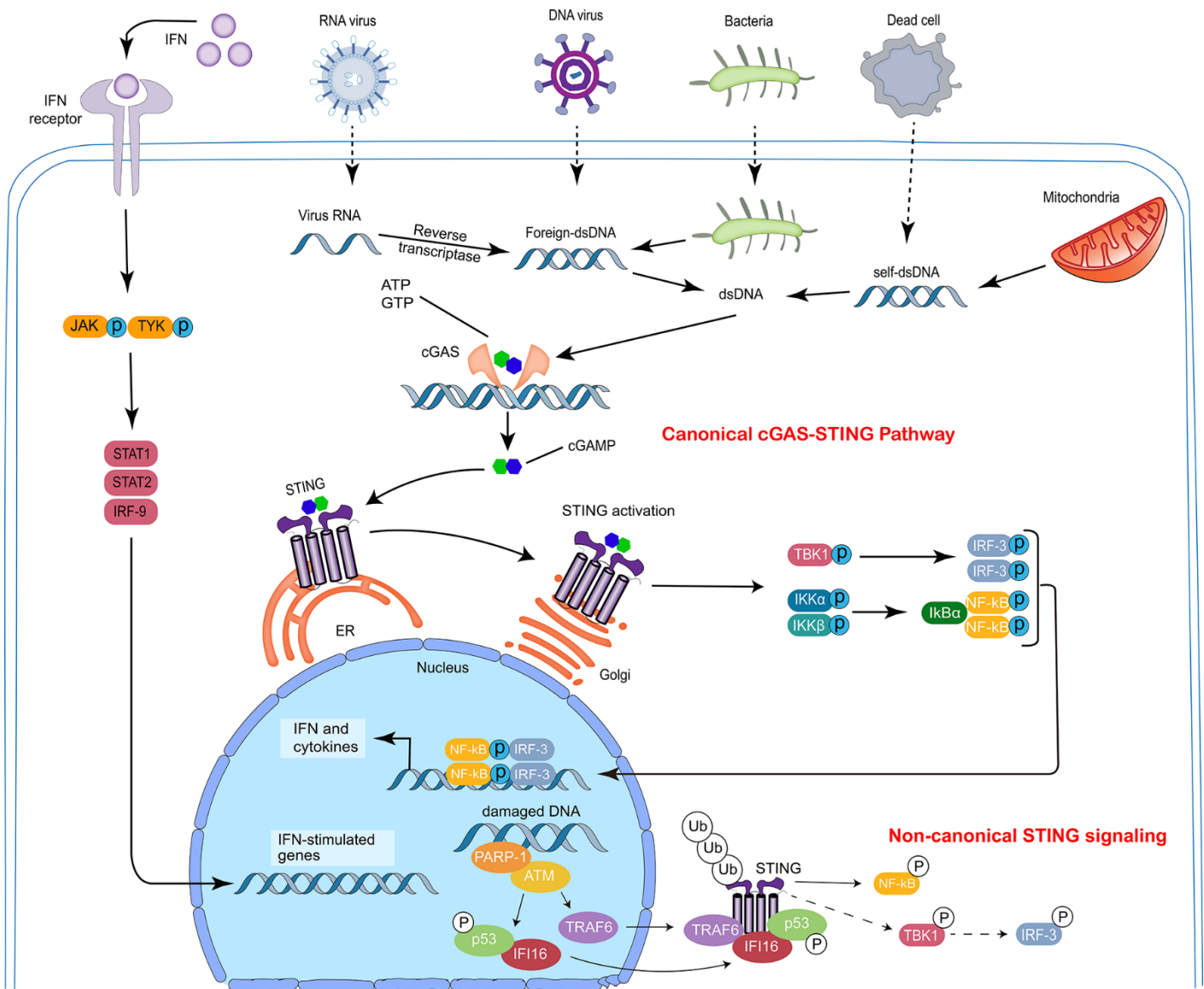
1.3 The cGAS -STING pathway in cancer

Tumour cells form as a result of normal cells developing mutations, transforming them into cancerous cells (215). Such cancerous cells develop a system to evade the immune system defence mechanism (215). The immune system defence mechanism can broadly be divided into innate and adaptive immunity (215). Innate immunity offers the spontaneous and first line of defence against invading pathogens and abnormal mutated or dysfunctional cells (215). Developing an understanding of how the innate immune system recognises invading pathogens and offending cells was greatly enhanced by the progress made on cell membrane Toll-like receptors (TLRs) over the last decade in the 20th century (216). This however did not provide an explanation as to how non-self-signals in the cytoplasm are recognised as an offending trigger (216). The discovery of STING in 2008 however, shed some light on how both self and non-self-derived DNA is recognised by the innate immune system (216).

Human STING is a 379 amino acid protein with a molecular weight of 42kDa (216). Murine and human STING are believed to share 68% sequence identity (217). STING is expressed in both immune and non-immune tissue. STING is found in a variety of cells including the immune cells of macrophages, dendritic cells, and lymphocytes (216). STING is widely but not universally expressed across immune cells, with expression noted to be low in cells like neutrophils and eosinophils (216). It is also expressed to a varying degree in non-dependent immune tissue such as the lungs, liver gastrointestinal system (216). Its expression level and activity vary depends on the cell type and condition that the tissue is under (216).

STING varies its activity levels depends on the physiological and pathological status of the tissue (216).

Cyclic GMP-AMP synthase (c-GAS) is a key sensor of intracellular DNA (218). DNA is confined to the nucleus of the cells when the cell is under a functional physiological condition (219). However, under pathological conditions such as infection, trauma, mitochondrial damage, genomic instability, invasion by bacteria, virus or non-self-cells, the cellular haemostasis is disrupted resulting in the DNA to now be situated in the cytosol (220-222). This is detected by the cGAS sensor which in turns results in the activation of STING resulting in the activation of the TBK1 kinase phosphorylating interferon regulatory factor 3 (IRF3) (223). This in turn leads to the production of interferons and downstream gene expression (223). STING activation also mediates the recruitment of IKK kinases and the activation of NF- κ B resulting in the release of proinflammatory cytokines including IL-6, IL-1 and TNF- α (223). The production of the interferons and release of such proinflammatory cytokines in this manner is known as the canonical cGAS-STING pathway (Figure 1.9) (215). Additionally, a non-canonical STING activation pathway also exists where an alternative STING signalling complex exists, consisting of IFI16 DNA-binding protein, p53 tumour suppressor protein, and the E3 ubiquitin ligase tumour necrosis factor receptor associated factor 6 (TRAF6) (215, 224). Within this signalling complex the TRAF6 results in STING ubiquitination mediating the activation of NF- κ B and the resulting gene expression (224).



1-9 Schematic of the Canonical and non-canonical cGAS-STING Pathway (215).

Cytosolic double-stranded DNA arising from various sources including pathogens and necrotic cells are detected and activate cGAS. The activation of cGAS triggers the synthesis of cGAMP from ATP. The formed cGAMP subsequently activates STING. STING activation results in the recruitment of TBK1 and IKK resulting in the phosphorylation of IRF3 and NF-κB with subsequent expression of the downstream inflammatory cytokines. Non-canonical STING Signalling results in an alternative STING signalling complex consisting of IFI16, p53, and TRAF6, this eventually leads to the activation of NF-κB (215).

1.3.1 The role of cGAS-STING pathway in cancer immunity

The primary role of c-GAS-STING pathway is to mediate an antitumour immune response whilst the tumour microenvironment (TME) is to facilitate tumour progression (215). The TME consists of a network of fibroblast, immune cells, extracellular matrix and vasculature all supporting the tumour cells (215).

The cGAS-STING signalling pathway once triggered functions to regulate immune cells within the TME to mediate an antitumour immune response (215). It achieves this within the TME by facilitating a cascade of events including the promotion of the differentiation of CD4⁺ T-cells into Th9 cells which produce IL-9 and recruit CD8⁺ T-cells to the tumour site (215). The cGAS-STING signalling pathway also activates natural kill cells (NK) by promoting NLRP3, and dendritic cells (DC) due to the production of type 1 interferons, further activating CD8⁺ T-cells all of which lead to tumour cell death (215, 225-227). Furthermore, STING signalling results in the secretion of interferon- γ through the IRF-3 promoting the differentiation of CD4⁺ T cells into Th1 cells (228).

The key elements of the regulatory role that cGAS-STING signalling plays in cancer immunity can be summarised into (1) enhanced antigen processing-presentation, (2) T-cell activation-infiltration mediating tumour cell death and the (3) reprogramming of the tumour microenvironment (215, 229). Each of these elements are discussed in depth here.

1.3.1.1 Enhanced antigen presentation

Dendritic cells the potent antigen presenting cells (APC), migrate to the draining nearby lymph nodes to present the tumour antigens that it has captured to the CD8⁺ T-cells initiating an adaptive immune response (230). The DCs are activated by either tumour derived DNA or tumour derived cyclic GMP-AMP (cGAMP) (231). The tumour derived cGAMP can directly activate the STING pathway, while the tumour derived DNA is first phagocytosed by the DCs which in turn leads to the activation of cGAS-STING resulting in type 1 interferons (IFNs) production, promoting DC maturation (231-233). Tumour derived vesicles are also known to transfer double-stranded DNA (dsDNA) from cancerous cells to dendritic cells triggering the cGAS-STING pathway (234-236). The IFNs released

can also stimulate the expression of co-stimulatory cells such as CD80, CD86, CD40 on DCs all of which results in the activation of T-cells (233, 234, 237).

1.3.1.2 T-cell activation and infiltration

STING activation and the resulting T cell activation of CD4⁺ and CD8⁺ plays a crucial role in mediating anti-tumour immunity (227, 228). In CD4⁺ T-cells, STING stimulation resulting in IRF3 activation and the release of type 1 IFNs results in the differentiation of CD4⁺ to Th1 cells, which is a subtype of T-helper cells (228). Th1 cells are crucial for cell mediated immune response, activating macrophages and cytotoxic T-cells to destroy invading pathogens and cells by releasing pro-inflammatory cytokines namely IFN- γ and IL-9 (228). In CD8⁺ T-cells STING activation mediates the persistent CD8⁺ T-cell immune response by upregulating the expression of TCF1 which is a transcription factor that allows for the persistence of CD8⁺ memory cells maintaining CD8⁺ T-cell activation (227). The activation of STING in DCs results in the production of the chemokines CXCL9 and CXCL10, both of which lead to the recruitment of T-cells and NK cells, enhancing anti-tumour immunity and tumour cell death (238-240). Interestingly, STING activation in the endothelial cells of the tumour, leads to the secretion of CXCL10 causing T-cells to transmigrate across the endothelial barrier (241, 242). STING activation in the endothelial cells of the tumour results in the upregulation and increased expression of adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) (242-244). This allows for T-cell extravasation, tumour vasculature normalisation and intra-tumoral T-cell infiltration (242-244).

1.3.1.3 Reprogramming of the tumour microenvironment

The immunosuppressive tumour microenvironment contains tumour associated immune cells such as tumour-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (245-248). All these cells have an immunosuppressive function facilitating the tumour immune escape phenotype (245-248).

STING activation re-programmes TAMs to exhibit an M1-like pro-inflammatory anti-tumour phenotype (243). This is believed to occur due to the upregulation of M1-like macrophage expressed genes such as the cytokines IL-6, and cell surface markers CD80, and TLR2, mediated by STING activation (243). The manipulation of the macrophage phenotype is also utilised by cancers to achieve an immunosuppressive environment for its survival (243). Tumour cells in peritoneal colon cancer were shown to stimulate the influx of the M2-like anti-inflammatory pro cancer phenotype mediating an immunosuppressive TME (243). This allowed for the inhibition of the effector T-cells, facilitating peritoneal metastasis (243). Additionally, STING activation inhibits the expansion of MDSCs and triggers the release of myeloid chemotactic factors such as IL-8 and CXCL12, which in turn attract a wide range of tumour suppressing cells including monocytes, macrophages and T-cells to the tumour site as well as reducing the presence of the regulatory T-cells that exist in the TME (248). Overall, this has the net effect of enhancing the anti-tumour immunity (248).

STING agonist activated CD11C+ dendritic cells *in vivo* and *in vitro*, show an up regulation of tertiary lymphoid structures (TLS) factors such as IL-36, type 1 interferons, inflammatory chemokines (249, 250). This highlights the crucial role that STING-signalling activation can play in counteracting and reversing the immunosuppressive tumour microenvironment mediated by tumour immune cells, enhancing cancer immunotherapy (249, 250). Once the cGAS-STING pathway is triggered it results in a cascade of events including the release and production of interferons and pro inflammatory cytokines, overall resulting in increased cellular immune activity (251, 252).

1.3.2 Non-immune anti-tumour effects of STING

Mounting evidence points to the link between cGAS-signalling and autophagy, the cells 'self-eating' process that functions to maintain cellular housekeeping, defence against pathogens and cellular renewal (253, 254). Autophagy is understood to have a tumour suppressive role in the early phases of oncogenesis (253, 254). Autophagy triggered by STING-signalling has been shown to prevent cell proliferation impacting tumorigenesis (255). This is supported by findings from studies that illustrated that the chemotherapy agent, Zalcitabine which is known to induce mitochondrial stress activating STING pathway (256). This activation led

to autophagy-iron-dependent death, ferroptosis through the lipid peroxidation in pancreatic cancer cells (256).

Additionally, STING-signalling is also understood to play a role that disturbs angiogenesis that is vital for cancer cells survival, proliferation and metastasis once the basement membrane is breached by the tumour mass (257). One of the most promising and potent agents in disturbing the tumour vasculature, known as 5,6-dimethylxanthenone-4-acetic acid (DMXAA), has now been identified as a target for murine STING (258, 259). DMXAA is a chemotherapeutic agent and now understood to be a potent murine STING agonist, has a selective ability to disrupt the tumour vasculature but not the vasculature of normal tissue (258, 259). It is effective in controlling solid tumour mass growth (258, 259). Other STING agonist such as cGAMP have also been identified for playing a crucial role in remodelling and normalising tumour vasculature, resulting in the infiltration of tumour by activated CD8⁺ T-cells mediating the suppression of tumour growth (243, 244).

Overall, the activation of cGAS-STING signalling pathway leads to the secretion of specific cytokines, chemokines and growth factors such as IL-6, VEGF. This is referred to as senescence-associated secretory phenotype (SASP) (260-262). SASP act as a barrier to tumour progression by promoting the recruitment of immune cells to inhibit and clear tumour cells (260, 263). This was evident *in vivo* where the inhibition of STING in melanoma cells as well as prostate cancer cells, lead to a reduction in immune cell infiltration allowing for the growth of the tumour (264, 265).

1.3.3 Tumour promoting role of STING activation

As well as having an anti-tumour immunosuppressive function, STING activation can also play a role in negatively regulating anti-tumour immune response, thereby exhibiting a tumour-promoting function (229). The role that dominates depends on several factors including whether it is an acute or chronic activation of the STING-signalling pathways (229).

Tumour cells treated with STING agonist show an increase in the expression of programmed cell death ligands-1 (PD-L1), which allows the cancerous cells to evade attack by T cells (266). This upregulation in PD-L1 is induced by the STING-

IRF3 signalling pathway resulting in the activation of the downstream of the NF- κ B pathway and INF-1 (267-269).

Additionally, STING-signalling is found to play a role in interfering with metabolic cellular activity (270). STING-activation mediates an upregulation of the expression of indoleamine 2,3-dioxygenase (IDO) in dendritic cells (270, 271). IDO catabolises tryptophan which T-cells require to execute their anti-tumour tumoricidal function (270). Similarly, IDO and tryptophan 2,3-dioxygenase (TDO) catabolise tryptophan to kynurenine which plays a crucial role in the generation of cellular energy (270). This accumulation promotes the polarisation of macrophages to the anti-inflammatory pro-cancer M2-like phenotype (271). The accumulation of Kynurenine also results in the infiltration of regulatory T-cells or Treg cells which inhibit immune cells such as APCs and T-cells from activating (270, 271).

A positive correlation was noted to exist between the Treg cells and STING expression in patients with squamous cell carcinoma of the tongue (272). This is understood to occur because the STING activation of the MAPK/AP-1 signalling pathway, leads to production of several immunosuppressive cytokines namely IL-10, IDO, CCL22 (272). This in turn results in an increase in the infiltration of Treg cells in human squamous cell carcinoma (272). As well as the MAPK/AP-1 pathway, STING-INF1 signalling in Tregs also promotes an increase in IL-10 secretion further enhancing the inhibitory Treg cells activity in mediating the negative anti-tumour immune response (273).

More recently regulatory B-cells (Bregs) have also been shown to play a role in contributing to the immune escape and treatment resistance exhibited by some tumours (274-277). Bregs promote the expression of the anti-inflammatory cytokines IL-10 and IL-35 (278, 279). The expression of IL-35 was found to be facilitated by the STING-IRF3-IL35 signalling, which acts to inhibit the NK cells activity against tumour cells (278). STING agonist, such as DMXAA, ADU-S100, were found to exhibit an increase in IL-10 and IL-35 B reg cells abundance (280). Whilst the knock down of the STING-1 gene in B-cells in tumour bearing mice was found to significantly decrease the tumour burden in such mice (280).

1.4 The cGAS-STING immune cells related role in melanoma

The activation of the cGAS-STING pathway and release of type 1 interferons especially IFN- β from macrophages results in a macrophage polarisation shift from an M2 protumour phenotype to an M1 antitumour macrophage phenotype (281). Furthermore, the release of IFN- β enhances CD8⁺-T-cell activity and reduces MDSCs, thus mediating an essential role in the immune response to melanoma (281). Furthermore, STING activation through the experimental intra-tumoral delivery of cGAMP was found to attract a population of M1-like macrophages and the release of TNF α and the chemokines CXCL10 and CXCL11 (282). This resulted in the recruitment of T-cells to the tumour site further enhancing the anti-tumour immune response (281, 282).

The cGAS-STING pathway has also been shown to be activated in dendritic cells (DC). Activation results in an increase in expression of IFN- β and the co-stimulatory molecule CD86 both mediating T-cell activation. Additionally, the activated DCs result in an enhancement in tumour antigen presentation to the CD8⁺-T-cells further enhancing the immune T-cell cytotoxic response to melanoma (283). Furthermore, natural killer (NK) cells cGAS-STING activation and the subsequent release of the type 1 interferons and pro-inflammatory cytokines mediate the recruitment and activation of NK cells (284). The production of chemokines, such as CCL5 from both the host and tumour cells facilitate the infiltration of NK to the TME resulting in cytotoxic action against melanoma cells (264).

With T-cells, cGAS-STING activation in CD4⁺-helper T-cells promotes its differentiation to TH9 cells resulting in anti-tumour activity observed in melanoma. This was illustrated in melanoma mouse models that received STING activated TH9 cells, which resulted in a decrease in the growth of the tumour mass and enhanced survival of the mice (228). cGAS-STING activation has an indirect effect on Tregs through its immunomodulation role within the TME (285). cGAS-STING activation alters the TME by increasing cytotoxic T-lymphocytes activity and reducing the immunosuppression activity mediated by Tregs (285). This overall results in an enhanced anti-tumour response. cGAS-STING activation resulting in type 1 interferon and pro-inflammatory cytokines release mediates an increase in the recruitment and activation of CD8⁺-cytotoxic cells within the TME

(281). STING activation also results in enhanced tumour antigen presentation further facilitating cytotoxic-T-cell activity against melanoma (284).

1.5 Chronic STING activation

cGAS-STING appears to have a dichotomic function depending on context. This is supported by evidence illustrating that both reduced and high cGAS-STING expression can be associated with poor patient survival (286). This indicates that cGAS-STING can exhibit a tumour suppressing or tumour promoting role, depending on several factors including the tumour type, and chronicity of STING activation (261, 287). It is generally agreed that chronic inflammation can mediate the formation and progression of tumours (288-290). This inflammation driven carcinogenesis is believed to occur due to the chronic activation of cGAS-STING signalling axis (291). This is endorsed by studies that have illustrated that skin tumorigenesis induced by chemotherapy agents, such as Cisplatin which mimic nuclear DNA injury and STING activation, was observed in wild type mice but not in STING^{-/-} mice (291). These mice were resistant to the skin tumorigenesis induced by such mutagenic agents (291). Additionally, in valvula melanoma chronic activation of cGAS-STING and the resulting chronic inflammation was found to be associated with poor patient outcomes and survival (292). The chronic activation of cGAS-STING observed here was due to chromosome instability (CIN) (292).

CIN is a key event in the initiation of tumour development triggering the cGAS-STING stimulation (293). In chromosomal unstable tumours, cGAS-STING is rarely inactivated or silenced, instead it tends to exhibit a tumour promoting role via its chronic activation (294). Typically, in a normal cell, DNA leakage into the cytoplasm is detected by the sensor cGAS-STING leading to its activation and a cascade of cellular innate immunity response, ultimately resulting in cell death (295). However, tumour cells utilise chronic activation to its advantage to allow for the tumour's survival and progression (295). It is thought that in tumours with CIN cGAS-STING is triggered stimulating the alternative non-canonical NF- κ B signalling pathway mediating tumour survival, growth, progression and metastasis (296). The stimulation of this alternative non-canonical NF- κ B is further supported by the finding that drug-induced CIN in triple negative breast cancer activated the IL-

6/STAT3 signalling pathway (296). The IL-6/STAT3 pathway is understood to be stimulated because of the downstream signalling of cGAS-STING and the non-canonical NF- κ B pathway (296, 297). Similarly other studies have shown that DNA damage induced by the chemotherapy drug, Doxorubicin stimulated the IL-6/STAT3 pathway via the cGAS-STING non-canonical NF- κ B pathway, allowing for the survival and progression of the tumour (298). It is believed that in tissue and tumour with high CIN, the STING mediated DNA damage pro-tumour response offsets its anti-tumour immunosurveillance response (229). This is further supported by the observation of high levels of STING expression in chemotherapy resistant breast cancer cells exposed to chemotherapy DNA damaging agents (297). These cells were not only resistant to therapy but also found to have higher rates of recurrence, indicating that cells with high STING expression allow for cancer cell survival and regeneration despite exposure to genotoxicity (297).

Chronic stimulation of cGAS-STING signalling can result in an immune-suppressive TME facilitating tumour growth, immune evasion, metastasis and treatment resistance (201, 260, 263, 299). Chronic cGAS-STING signalling activation results in the suppression of type 1 IFN release and up regulation of the noncanonical NF- κ B signalling allowing for tumour survival and metastasis (300). Additionally, whilst acute stimulation of STING results in cell-cycle arrest through SASP, chronic STING stimulation leads to cancerous cells developing tolerance and loss of this cell-cycle regulation (201). The exact mechanism of how SASP can induce pro-tumour survival is not fully understood, but it is thought to occur due to changes in the components of SASP to include pro-inflammatory immune suppressive cytokines through alternative splicing and oncogenic events (301, 302). The net effect of this chronic stimulation of STING is that a pro-tumour phenotype is developed, enabling cancer cells to evade the immune response and continue to proliferate, survive and metastasise.

In addition to the cancer-cell autonomous role of chronic cGAS-STING stimulation, chronic stimulation results in increased expression of cGAMP, a STING activator (303). The transfer of this cGAMP to neighbouring normal tissue and cells through gap junctions allows for cancer cell communication with healthy neighbouring cells (303). This in turn stimulates STING in that cell, amplifying the cGAS-STING signalling cascade in a wide range of cells in both cancerous and

non-cancerous tissue. Thus, further facilitating metastasis to distant organs (303). This was observed in astrocytes and brain metastatic cells (303). Here the transfer of cGAMP into astrocytes via gap junctions triggered the cGAS-STING signalling pathway, mediating the release of the the cytokines TNF and IFN- α (304-305). This would typically play a role in promoting inflammation and tumour regression (303-305). Yet the release of these inflammatory cytokines led to the activation of pathways, such as STAT1 and NF- κ B in the metastatic brain cells in a paracrine signalling manner (303). The net effect of this was tumour growth, survival and treatment resistance (306).

Autophagy can also play a role in promoting the development of tumour progression, depending on the tissues type, health status and microenvironment architecture (253, 254). In normal tissue, autophagy acts to inhibit tumour formation, whilst some tumours utilise autophagy to obtain substrates for their metabolic activity to progress and survive (307). The STING driven autophagy association is highlighted by the finding of mtDNA activated cGAS-STING signalling pathway, which resulted in the up regulation of autophagy and growth and progression of oesophageal squamous cell carcinoma (308). The opposite of this was observed in cGAS-STING depletion which diminished autophagy, halting the growth of the oesophageal squamous cell carcinoma (308). Thus cGAS-STING signalling can potentially play a role in regulating autophagy and tumour progression (307, 308).

This link between chronic cGAS-STING activation, tumour growth and metastasis may provide an explanation as to why there has been a lack of meaningful clinical benefit observed from using STING agonists in the management of advanced human tumours (201, 229). Chronic persistent cGAS-STING signalling activation appears to inhibit the anti-tumour immune surveillance observed in early tumorigenesis when the stimulation is of acute nature (201).

1.6 cGAS-STING and metastatic melanoma

Most melanoma cases are diagnosed in the early stage especially with the rising public health awareness of melanoma risk factors of sun exposure and the importance of 'mole checking'. Nevertheless 2-5% of patients will have metastatic

melanoma at the time of diagnosis, with 15-20% of all melanoma patients developing metastatic melanoma eventually if not at the time of diagnosis (309). Melanoma tends to spread via the lymphatics rather than the hematogenous system initially. Hence the most frequent spread sites are the subcutaneous tissue in what is referred to as 'in-transient' satellite metastasis and regional lymph nodes (309). At a later stage distant metastasis can be seen in the lungs, liver, brain and bone (Table 1.4). Metastases to the liver is very common, seen in 1 in 4 patients (309). Metastatic melanoma spread to the liver results in poor prognosis due to the livers' essential role in metabolism and immune tolerance (309). Liver metastases can either be diagnosed at the time of initial presentation or can appear months after the primary tumor is removed (310). In most cases there is no cure for liver metastases, although treatments to shrink the tumor mass and manage symptoms are utilised (310, 311). The prognosis of metastatic melanoma is poor, the 5-year disease free survival is only 5% (309).

Table 1.4 Common metastatic melanoma spread sites.

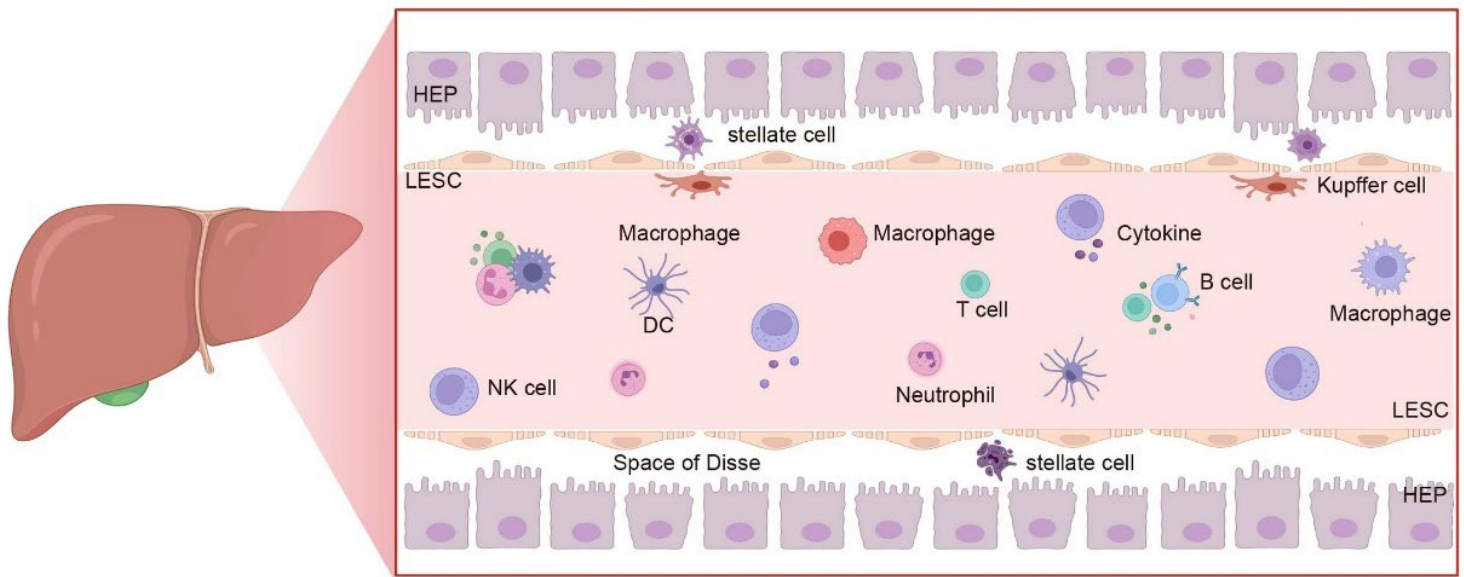
Metastatic site frequency (309)	
Lungs	60-70%
Brain	40-60%
Liver	15-30%
Bone	20-30%

As well as melanoma, the liver is a known common metastatic site for other cancers such as colorectal, pancreatic, gastric, breast, lungs and neuroendocrine tumors (309). This organotropism is not fully understood at a molecular level (312). The 'seed and soil' hypothesis was presented by Steven Pagets back in 1889 (312). He hypothesised that the spread of tumor cells is facilitated by the interactions between the seed, tumor cells, and the soil, the receptive organ microenvironment (312). Since then, signaling mechanisms implicated in facilitating and mediating this metastatic tumor seeding to the liver have come to light (313). The activation of the STAT3 signaling pathway in hepatocytes in mice with pancreatic ductal adenocarcinoma was noted to be active in all hepatocytes (313). Whereas STAT3 activation was noted in only 2% of hepatocytes in control mice models with no cancer (313). Additionally, the knockout of STAT3 in mouse hepatocytes prevented the development of this pro-metastatic niche in the liver

(313). Furthermore, the release of the cytokine IL-6 was found to trigger the STAT3 signaling pathway in the hepatocytes resulting in the accumulation of amyloid (313). This in turn attracted myeloid inflammatory cells to the site, facilitating inflammation and fibrosis in the liver (313). This subsequently resulted in the seeding and growth of cancerous cells to a distant site, the liver (313).

The liver is the largest solid organ in the body accounting for approximately 2% of total adults body weight (314). The liver is recognised as a 'lymphoid organ' amongst immunologists (314). The liver is composed of parenchymal and non-parenchymal cells (314). The parenchyma cells, the hepatocytes, are the most abundant cell type in the liver, accounting for 60-70% of all cells in the liver (314). The non-parenchymal cells account for the remaining cells and include the endothelial sinusoidal cells, resident macrophages Kupffer cells and the hepatic stellate cells (Figure 1.10) (314, 315). The endothelial sinusoidal cells regulate the blood flow, maintaining the portal pressure and allow for exchange of substances between the income blood and the highly metabolically functioning hepatocytes (315). Kupffer cells (KC) are the resident macrophages in the liver that are present during embryonic development (316). KCs are specialised cells and are specific to the liver's immune system (315, 316). KCs form the first line of immune defense, in addition to playing a crucial role in the inflammatory response in the liver (316, 317). The hepatic stellate cells (HSCs), the 'star shaped' cells of the liver play a role in Vitamin A metabolism. In a pathological state HSCs are activated to become myofibroblasts, producing collagen which can eventually lead to liver fibrosis (317).

Cellular Components



1-10 The cellular composition of the liver (315).

The liver is composed of parenchymal and non-parenchymal cells. The parenchyma cells, the hepatocytes are the most abundant cell type in the liver. The non-parenchymal cells account for the remaining cells, non-parenchymal cells include the endothelial sinusoidal, resident macrophages Kupffer, and the hepatic stellate cells. KC are the resident macrophages in the liver and are specific to the liver's immune system (315).

1.6.1 STING and liver metastases

In the liver the cGas-STING signaling system is expressed in non-parenchymal cells, mainly Kupffer, sinusoidal endothelial and HSCs (318). When the cGAS-STING IRF3 and NK-kB is triggered, the IFNs, pro-inflammatory factors and chemokines are released (318). This can result in both anti-tumor effects initially, followed by tumor survival and immune evasion when chronically stimulated (318). The effector immune cells resulting from the STING signaling pathway activation, are known to play an essential role in the pre-metastatic and metastatic niche in liver metastases (318).

1.6.2 The liver metastatic niche and the immune cells role

Immune cells playing a role in liver metastatic niche can broadly be divided according to their lineage of origin, into the myeloid and lymphoid lineage cells (319, 320). The myeloid lineage cells include macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs) (319, 320). The lymphoid lineage cells include T, B, natural killer (NK), and natural killer T cells (319, 320).

1.6.2.1 Macrophages

In the liver metastatic niche, macrophages associated with tumour development, tumour associated macrophages (TAM) are divided into monocyte-derived macrophages (MoMs) and resident macrophages, Kupffer cells (KC) (321, 322). MoMs originate from circulating monocytes derived from bone marrow, while KC are liver resident macrophages (316, 323, 324). The difference between the two types of macrophages does not just lie in the origin but also includes the phenotype of the cells (316, 323).

1.6.2.2 Monocyte-derived macrophages (MoMs)

Macrophages are known to exhibit an M1 anti-tumour or M2 Pro-tumour phenotype, owing to their plasticity that allows for various factors to induce this phenotype polarisation (325). This classification of macrophage phenotype may not strictly be true in MoMs in liver metastases (325). The finding that M1 like macrophages produce tumour necrosis factor which promoted liver metastases challenged this broad macrophage classification of M1 and M2 in liver metastases (326). As a result of this, alternative macrophages classification in liver metastases has been suggested including subgrouping of MoMs based on expression of proteins known to play a role in metastasis, or subgrouping of MoMs based on the macrophages morphology (327, 328). Subgrouping based on the expression and the presence of certain molecules was supported by the finding that the expression level of the SPP1, which a protein implicated in mediating liver metastases in cancers such as gallbladder and colorectal cancers (329, 330). The subgrouping based on the morphological size was highlighted by the finding of a strong correlation between macrophage morphological size and liver metastases in colorectal cancer (328). This topic of classification of MoMs in liver metastases is ongoing. What is known thus far is that compared to healthy liver, tissue from livers with metastases have a significantly higher number of macrophages present and have immunosuppressive features (331, 332). Currently it is understood that MoMs facilitate liver metastases via several mechanisms namely promoting an immunosuppressive and fibrotic environment in the liver, as well as mediating angiogenesis, facilitating tumour cells survival and growth (333). The fibrotic environment in the liver is promoted by the MoMs secreting proteins, such as granulins and progranulins in pancreatic ductal adenocarcinoma for example (334, 335). This then activates the liver HSCs to differentiate into myofibroblasts releasing periostin, a protein involved in tissue remodelling, subsequently resulting in a fibrotic liver environment that is ideal for the growth of the metastatic tumour (335). The immunosuppressive environment in liver occurs due to liver MoMs interacting with the circulating cytotoxic T-cells resulting in apoptosis of these cells which would otherwise have had a cytotoxic effect on tumour cells in the liver (336). Angiogenesis by the MoMs in the liver pre-metastatic niche is understood to occur due to MoMs expressing pro-angiogenic factors namely VEGF (337). Additionally, MoMs can interact with the tumour derived VEGF via

VEGFR1, as observed in colorectal cancer facilitating colorectal cancer liver metastases (337, 338).

While MoMs can facilitate the development of liver metastases, they can also promote an anti-liver metastases behaviour (226, 321). This was evident in colorectal liver metastases mice models, where STING activation in MoMs triggered an anti-tumour response via the NK cells, due to the excessive secretion of IL-18 and IL-1 β (226). It is understood that tumour cells also play a role in promoting MoMs to exhibit a pro liver metastases phenotype (339). Colorectal cancer cells for example promote an M2-like polarisation of MoMs via the secretion of the chemokine CCL2 (339). Additionally, recently the metabolic activation of macrophages in liver metastases has come to light (340). Here the highly metabolically activate macrophages in colorectal liver metastases were identified through single-cell and spatial mapping (340). It is believed that long-chain fatty acids derived from the tumour cells are phagocytosed by MoMs via the scavenger CD36 receptor (341). Thus, providing energy to further trigger a pro-tumour activity at the liver metastases site (341). Signalling pathways within MoMs can also be manipulated to promote liver metastases. An example of this included colorectal tumour cells secreting VEGF triggering the NF- κ B pathway, which in turn promoted the polarisation of MoMs to the pro-tumour M2 like macrophages (342, 343).

1.6.2.3 Liver resident macrophages

Kupffer cells play a role in the immune defense, inflammatory response and repair in the liver tissue (344). In the context of liver metastases, KCs can play a dual role, an inhibitory and a promoter role in liver metastases (316, 344). This depends on the stage of liver metastases niches, whether its early or late stages (316, 344). In the early stages of liver metastases development, KCs exhibit anti-tumour activity mainly through their phagocytic activity against the tumour cells (316). The phagocytic activity is mediated by the presence of specific receptors on KCs, such as the Dectin-2 receptor that acts as a pattern recognition receptor (345). Dectin-2 receptors bind to the tumour cells resulting in phagocytosis and clearance of the liver metastases (345). Phagocytic clearance of tumor cells also occurs via antibody dependent mechanisms (346). Kupffer cells were found to robustly

phagocytose and eliminate tumor cells in the presence of monoclonal antibodies therapy (346). In contrast to only recognising and capturing tumor cells but not eliminating tumor in the absence of a monoclonal antibody (346). KCs also collaborate with other innate immune cells such as the NK cells to eliminate tumor cells in the early stage of liver metastases development (347). This was illustrated when the depletion of NK cells resulted in reduced KCs phagocytic activity against the tumor cells, as the cytotoxic effect of the NK cells that are facilitated by the KCs is reduced (347). Additionally recent research has added to the understanding of this collaborative suppressive activity between effector cells (348). KCs were found to release IL-12 activating NKs to release IFN- γ , which in turn stimulates effector T-cells (348). It is also now understood that KCs combat and eliminate metastatic tumour cells by releasing several molecules namely, TNF- α and nitric oxide inducing hostility and elimination of tumour cells (349). Whole genome analysis has also illustrated that the expression of certain tumour suppressor genes plays a role in mediating the tumour inhibitory role observed early on in liver metastases (350). It was noted that the expression of the tumour suppressor gene *BRACA1* in KCs in mouse and patient samples of colorectal liver metastases, was associated with the anti-tumour phenotype observed in the early stages of colorectal liver metastases (350).

In the late stages of liver metastases, the KCs exhibit a pro-tumor phenotype (333). KCs appear to lose the core and peripheral areas of the tumor as observed in intravital microscopy studies, thus impacting their ability to eliminate tumor cells (351). Additionally in the late stages of liver metastases, persistent oncogenic KCs release several pro-carcinogenic molecules such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), matrix metalloproteinases (MMPs), all of which facilitate tumor proliferation and angiogenesis (326, 348). In contrast to the collaboration with other immune effector cells to eliminate tumor cells that are observed in the early stages, in the later stages of liver metastases KCs attract immune cells that exhibit a pro-metastatic function (352). KCs produce IL-10 and CXCL5, recruiting Tregs and MDSCs respectively (352, 353). Additionally, tumor cells also exhibit features that enable it to evade phagocytotic activity (354). This is illustrated in the study looking at gastric cancer liver metastases (354). They found a high expression of the CD47, 'don't eat me' signal on tumor cells thus evading the phagocytic activity of KCs (354). Whilst such findings enhance our understanding of the anti and pro tumor phenotype in KCs,

further exploration is required to fully understand what triggers the switch or acquisition of a pro-tumor phenotype in KCs in the late stages of liver metastases.

1.7 Rationale

In melanoma current diagnostic tools are invasive, anxiety inducing and costly. The clinical utility of biomarkers in melanoma remains unclear. Much of the current research on melanoma biomarkers has in the main focused on nuclear DNA. This has not to date materialised or translated into clinical practice. mtDNA has been identified as a potential biomarker for cancers due to its ease of detection in blood and non-invasive sample collection. mtDNA offers several advantages over nuclear DNA, including a substantially higher copy number (approximately 1,000–10,000 copies per cell), a simpler structure, and a shorter genome. These characteristics facilitate its extraction, and processing in molecular analyses. Thus, mtDNA could potentially be a preferable choice for a non-invasive biomarker. Mitochondrial DNA could potentially be utilised in the outpatient department skin clinics to correlate levels of mtDNA with clinical severity, recurrence, progression, and response to treatment.

There is a paucity of research investigating mtDNA in melanoma. Epidemiological studies have illustrated that there is a significant association between mtDNA copy number and an increase in risk of cancers such as breast, non-Hodgkin lymphoma, renal cell carcinoma, lung and pancreatic cancer. In melanoma a study showed that the mean mtDNA copy number was significantly higher in melanoma patients compared to healthy controls (n=500 patients). This study however looked at mtDNA copy number in patients with early-stage melanoma and not metastatic melanoma. Furthermore, it was a retrospective study, hence no tracking of mtDNA level and monitoring of response to treatment or progression was investigated. There has been a lack of studies investigating if circulating mtDNA could be used as a biomarker to determine melanoma disease status. Moreover, no work has investigated the role and significance of cell free mtDNA in metastatic melanoma and its progression.

1.8 Hypothesis

I hypothesise that elevated circulating mtDNA can be used as a potential biomarker to monitor metastatic melanoma. I further hypothesise that elevated mtDNA in melanoma can promote pre-metastatic niche development in the liver leading to metastatic progression.

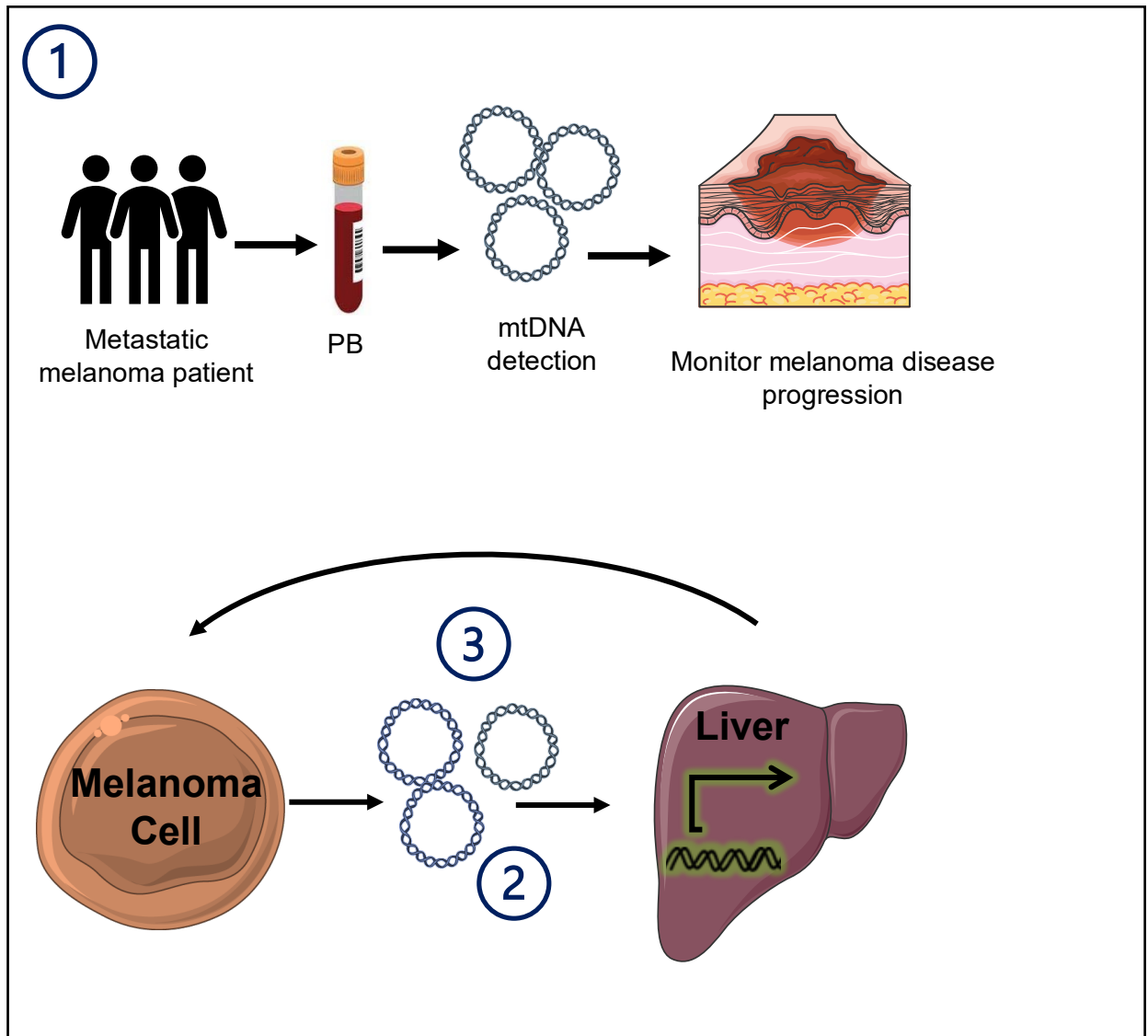
1.9 Objectives

To achieve this, I have three main objectives (Figure 1.11):

Objective 1: Investigate patient serum levels of cell-free mtDNA in metastatic melanoma, its changes in response to disease progression and its potential role as a novel non-invasive biomarker of metastasis.

Objective 2: Investigate in mouse models of metastatic melanoma if cell-free mtDNA can promote changes in the liver immune cell function which leads to increased melanoma engraftment.

Objective 3: To understand the mechanism underlying mtDNA induced changes in liver immune cells and how this allows for metastasis of melanoma



1-11 Graphical representation of aims and objectives.

Using a combination of both *in vivo* and *in vitro* (human and mouse) method

The aims of this thesis to investigate:

- (1) Investigate serum levels of cell-free mtDNA in metastatic melanoma patients as a potential biomarker and its changes in response to therapy and disease progression.
- (2) The signalling mechanism that mediates the interaction between melanoma derived mtDNA and the liver tissue, a known melanoma metastatic site.
- (3) How mtDNA activated liver tissue facilitates melanoma disease survival and progression.

2 Materials and Methods

2.1 Materials

All materials and reagents utilised in this study are included in the table below and the subsequent Methods section.

Table 2.1 Regents and materials utilised in this study.

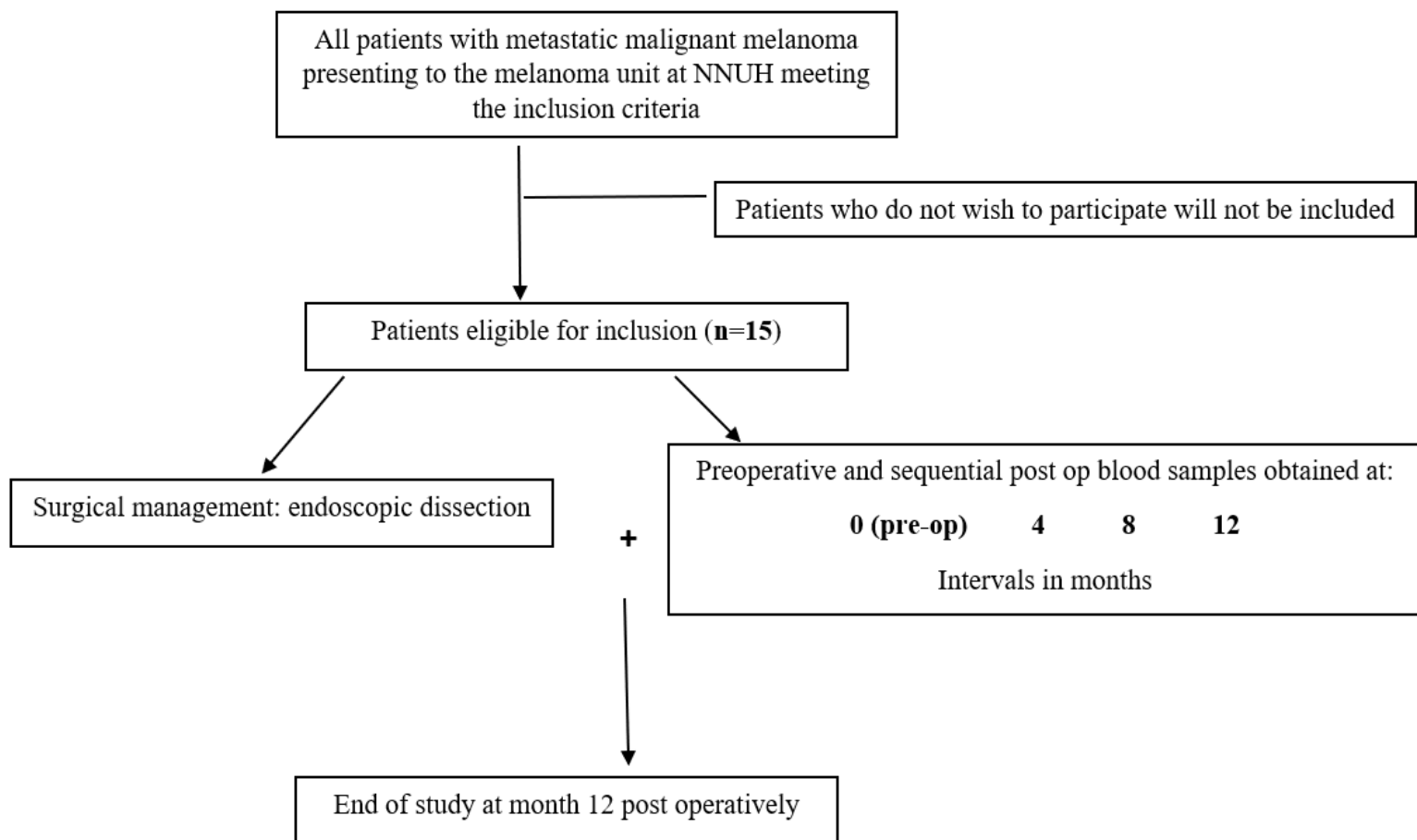
Product	Manufacturer	Catalogues number
ND1 TAQMAN Gene Expression Assay Human	ThermoFisher	4331182_Hs02596873
ND1 TAQMAN Gene Expression Assay Mouse	ThermoFisher	4331182_Mm04225274
8µm Transwell 24-well plate	Fisher Scientific	10167000
26G Butterfly Needles	Medisave UK Ltd	2674829
26G Needles	Fisher Scientific	12349189
Amicon® Ultra-15 Centrifugal Filter	Merck Life Science	UFC910008
Bovine Serum Albumin	Fisher Scientific	BP1600-100
Cellometer SD100 counting chamber	Nexcelom Bioscience	CHT4-SD100-002
CellTrics 40µm filter	Wolf Laboratories	04-0042-2316
DMEM Medium	ThermoFisher	10566016
EDTA	Sigma Aldrich	E9886
Fetal Bovine Serum (FBS)	ThermoFisher	105000056
MicroBeads UltraPure	Miltenyi Biotec	130-110-443
MACS BSA Stock Solution	Miltenyi Biotec	130-091-376
autoMACS® Rinsing Solution	Miltenyi Biotec	130-091-222

MACS separator (QuadroMACS)	Miltenyi Biotec	130-091-051
LS columns	Miltenyi Biotec	130-042-401
Invitrogen™ PURELINK™ Genomic DNA Mini Kit	Fisher Scientific	K182002
Mitochondria Isolation Kit for Tissue	ThermoFisher	89801
OneComp eBeads™ Compensation Beads	ThermoFisher	01-111-42
PCRBIO 1-Step Go RT- PCR Kit	PCR Biosystems	PB10.53-10
Penicillin-Streptomycin	GE-Healthcare	SV30010
qPCRBIO cDNA Synthesis Kit	PCR Biosystems	PB30.11-10
qPCRBIO SyGreen Mix	PCR Biosystems	PB20.12-51
ReliaPrep RNA Cell Miniprep System	Promega	Z6012
RPMI-1640 Medium	ThermoFisher	11875093
TaqPath ProAmp Mastermix	ThermoFisher	A30865
Trypsin-EDTA	ThermoFisher	25200056
Trypan Blue	Sigma Aldrich	T8154

2.2 Patient recruitment

This study is a non-randomized, part-blinded observation single-arm single center feasibility study, investigating circulating peripheral mitochondrial DNA as a potential non-invasive biomarker for surveillance of high-risk cutaneous melanoma.

16 Patients presenting to the melanoma skin unit at the plastic surgery department in the Norfolk and Norwich University Hospital (NNUH), with biopsy proven metastatic melanoma due to undergo resection, were identified for the study (Figure 2.1). Ethics (ETH2122-2327) was obtained from the Norwich research park biorepository. Consenting eligible patients underwent venepuncture pre-operatively with the tumour still in situ, and subsequently had serial follow ups four months post operatively at 4, 8, and 12 months. At follow up, blood samples were obtained during these visits. In this study the exclusion criteria included patients with known metastasising cancer other than melanoma and patients on systemic immunosuppressive therapy. Patients with no known underlying conditions were recruited as the healthy control group.

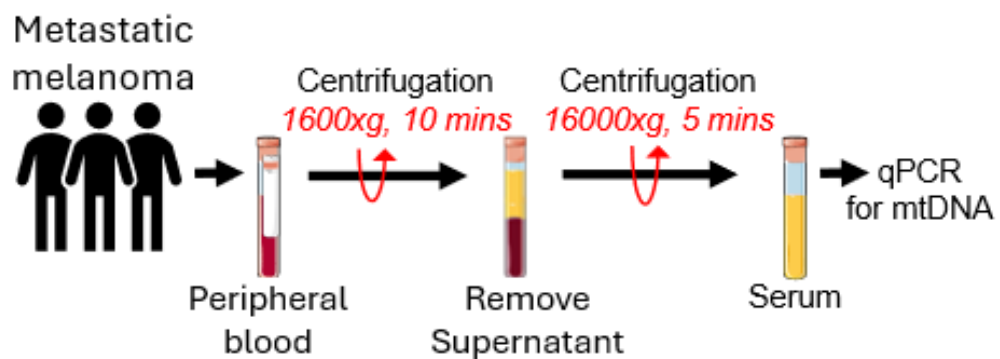


2-1 The human feasibility study design protocol and follow up timeline period.

Patients with biopsy proven metastatic melanoma were recruited in this study. A blood sample was obtained on the day of surgery before tumor resection. Subsequently the patients were followed up at 4, 8, and 12 months post-surgery. A repeat blood sample was obtained at each of these follow up visits to monitor mtDNA levels.

2.2.1 Human metastatic melanoma blood

Blood in standard EDTA blood bottles were obtained from consenting eligible patients at Norfolk and Norwich University Hospital. On receipt of the peripheral blood samples freshly obtained on the day within an hour of venepuncture, the samples were centrifugated at 1600xg for 10 minutes, removing the supernatant, then centrifuging the supernatant at 16,000xg for 5 minutes to pellet any cell debris. The serum was then either stored in the -80°C for processing later or further processed to mtDNA extraction and analysis (Figure 2.2).



2-2 Schematic of the serum extraction process from the peripheral blood (PB).

2.3 Animal maintenance and models

In this thesis all *in vivo* experiments were carried out in accordance with the Animal Scientific Procedure ACT 1986 adhering to regulations set by the UK Home Office under project license PP023671 (Professor Stuart Rushworth). Animals were housed in the Disease Modelling Unit (DMU) at the University of East Anglia in a pathogen free, containment level 3 laboratory. Breeding pairs were set up at 6-8 weeks and maintained for 6 months before separation. The offspring was weaned at 3 weeks after birth. Both male and female mice, aged 8-12 weeks were used for the experiments presented in this thesis.

2.3.1 Wildtype C57BL/6 mice

In this thesis C57BL/6 mice (Jackson Laboratory, Bar Harbour, ME, USA) were used throughout. These mice are widely used inbred mouse strain.

C57BL/6 mice were used to develop the melanoma primary and metastatic model and to investigate mtDNA content in peripheral blood, described in section 4.2. These models were also used to investigate the impact of melanoma engraftment on STING signalling. C57BL/6 were also used to obtain bone marrow derived macrophages (BMDM).

2.3.2 C57BL/6J-Sting1^{gt}/J

This strain commonly known as Goldenticket (Gt) or Sting^{Gt} was purchased from the Jackson laboratory (JAX #017537) has a chemically induced mutant allele of the Sting gene (355). The manufacturer states that in this strain, type 1 interferons such as IFN- β are not produced when the BMDM are stimulated with cyclic dinucleotides or *Listeria monocytogenes* infection (356). They state that this strain can be used to investigate the detection of cytosolic DNA pathogen, the innate immune response and tumour microenvironment. In this thesis, I will refer to this strain of mice when used in experiments as STING-/-.

2.4 Animal procedures

Animal handling and procedure training was provided by Mr. Richard Croft (IGEBEFB87). Experimental procedures were conducted by me, having obtained the UK Home Office personal licence (I39252493) with the help of Dominic Fowler-Shorten (I84770732) and supervision of Professor Stuart Rushworth (ICD3874DB).

2.4.1 Subcutaneous injections (SC)

1×10^6 Melanoma cells (B16F1) suspended in 200ul of PBS were subcutaneously injected once into a selected flank per animal. This was kindly performed by Professor Stuart Rushworth. After the injections were administered, the mice were placed in a clean cage and monitored for a short recovery period before being returned to their home cage.

2.4.2 Intravenous injections (IV)

1×10^6 Melanoma cells (B16F10) suspended in 200ul of PBS were intravenously injected once into each animal using a sterile 26-gauge needle. Prior to injection, the tail vein was vasodilated by placing the mice in a hot box at 37°C for 10 minutes. The mice were then transferred to a benchtop restraint whilst the injection was taking place. This was kindly performed by my colleague Dominic Fowler-Shorten. After the injections were administered, the mice were placed in a clean cage and monitored for a short recovery period before being returned to their home cage.

2.4.3 Melanoma mice model

Primary melanoma

C57BL/6 mice received a single subcutaneous injection of melanoma cells (B16F1) in either flank, this model mimics primary Melanoma.

Metastatic Melanoma

C57BL/6 mice received a single intravenous injection of melanoma cells (B16F10) in the tail vein; this model mimics metastatic Melanoma.

For both models either at end point or at the first sign of ill health, schedule 1 was performed and the animal sacrificed. The primary melanoma in the flanks was dissected. For the metastatic melanoma models the liver was examined macroscopically for metastatic melanoma nodules. For both primary and metastatic melanoma models at end point blood samples were obtained, serum extracted and analysed for the presence for murine mtDNA by qPCR. Liver RNA was extracted and investigated for alterations in specific genes of interest, Stimulator of interferon (STING)-associated genes. The liver was also analysed for immune cell infiltration.

2.4.4 Schedule 1

Mice were humanely euthanised at end point of experiments or at the first signs of ill health such as weight loss, hunched posture, piloerection, and reduced motility. The animals were monitored closely, with weights recorded twice a week at least and more frequently closer to the end point. The schedule 1 method used was asphyxiation by rising CO₂ concentration followed by dislocation of the neck as a confirmation of death.

2.5 Cell culture

All cells utilised in this study were cultured and maintained at 37°C with 95% air/5% CO₂ humidity.

2.5.1 Murine Melanoma B16 cell line

Two variants of the B16 melanoma cell line were utilised in this study, B16F1 and B16F10. B16F1 cells have a lower metastatic potential compared to the B16F10 that have a high metastatic potential (357). The B16F1 cell line was utilised for the primary melanoma model, while the B16F10 was used for the metastatic melanoma models. The cell lines were cultured in DMEM media supplemented

with 2mM glutamine, 1mM pyruvate, 10% Fetal Bovine Serum and 1% penicillin/streptomycin.

2.5.2 Alpha Mouse Liver 12 (AML12) cells

The AML12 cell line was purchased from ATCC (Manassas, VA, USA). This cell line is obtained from hepatocytes isolated from healthy adult male mouse aged 12 weeks. The manufacturer states that the AML12 cells mimic typical hepatocytes and can therefore be utilised as healthy control hepatocytes. The media used to culture AML12 cells consisted of Dulbecco's Modified Eagle Medium F12 (DMEM F12) supplemented with 10% FBS, 1% Penicillin-Streptomycin, 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium and 40 ng/mL dexamethasone. The insulin, transferrin and selenium were purchased as a combined Insulin-transferrin-sodium selenite (ITS) media supplement. The AML12 cells were maintained in a T75 flask in an incubator at 37 °C, with fresh media added every 2-3 days.

2.5.3 Cell cryopreservation

The melanoma cell lines were cryopreserved to allow for long term storage. To do this, the cells were pelleted, and resuspended in freezing mix (10% dimethyl sulfoxide/DMSO in FBS) at a density of 5×10^5 cells/ml. The cells were then transferred into a cryotube and placed in a Mr Frosty container, maintaining the cells at -1°C/minute preserving cell integrity. The cryotubes were then stored in the -80°C freezer.

To thaw the cells, the cryotubes were removed from the -80°C freezer and defrosted at 37°C. The cells are transferred to a 15ml Falcon tube with 10ml of pre-warmed medium added in a drop-by-drop manner to dilute the DMSO and prevent disruption to the cells. Cells were centrifuged at 1400xrpm for 5 minutes; the pellet was then resuspended in fresh medium and cultured as required.

2.6 Murine tissue collection and processing

2.6.1 Serum extraction

The peripheral blood obtained at end point after schedule 1 was used to extract the serum, by centrifuging the blood sample at 1600xg for 10 minutes, followed by a second spin at 16000xg for 5 minutes of the supernatant to discard cellular debris from the sample, based on methods previously described by similar studies (358). The serum sample was then either stored in the -20°C or was processed onto the next stage of DNA extraction.

2.6.2 Murine liver isolation

At end point after schedule 1, to harvest the livers from *in vivo* mice models an incision was made below the sternum and liver lobes dissected out. The gallbladder was separated from the rest of the liver. The largest lobe is obtained and immediately snap frozen in cryopreservation tubes and stored in dry ice to be used for RNA extraction. Two smaller liver lobes were also obtained and placed in Eppendorf containing 1ml PBS supplemented with 2% foetal bovin serum. The Eppendorfs were placed on wet ice ready for the liver immune cell isolation.

2.6.3 Bone marrow isolation

Bone marrow was obtained from the femur and tibia of either wildtype C57BL/6 or STING^{-/-} mice depending on the experiment. Each of the bones were cut in half and placed upside down in a 0.5ml Eppendorf tube containing a puncture at the bottom. This Eppendorf was placed in a 1.5ml Eppendorf. A short spin centrifugation for roughly 5 seconds was performed with the bone marrow (BM) pellets collecting at the bottom of the 1.5ml Eppendorf. The BM pellets were pooled, washed with MACS buffer (1XPBS, 0.5% BSA, 2mM EDTA) and filtered using 40µm CellTrics filter.

2.6.4 Murine bone marrow derived macrophages

Freshly isolated mouse bone marrow was utilised to derive macrophages. 1×10^7 bone marrow cells are plated onto 10cm tissue culture dishes containing RPMI-1640 (Thermofisher) media supplemented with 20% FBS, 1% Penicillin-Streptomycin, and 30% of L929 murine fibroblast cells. 5ml of fresh media was added to the dishes after 3-4 days. Once cells are adherent and confluent, i.e. differentiated into macrophages, media was changed every 2-3 days. The presence of BMDM was confirmed via the characteristic morphology of macrophages examined by microscopy (EVOS M5000 Imaging System). On day 6-7 cells were washed with PBS, EDTA added, and cells scrapped. The BMDM were then seeded in RPMI-1640 media supplemented with 20% FBS, 1% penicillin-Streptomycin, and 30% of L929 murine fibroblast cells.

2.6.5 Mitochondrial DAMPs (mtDAMPs) isolation

The mitochondria isolation kit for cultured cells (Thermoscientific) was utilised to extract mitochondria from the melanoma cell line (B16F1/B16F10). The reagent-based method was used, where 2×10^7 cells were centrifuged at $\sim 850 \times g$ for 2 minutes. 800 μ L of Mitochondria Isolation Reagent A was then added to the isolated pellet, and vortex for 5 seconds, and placed on ice for 2 minutes. 10 μ L of Mitochondria Isolation Reagent B was then added, vortexing at maximum speed for 5 seconds. The tube was then placed on ICE for 5 minutes, vortexing every minute. 800 μ L of Mitochondria Isolation Reagent C was then added to the tube followed by the inversion of the tube few times to mix the reagents. The tube was then centrifuged at $700 \times g$ for 10 minutes at 4°C . The supernatant was then transferred to a new 2.0ml tube and centrifuged at $12,000 \times g$ for 15 minutes at 4°C . 500 μ L of Mitochondria Isolation Reagent C was then added to the pellet, and centrifuged at $12,000 \times g$ for 5 minutes. The pellet containing the mitochondria was either disrupted by sonication to create mtDAMPs or subject to mtDNA isolation described below. The resultant mtDAMPs was then stored in the -20°C for use when required.

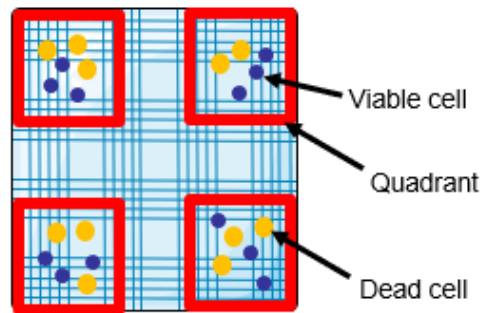
2.6.6 Mitochondrial DNA (mtDNA) isolation

The mtDNA was extracted using the PURELINK Genomic DNA Mini Kit (Thermofisher). The following steps were used for the extraction as per the manufacture's manual. The extracted mitochondria pellet was added to 200ul PURELINK Genomic Lysis/Binding Buffer and vortexed. 200ul of 96–100% ethanol was then added to the lysate and vortexed. 640ul of the lysate was transferred to a PURELINK Spin Column in a collection tube. The column was centrifuged at 10,000xg for 1 minute at room temperature. The spin column was then placed in a clean collecting tube. 500ul of PURELINK wash buffer 1 was added, centrifuging the tube at 10,000xg for 1 minute. 500ul of PURELINK wash buffer 2 was added, centrifuging the tube at maximum speed for 3 minutes. The spin column was then placed in a sterile 1.5ml collecting tube, and 50ul of PURELINK® Genomic Elution Buffer added to the column. This was Incubated at room temperature for 1 minute. The column was then centrifuged at maximum speed for 1 minute. This was then stored in -20°C or utilised for downstream application.

2.7 Cell culture assays

2.7.1 Cell counting

To determine viable cells and seed the correct number of cells, the Trypan blue exclusion method was used. Here viable cells with an intact membrane do not take up the trypan blue dye and remain unstained appearing bright under the microscope. Non-viable cells with a distributed membrane take up the blue dye and can therefore be identified and discounted from the cell count. 10µl of cell suspension is diluted with Trypan blue in a 1:1 ratio. The suspension is loaded onto a haemocytometer, cells are counted in each of the four outer quadrants, and the total number of cells per ml can be calculated as illustrated in Figure 2.3.



Cell number(cells/ml)= Viable cells/number of quadrants counted X dilution factor X 10^4

2-3 cell counting the Trypan blue exclusion method

Haemocytometer with the four outer quadrants containing viable (yellow) and non-viable (blue) cells. The formula is then used to calculate the number of cells per 1 ml.

2.7.2 BMDM stimulation with mtDNA

BMDMs were seeded at a density of 250, 000 in 1ml of RPMI-1640 containing 10% FBS and 1% Penicillin-Streptomycin in a 24-well plate. BMDM were cultured with 53µl of mtDNA in a 2ml final volume of media. Depending on the experiment, cells were treated with 100ng/ml of mtDNA once for the 1-day time point, or daily for the 7-day time point experiment. At end point, BMDM RNA was extracted for subsequent analysis of STING-associated genes via qPCR. The same approach was utilised for the AML12 cell line stimulation with melanoma-derived mtDNA experiment.

2.8 Flow cytometry

In this thesis flow cytometry was performed using a BD FACSymphony A1 flow cytometer, located in the Bob Champion Research and Education Building at the University of East Anglia. The flow cytometer has a violet, blue, yellow-green, and red laser allowing up to 16 colours to be used at one time. Flow cytometry was utilised to identify different cell populations, macrophages (M1 vs M2) and liver immune cells. That is made possible using antibodies to stain the cell surface receptors that are conjugated with a detectable fluorophore.

Table 2.2 contains the antibody panels utilised in this study. Compensation for each antibody panel was set up using the OneComp eBeads™ Compensation Beads. To identify positive and negative populations fluorescence minus one were run for each panel to ensure optimal gating strategies were used. Data was analysed using FloJo software version 10.10.0 software (FlowJo LLC, Ashland, OR, USA). All antibodies were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany), or BioLegend (San Diego, CA, USA).

Table 2.2 The antibody panels utilised for flow cytometry.

Panel	Antibody	Fluorophore
Liver immune cells	CD45	APC-Cy7
	CD11b	APC
	Ly6C	PE
	Ly6G	PE-Cy7
	F4/80	FITC
Bone marrow derived macrophages	F4/80	RE-Cy7
	CD11B	APC
	CD163	APC-Cy7
	CD86	42
	CD206	PE

2.8.1 Bone marrow derived macrophage panel

At end point to prepare the samples for flow cytometry the following steps were used. Media was removed from each well and washed with PBS. 500 µl of 0.25% Trypsin-EDTA was added, a cell scraper was used to scrape the cells from each well. 1 ml of PBS was added to each well after this, the cell suspension was collected in 1.5 ml Eppendorf. Samples were centrifuged at 1000 RPM for 5 minutes. The pellet was resuspended in 400 µl of the master mix containing MACS buffer and the relevant antibody stated in Table 2.2. The master mix consisted of 400 µl MACS per sample and 0.5 µl of each antibody per sample.

2.8.2 Liver immune cell isolation panel

Liver lobes harvested after schedule 1 were placed in aliquots containing PBS supplemented with 2% FBS (PBS/FBS). This was then transferred into 1ml of pre-warmed 6.25µg/ml collagenase IV in PBS/FBS. The lobes were chopped into multiple smaller pieces, placed into a chamber at 37°C for 30 minutes. The parts of the livers that contain metastatic melanoma nodules were not included, and that part of the tissue was excluded in this preparation. After this the liver tissue was passed up and down through a 3 ml syringe further aiding digestion. This was then filtered through a 70µm filter into 15ml tubes. Tubes were centrifuged at 1300 RPM, 4°C for 10 minutes. The pellet was resuspended in 5 ml of 35% Percoll® in PBS/FBS. This was then centrifuged at 1700 RPM for 40 minutes, room temperature.

The supernatant was then discarded, and pellet resuspended in 1 ml of red blood cell lysis and incubated for 3 minutes. The samples were then diluted in 5 ml of PBS/FBS, before centrifugation at 1300 RPM, 4°C for 10 minutes. After that, the supernatant was discarded, and cells/pellet resuspended in 50 µl of the antibody mix stated in Table 2.2. 0.5 µl of each of the listed antibodies was used per sample diluted in PBS/FBS. Antibodies were not added to the samples undergoing the F4/80 enrichment protocol detailed next in 2.8.3. After the addition of the antibodies the samples were incubated for 30 minutes in the dark and left to stain. 200 µl of sheath fluid was then used to top up the samples, which were then transferred into flow tubes ready for flow. 50, 000 events were collected for each sample.

2.8.3 F4/80 enrichment

F4/80 is a specific cell surface murine macrophage marker (359, 360). High expression of the F4/80 cell surface marker is present in tissue resident macrophages, such as the liver resident macrophages, Kupffer cells (359, 360). Once the liver immune cell isolation was carried out, F4/80+ macrophages were purified utilising the magnetic MicroBeads UltraPure (Miltenyi Biotec, Bergisch Gladbach, Germany) separation technique. Here the F4/80+ cells are labelled magnetically with Anti-F4/80 MicroBeads UltraPure. The cells are loaded onto Magnetic-activated cell sorting (MACS®) columns placed in MACS separator magnetic field. The F4/80+ cells magnetic labeled cells are retained within the columns, this can then be eluted as the purified positively selected cell fraction for downstream processing, RNA extraction in this case. To start the F4/80 enrichment process, first a buffer solution was made consisting of PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA, diluting MACS BSA Stock Solution (130-091-376) 1:20 with autoMACS® Rinsing Solution (130-091-222). The buffer was maintained at 2-8°C. Cell suspension obtained from the immune liver cell isolation step was centrifuged at 300g for 5 minutes. The supernatant was aspirated; the pellet was resuspended in 90 µl of the buffer. 10 µl of the Anti-F4/80 MicroBeads UltraPure was added. This was mixed and incubated in the refrigerator (2-8°C) for 15 minutes in the dark. The cells were then washed with 2ml of buffer and centrifuged at 300g for 10 minutes. The supernatant was then aspirated, pellet resuspended in 500 µl of buffer. For the magnetic separation step, the MACS LS columns were placed in the MACS separator (QuadroMACS) and rinsed with 3ml of buffer. The cell suspension was added to the LS column, the flow through the columns (unlabelled cells) was collected. The column was then washed 3 times with 3 ml of buffer, continuing to collect the flow through. The LS columns were then removed from the MACS separator and placed in a collecting tube; 5 ml of buffer was used to flush out the magnetically labelled F4/80+ cells. The F4/80+ cells were then used to extract RNA detailed in Section 2.9.2.

2.9 Molecular biology

2.9.1 DNA extraction

Extracted serum was subjected to DNA extraction to detect presence of mouse and /or human mitochondrial DNA by qPCR. DNA was extracted using the PURELINK Genomic DNA Mini Kit (Thermofisher). The following steps were used for the extraction as per the manufacture's manual. 250ul of serum was added to 200ul PURELINK Genomic Lysis/Binding Buffer and vortexed. 200ul of 96–100% ethanol was then added to the lysate and vortexed. 640ul of the lysate was transferred to a PURELINK Spin Column in a collection tube. The column was centrifuged at 10,000g for 1 minute at room temperature. The spin column was then placed in a clean collecting tube. 500ul of PURELINK wash buffer 1 was added, centrifuging the tube at 10,000g for 1 minute. 500ul PURELINK wash buffer 2 was then added, centrifuging the tube at maximum speed for 3 minutes. The spin column was placed in a sterile 1.5ml collecting tube, 50ul of PURELINK® Genomic Elution Buffer was added to the column. This was incubated at room temperature for 1 minute. The column was then centrifuged at maximum speed for 1 minute. The purified extracted DNA was then either stored in -20°C or processed for downstream application.

2.9.2 RNA extraction

To assess the STING gene expression in BMDM, and hepatocytes/AML12 used in the *in vitro* experiments and livers from in the *in vivo* experiments, real-time qualitative polymerase chain reactions were carried out. To do this RNA was extracted using the Reliaprep RNA Cell Miniprep extraction kit following the manufacturer's instructions. For *in vivo* experiments, a small grain, rice-sized piece of the snap frozen liver was finely placed into homogeniser tubes containing 3 homogeniser beads. After that 500µL RNA lysis buffer was added to the homogeniser tubes and homogenised utilising a Precellys 24 tissue homogeniser (Bertin Technologies SAS, France), settings 2 for 30 seconds at 6000 RPM. For the *in vitro* experiments, plates of AML12 or BMDM cells frozen in RNA lysis buffer were defrosted and collected into Eppendorf tubes ready for RNA extraction. From here both *in vitro* and *in vivo* samples underwent the same processing steps as

follows. 170µl of absolute Isopropanol was added, vortexing the solution for 5 seconds. This was then transferred to a Reliaprep minicolumn in a clean collection tube and centrifuged at 13,000g for 30 seconds. The supernatant was discarded, and 500µl RNA wash added, and centrifuged at 13,000g for 30 seconds. 200µl of Column Wash Solution was then added, and centrifuged at 13,000g for 15 seconds. This was then washed with 500µl of RNA Wash Solution and centrifuged at 13,000g for 30 seconds, followed by a second wash with 300µl of RNA Wash Solution, this time centrifuging at 13,000g for 2 minutes. The minicolumn was transferred to an elution tube, and 20µl of Nuclease-free water added for *in vitro* samples, for the *in vivo* samples using whole-liver lysates 50 µL of nuclease free water was instead added. This was then centrifuged at 13,000g for 1 minute to elute the RNA. The samples were then placed on to ice for quantification and subsequent cDNA synthesis or stored in the -20°C.

2.9.3 Quantification of RNA and DNA

NanoDrop 2000 spectrophotometer (ThermoFisher, Waltham, MA, USA) was used to quantify the extracted RNA and DNA. The NanoDrop was first cleaned with 1 µL of nuclease free water. 1 µl of the sample was then loaded onto the NanoDrop and RNA/DNA concentration (ng/µl) determined. The purity of the sample was determined using the A260/230 ratio. A detected value of 1.8 and 2. was deemed sufficiently pure.

2.9.4 cDNA extraction

The extracted RNA was utilised to obtain cDNA using qPCRBIO cDNA synthesis Kit as per the manufacture's manual. 2µl of RNA was added to an 8µl master mix consisting of 2µl of 5x cDNA synthesis mix, 0.5µl of 20x RTase, and 5.5µl of RNase free water. The samples were incubated with the Bio-Rad T100 Thermal Cycler, where the reactions were incubated at 42°C for 30 minutes, followed by a 10-minute incubation at 85°C to denature RTase followed by an indefinite hold step at 4 °C until samples are removed. cDNA was then diluted in 100 µL of nuclease free and stored in the -20°C until use or processed immediately.

2.9.5 Real-time quantitative PCR (qPCR)

qPCR was carried out using the Roche Lightcycler 480 (Roche, Basel, Switzerland) in 384-well Roche Lightcycler reaction plates. qPCR allowed for analysis of gene expression using the SYBR-GREEN technology. Analysis of mtDNA was carried out using TAQMAN technology. Primers used in this thesis are outlined in Table 2.3.

Table 2.3 A list of SYBR GREEN and TAQMAN primers used in this thesis.

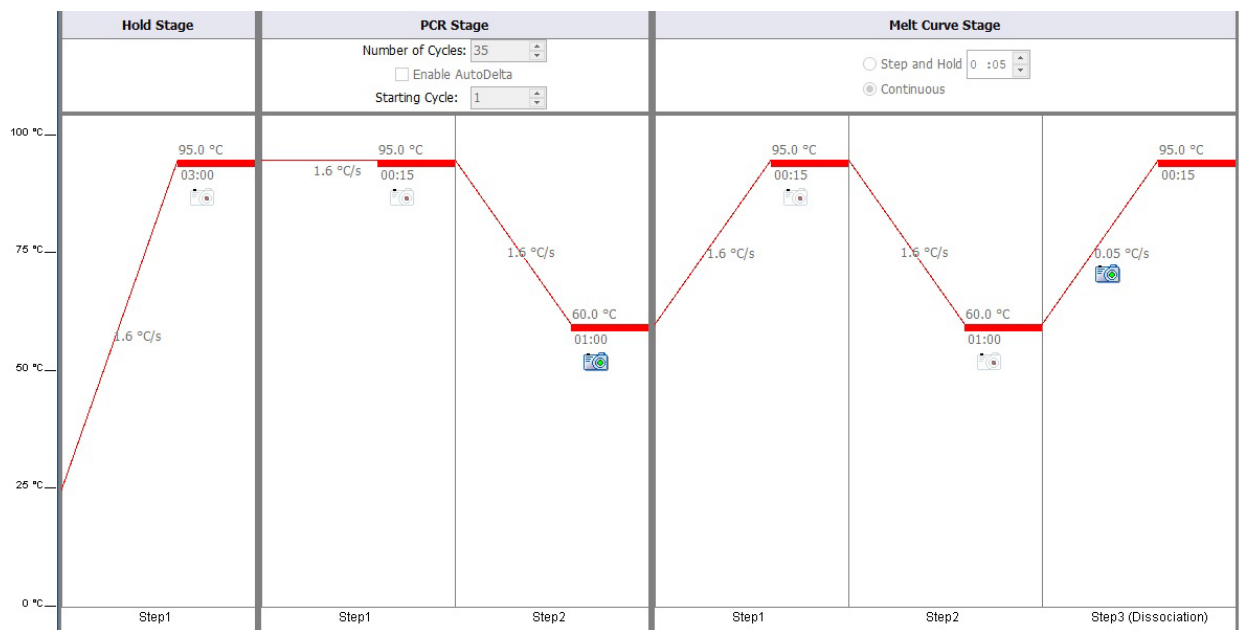
QuantiTect SYBR-GREEN Primers (Qiagen)		
Gene	Primer Assay Name	Gene Globe ID
GAPDH	Mm_Gapdh_3_SG	QT01658692
STING-associated genes (361)		
IRF3	Mm_Irf3_1_SG	QT00108759
STAT1	Mm_Stat1_1_SG	QT00162183
CXCL2	Mm_Cxcl2_1_SG	QT00108759
IRF7	Mm_IRF7_1_SG	QT00104006
Macrophages markers		
CD163	Mm_Cd163_1_SG	249900
CD80	Mm_Cd80_1_SG	249900
TAQMAN® Primers (ThermoFisher)		
Gene	Assay name	Assay specifics
Human ND1	Hs02596873_S1	109116
Mouse ND1	Mm04225274_S1	4331182

2.9.6 SYBR Green gene analysis

The gene expression in the experimental samples was determined by SYBR Green qPCR. A master mix consisting of 2.5µl of 2x SYBR® Green and 0.5µl gene-of-interest primer was added to 2µl of cDNA. The reaction was added to a Lightcycler 480 Multiwall plate384. The plate sealed and centrifuged at 300g for 15 seconds and inserted into the RT-qPCR machine and run on the pre-programmed cycle illustrated in Figure 2.4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilised as the housekeeping gene for all

experiments. As GAPDH is expressed in all cell types and remains relatively constant, genes of interest were normalised to GAPDH (362).

The cycle threshold (Ct) value, the raw output value obtained from the lightcycler of GAPDH was subtracted from the Ct value obtained for the gene of interest, this value known as delta (Δ) Ct. The Δ Ct of the treatment condition was then subtracted from the control condition Δ Ct, generating the $\Delta\Delta$ Ct value. The fold change in expression was then determined relative to untreated samples by calculating $2^{-\Delta\Delta Ct}$.

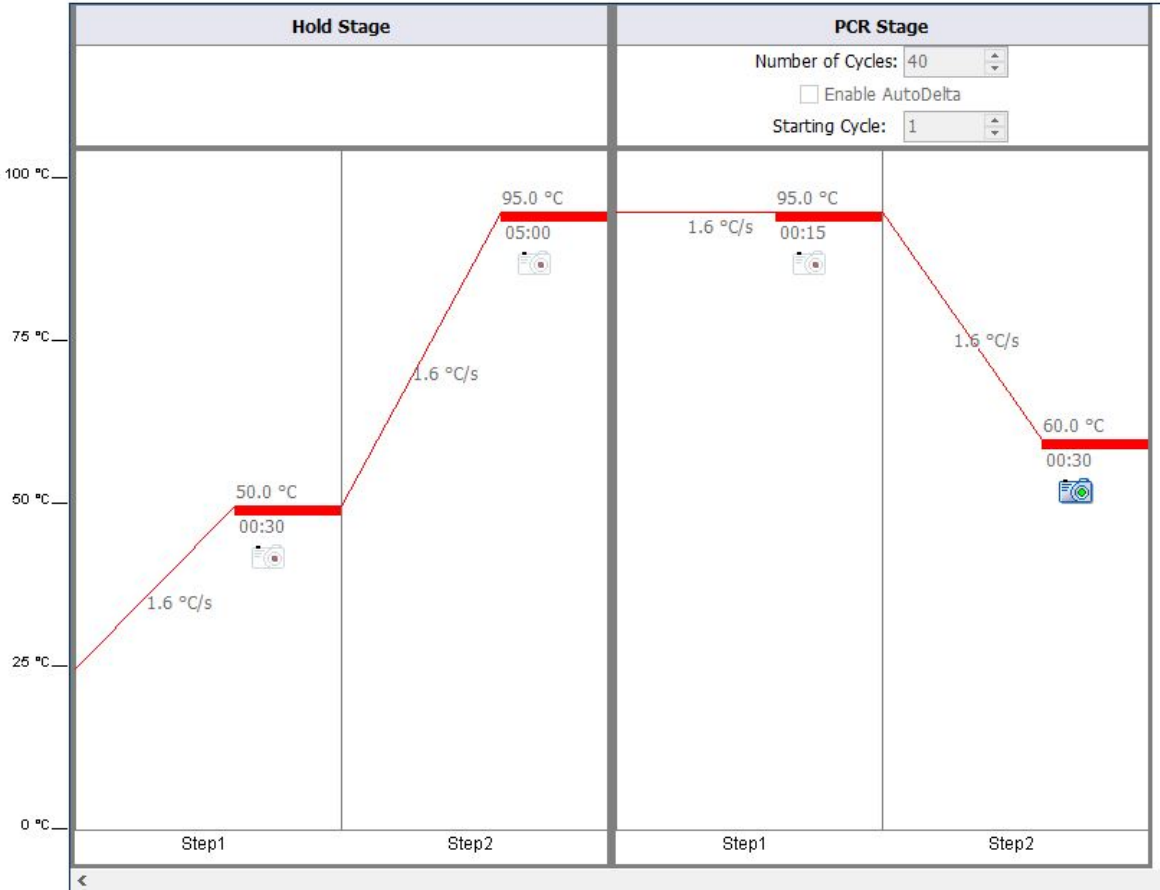


2-4 RT-qPCR SYBR-Green programme settings.

2.9.7 TAQMAN mtDNA analysis

The quantification of mtDNA isolated from the human or mouse serum or melanoma cell line was analysed by TAQMAN® qPCR assay. Probes for the genes of interest contained either 2'- chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) or 6- Carboxyfluorescein (FAM) fluorophores. To prepare the PCR plates, the following master mix was made, 0.25 μ l primer (ND1), 2.5 μ l of TaqPath ProAmp enzyme (Thermofisher), and 0.25 μ l nuclease-free water. 3 μ l of the master mix was then added to 2 μ l of the mtDNA in a Lightcycler 480 Multiwell plate 384, plate sealed and centrifuged at 1000rpm for 300 x g for 15 seconds to mix the reaction, then loaded into the Roche Lightcycler 480 (Roche, Basel,

Switzerland). The PCR was run with a pre-programmed setting shown in Figure 2.5. The generated cycle threshold (Ct) values were utilised to calculate the fold change in the gene expression of mtDNA. $\Delta\Delta C_t$ value of each sample was determined by subtracting the average Ct value of the control samples from the test samples. The fold change was then determined by calculating $2^{-\Delta\Delta C_t}$



2-5 TAQMAN® assay Lightcycler program.

2.10 Statistical analysis

GraphPad Prism software version 10.1.0 (GraphPad Software, San Diego, CA, USA), Microsoft Excel (Albuquerque, NM, USA), and FlowJo software FloJo software version 10.10.0 software (FlowJo LLC, Ashland, OR, USA) were used to analyse the data obtained from the experiments carried out in this thesis. A Shapiro-Wilk test confirmed that the data were not normally distributed, therefore a non-parametric Mann-Whitney U test was used to assess differences between groups. The difference between groups were considered statistically significant if the probability value (p) was less than 0.05. Statistical significance is presented as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data presented in this thesis represent the mean $\pm$ standard deviation, with n denoting the number of biological replicates. The graphs presented in this thesis were produced using GraphPad Prism software version 10.1.0 (GraphPad Software, San Diego, CA, USA). No statistical methods were used to pre-determine the sample size.

3 Results - Circulating cell free mitochondrial DNA is elevated in metastatic melanoma human cases

3.1 Introduction

The currently used diagnostic and prognostic tools in melanoma are invasive (76-79). The role of biomarkers in melanoma remains unclear (88-89). This is a recognised gap in the field of diagnosis and monitoring of melanoma patients (88-89). It has also been identified as one of the National Institute for Health and Care Excellence (NICE) Melanoma future research recommendations (90). A non-invasive biomarker obtained via a simple blood sample for example in the outpatient department setting could potentially have a significant and positive impact in monitoring melanoma disease progression in real time. Several biomarkers such as CfDNA, CtDNA have been investigated as a potential non-invasive biomarker for melanoma, however non-have materialised into clinical practice (112). Nuclear DNA has received the most attention in the search for a melanoma and other cancer biomarker, while mitochondrial DNA has been overlooked somewhat. This could potentially be an oversight, as cell free mitochondrial DNA can also be detected and is known to have a significantly higher copy number per cell, shorter length and simpler structure than nuclear DNA (161-162). Furthermore, studies have illustrated that there is an association between mtDNA copy number and an increase in risk of cancers, such as breast and non-Hodgkin lymphoma (167-170). In melanoma, one study back in 2015 involving 500 melanoma patients, found that the mtDNA copy number was found to be significantly higher in melanoma patients compared to control (167). This study recruited patients with early-stage melanoma and not advanced melanoma. It was also a retrospective study and therefore lacked the ability to track the levels of mtDNA in such patients in response to treatment, and disease progression.

In this chapter I therefore set out to investigate patient serum levels of cell-free mtDNA in advanced metastatic melanoma patients, and its changes in response to surgery and disease progression, as well as its potential role as a novel non-invasive biomarker of metastasis. I hypothesise that elevated circulating mtDNA can be used as a potential biomarker to monitor metastatic melanoma. The aims of this chapter were to investigate serum levels of cell-free mtDNA in metastatic

melanoma patients as a potential biomarker, and it changes in response to therapy and disease progression.

3.2 Cell-free mtDNA is elevated in serum samples from patients with metastatic melanoma

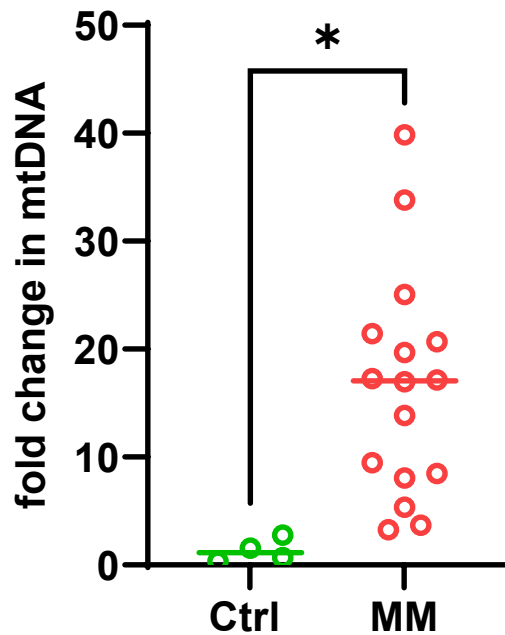
To investigate if mtDNA could potentially act as a non-invasive liquid biopsy or a biomarker in aggressive melanoma, I first carried out a proof-of-principle study. In this study, 16 patients with biopsy proven metastatic melanoma were recruited from the Norfolk and Norwich University hospital, under the Norwich research park ethics (ETH2122-2327). These patients were listed for the standard usual debulking resection surgery for the metastatic melanoma. Table 3.1 shows the demographics and histological diagnosis of each of the recruited patients. The exclusion criteria included patients with known metastasising cancer other than melanoma and patients on systemic immunosuppressive therapy. Patients with no known underlying conditions were recruited as the healthy control group. The commonest diagnosis was the superficial spreading melanoma, which is the commonest type of melanoma. This was closely followed by nodular melanoma which is the second most common type.

Table 3.1 Patient demographics and histological diagnosis.

Patient No.	Age	M/F	Diagnosis	Primary site	Secondary site
1	75	F	Nodular Melanoma	Left upper back	Left axilla
2	86	M	Nodular Melanoma	Right shoulder	Right neck
3	58	F	Superficial spreading Melanoma	Left leg	Left groin
4	63	M	Unknown, Melanoma type	Unknown	Left axilla
5	76	F	Unknown Melanoma tumour type	Left arm	Left axilla
6	68	M	Superficial spreading Melanoma	Midback	Left axilla
7	83	M	Superficial spreading Melanoma	Left lower back	Left axilla
8	52	M	Superficial spreading Melanoma	Right arm	Right axilla
9	57	F	Superficial spreading Melanoma	Right lower back	Right axilla
10	80	M	Nodular Melanoma	Left forearm	Left elbow
11	56	F	Superficial spreading Melanoma	Left arm	Left axilla
12	83	M	Nodular Melanoma	Right arm	Right axilla
13	57	F	Nodular Melanoma	Right arm	Right axilla
14	67	M	Nodular Melanoma	Left chest	Left axilla
15	73	F	Superficial spreading Melanoma	Right leg	Right groin
16	55	M	Superficial spreading Melanoma	Upper back	Right axilla

A peripheral blood sample was obtained for serum from each of the recruited patients on the day of surgery. The blood sample was obtained from the patient with the tumour in situ before the start of surgery. Serum was extracted using the protocol described in the Methods (Section 2.2.1). The blood sample underwent a series of centrifuging steps to remove both blood cells and any remaining large vesicles that may contain mitochondria. DNA was extracted and TAQMAN PCR utilised to quantify mtDNA.

The total DNA was extracted utilising an established kit as described in the Methods section. TAQMAN PCR was then used to quantify the content of mitochondrial DNA (mtDNA). I found that patients with biopsy proven metastatic melanoma had significantly higher levels of circulating cell free mtDNA compared to healthy controls ($p < 0.0106$), as illustrated in Figure 3.1.

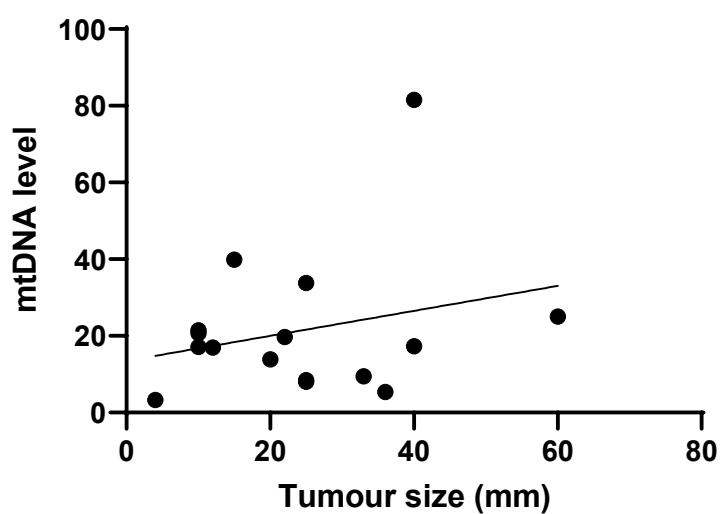


3-1 Cell-free mtDNA is significantly elevated in PB serum of patients with metastatic melanoma.

TAQMAN qPCR was utilised to quantify mtDNA from the isolated sample. mtDNA fold change in serum mtDNA in 4 healthy subjects vs 16 metastatic melanoma patients. Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of control (healthy) samples subtracted from the metastatic melanoma samples. Data indicate mean $\pm$ SD. Data were analysed using Mann-Whitney U test. * $p < 0.0106$.

3.2.1 Tumor size and mtDNA level

To ascertain if there was an association between mtDNA and tumor characteristics such as size and mutations, the histological features were correlated with mtDNA detection. There was no significant correlation between tumor size and mtDNA level ($p=0.339$) illustrated in Figure 3.2.



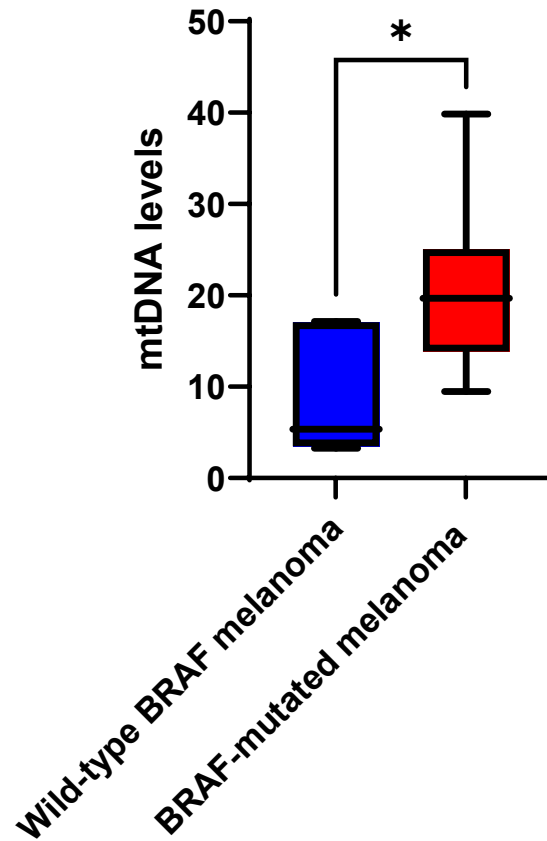
3-2 Tumor mass size and mtDNA level.

The correlation between tumor size and mtDNA level, $R^2=0.06534$ ($P=0.339$).

3.2.2 *BRAF* mutation associated with higher mtDNA

BRAF is a proto-oncogene that encodes a protein called B-Raf, involved in the regulation of cellular growth and differentiation (39, 40). If mutated as is the case in 40-60% of melanoma, it results in uncontrolled cellular growth and cancer development (39, 40). *BRAF*-mutated melanoma has more aggressive behaviour in advanced melanoma (40).

I found that higher levels of mtDNA were associated with the presence of a *BRAF* mutation ($p < 0.0490$), illustrated in Figure 3.3.

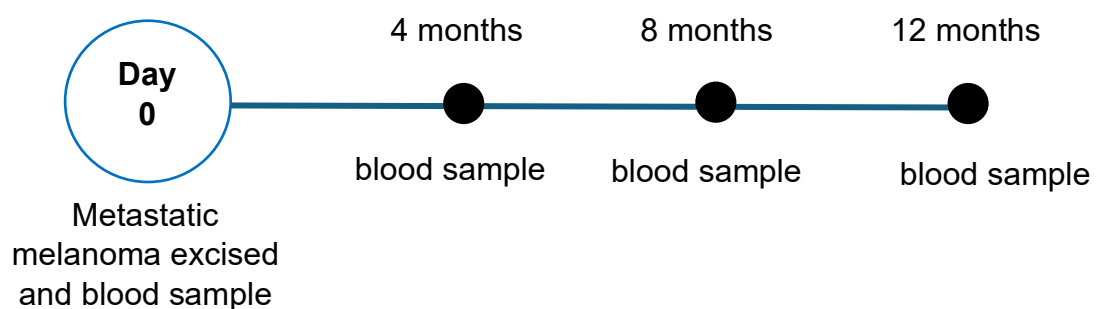


3-3 BRAF mutation is associated with high mtDNA.

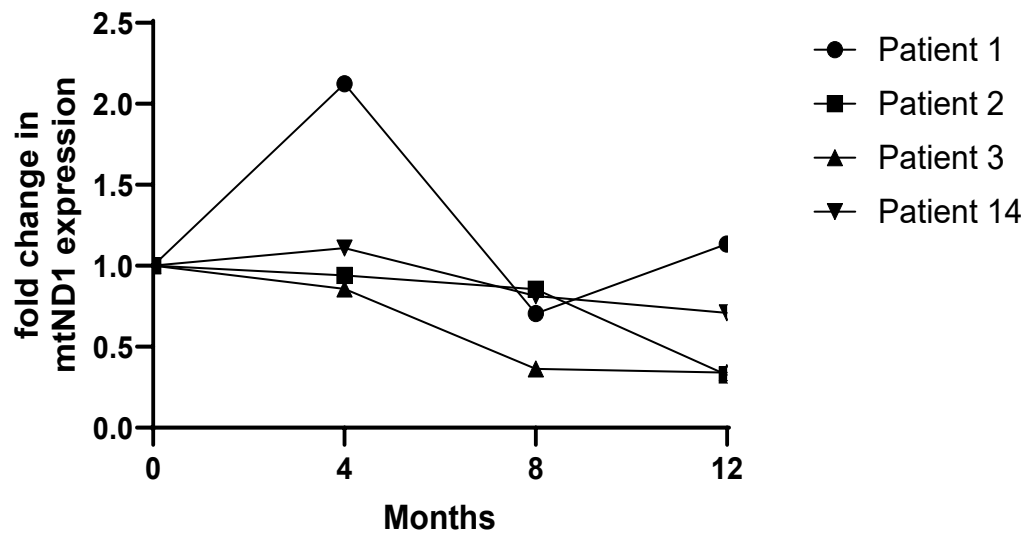
TAQMAN qPCR was utilised to quantify mtDNA from the isolated sample. mtDNA fold change in serum mtDNA in 4 healthy subjects vs 16 metastatic melanoma patients. Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of control (healthy) samples subtracted from the metastatic melanoma samples. Data indicate mean $\pm$ SD. Data were analysed using Mann-Whitney U test. * $p < 0.0490$.

3.3 mtDNA detected distinguished remission and relapsing groups

To investigate the potential of mtDNA as a tool to monitor response to treatment or disease progression, the patients were tracked for 12 months post-surgery. Sequential blood samples were obtained at 4, 8 and 12 months after surgery (Figure 3.4). mtDNA was extracted and quantified in the same manner as the pre-operative baseline mtDNA was measured. Having reviewed the patient's clinical data, I found that 4 of the 11 tracked patients over 12 months were diagnosed with relapsing metastatic melanoma. This relapse was reflected in an increase in the mtDNA detected at the corresponding month of relapse (Figure 3.5), compared to the levels remaining low and not returning to baseline or higher after surgery at months 4, 8 and 12 after surgery in patients who remained in remission (Figure 3.6). In the patient cohort of four who developed a relapse, patient 1 developed an obvious increase in mtDNA measured at month 4, whilst in the remaining 3 patients mtDNA levels did not decrease post operatively (Figure 3.5). In the patient cohort of seven who remained in remission, mtDNA remained low at the sequential 4, 8 and 12 months post operatively follow up bloods (Figure 3.6). There were fluctuations in mtDNA, but mtDNA did not return to the initial pre-operative level measured at month 0.

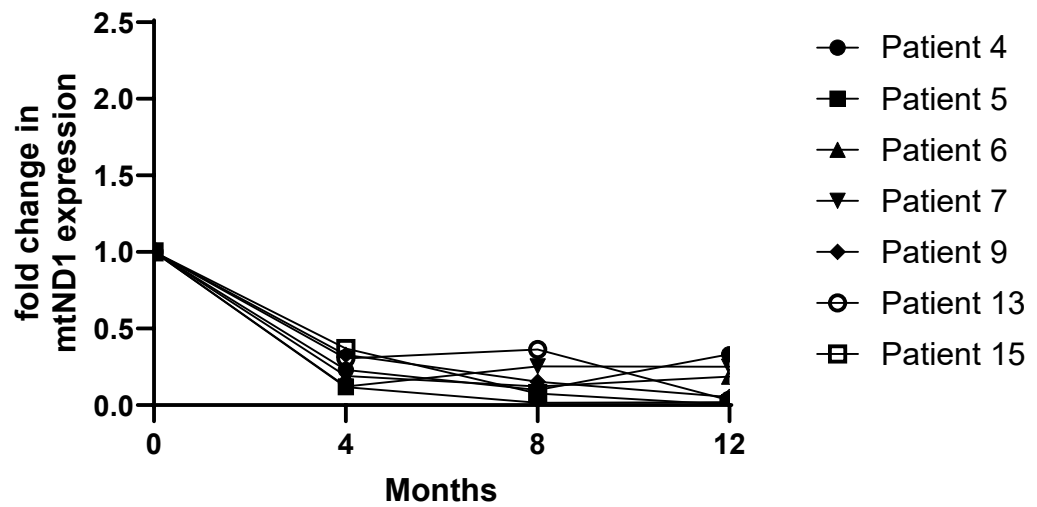


3-4 Schematic of the timeline illustrating when PB was obtained from patients.



3-5 mtDNA levels in four patients developing a relapse.

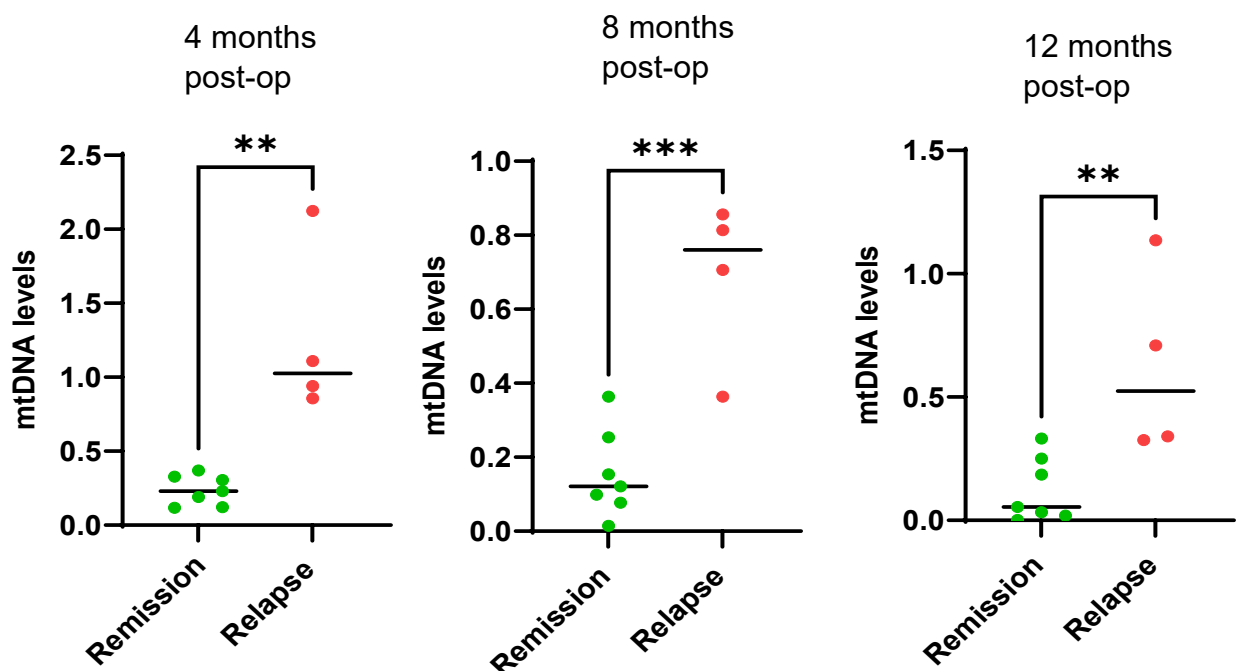
mtDNA fold change in serum mtDNA in four patients with metastatic melanoma who developed a relapse in disease (n=4) out of the 11 patients tracked over a one-year period post operatively. Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of baseline (month 0) samples subtracted from the follow up samples. (n=4).



3-6 mtDNA levels in patients in remission.

mtDNA fold change in serum mtDNA in seven patients with metastatic melanoma who remained in remission (n=7) out of the 11 patients tracked over a one-year period post operatively. Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of baseline (month 0) samples subtracted from the follow up samples. (n=7).

mtDNA detected distinguished between the remission and relapse groups at the 4 months ($p= 0.0012$), 8 months ($p= 0.0005$), and 12 months ($p= 0.0097$) sequential blood tests (Figure 3.7). The 4 patients who developed a relapse all developed metastasis to sites additional to that of the original site of metastasis which required the surgery initially. The metastatic sites included the lungs and brain. The 4 relapsing patients had different outcomes, with one requiring radiotherapy for the brain metastasis, two died at 16 and 18 months from the time of surgery, and one was placed on end-of-life care. Table 3.2 details a breakdown of the patient demographics and outcomes of the four patients who developed a relapse detected by mtDNA.



mtDNA levels compared between the remission and relapse groups at the 4 months ($p= 0.0012$), 8 months ($p= 0.0005$), and 12 months ($p= 0.0097$) sequential blood tests. (remission $n=7$, relapse=4). Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of baseline (month 0) samples subtracted from the follow up samples. Data indicate mean $\pm$ SD. Data were analysed using Mann-Whitney U test. ** $p<0.01$, *** $p<0.001$

Table 3.2 The demographics, diagnosis and outcomes of the patients developing a relapse

Patient No.	Age	Sex	Diagnosis	Progression site	Outcome
1	78	F	Metastatic melanoma lymph node left axilla	Brain metastasis	Radiotherapy required at 4 months post-op
3	57	F	Metastatic melanoma left groin	Brain metastasis	Death at month 18 post-op
2	66	M	Metastatic melanoma left axilla	Brain metastasis	End of life palliative care at 20 months post-op
14	76	F	Metastatic melanoma lymph node right elbow	Lung metastasis	Death at month 16 post-op

3.4 Summary

In this first results chapter, I set out to investigate the association between metastatic melanoma and circulating cell-free mtDNA in peripheral blood. Here, I have found that elevated mtDNA levels can be detected in the peripheral blood serum of patients with metastatic melanoma. I also investigated if there was an association between mtDNA level and tumor features, namely tumor size and mutation. I illustrate that there was no correlation between levels of mtDNA detected and size of tumor. However, the presence of a *BRAF* mutation was associated with much higher mtDNA levels. *BRAF*-mutated melanoma exhibits aggressive behaviour in advanced melanoma.

Investigating mtDNA levels post operatively over 12 months, I show that the four patients who developed a relapse were found to have an increase in mtDNA detected at the sequential post-operative follow up blood samples. Whilst the patients who remained in remission were found to have lower mtDNA levels than the initial pre-operative level detected prior to the resection of the tumour.

4 Results - Establishment of *in vivo* Melanoma Mouse Models and Circulating mtDNA Levels

4.1 Introduction

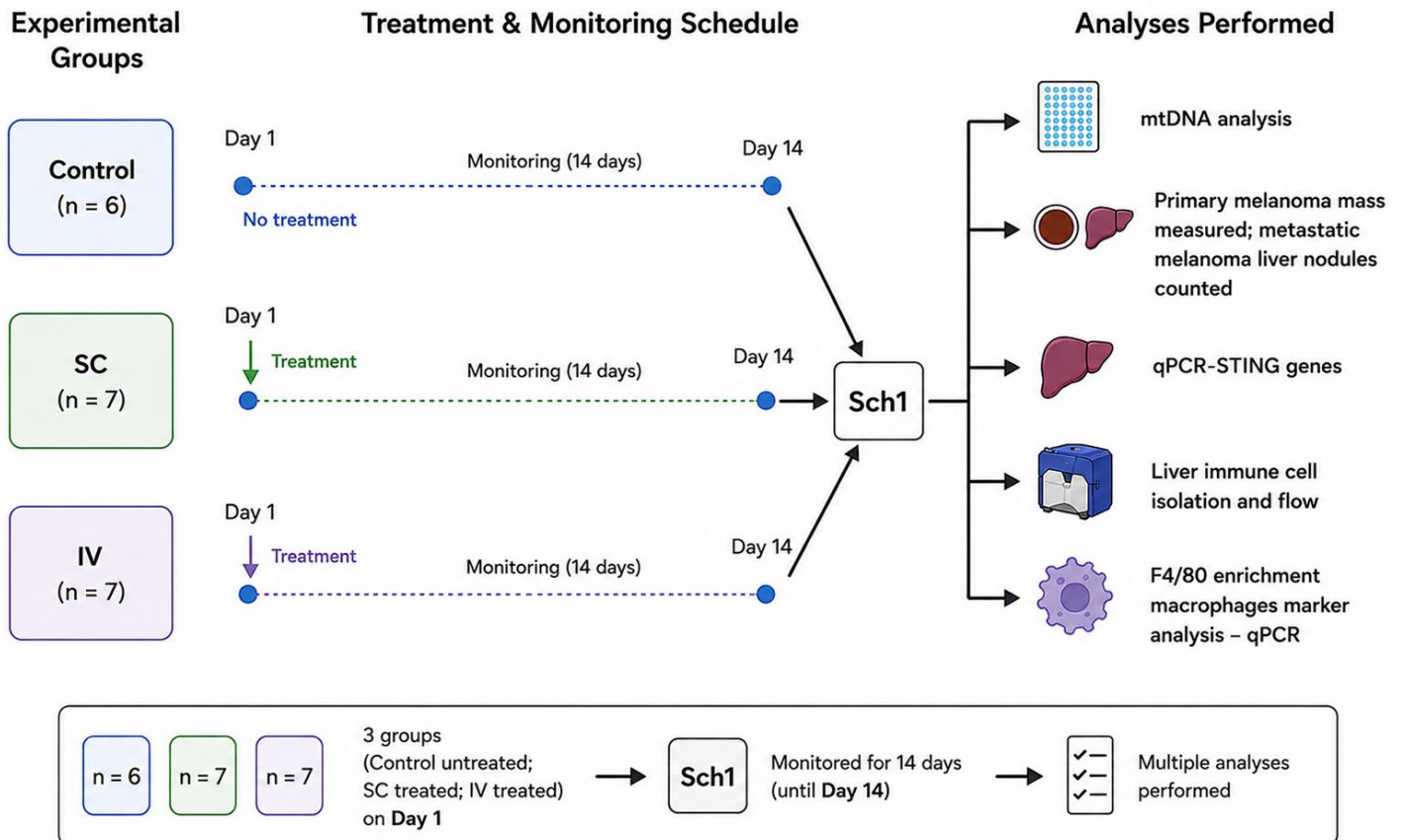
Research examining mtDNA in melanoma remains limited, and if circulating mtDNA could be used as a biomarker to determine disease status. Moreover, no work has investigated the role of cell free mtDNA in metastatic melanoma and its progression. Better outcomes can be realised through new therapies or improved diagnosis, developed from a better understanding of the biology of the disease.

To address this gap in the field, and to allow me to investigate the significance of circulating cell-free mtDNA on melanoma progression, I first set out to develop and establish the melanoma mice models mimicking both primary in situ early-stage melanoma and advanced disseminated metastatic melanoma. This is discussed in this chapter.

4.2 Cell-free mtDNA levels are elevated in primary and metastatic mouse models of melanoma

To test and verify the observation of elevated mtDNA made in patients with biopsy proven metastatic melanoma, two mouse models mimicking primary and metastatic melanoma were first developed. For the primary melanoma model, C57BL/6 mice received a single subcutaneous (SC) injection of 1×10^6 murine B16F1 cells. For the metastatic melanoma model, C57BL/6 mice received a single intravenous (IV) injection of 1×10^6 murine B16F10 cells. At end point on day 14, after schedule 1 the following was obtained namely peripheral blood, tumor mass dissected from the flank site of the SC injection, and intact livers. Schematic of the experimental is depicted in Figure 4.1.

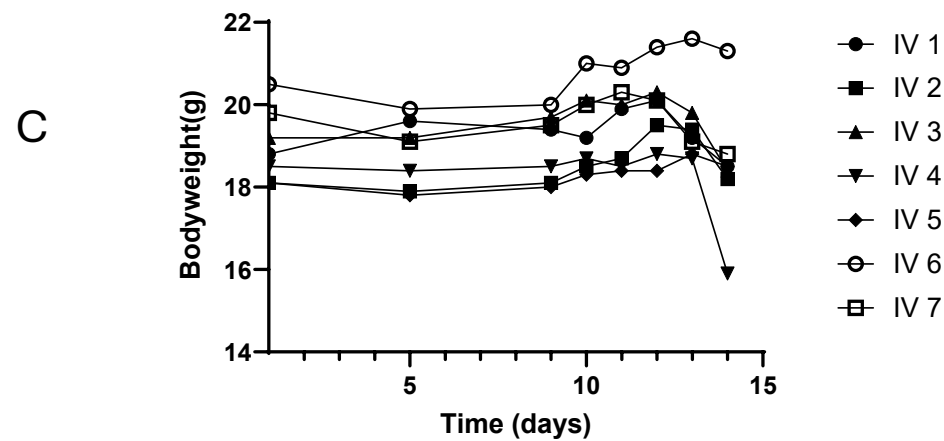
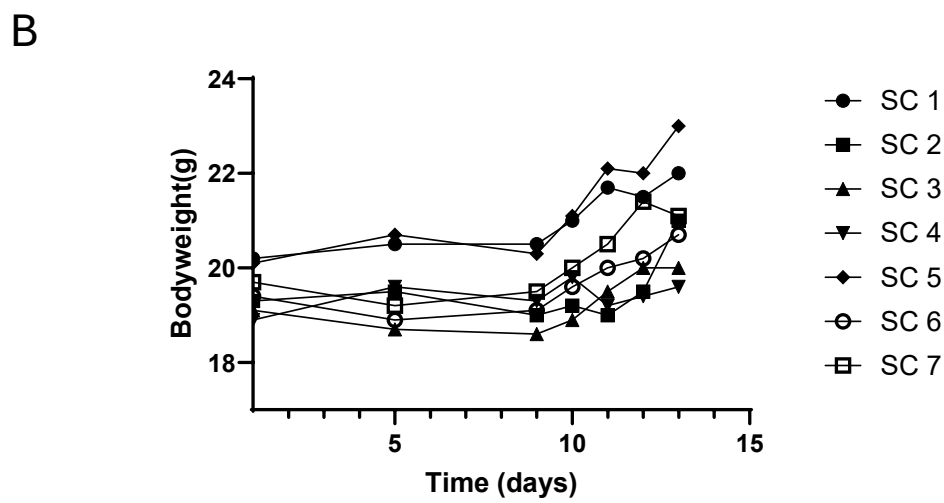
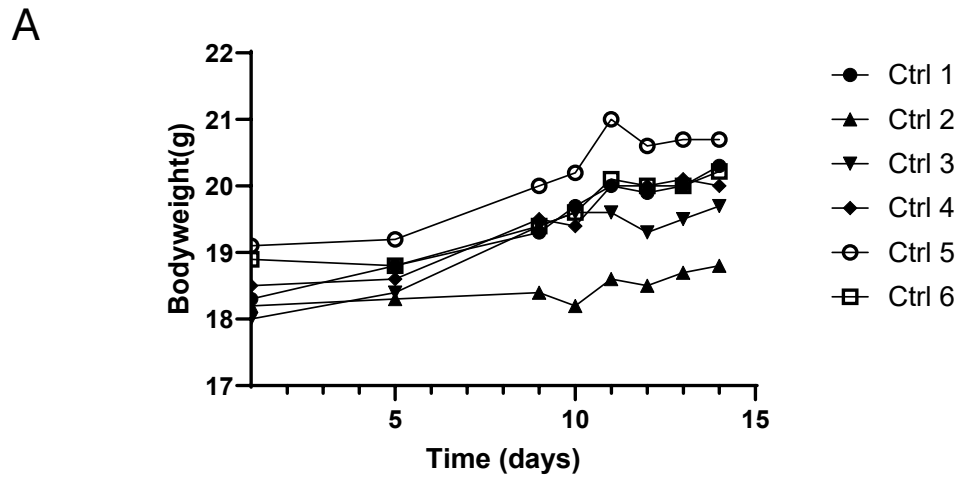
Mice weight was closely monitored throughout; Figure 4.2 compares changes in weight in all three experimental groups. The IV metastatic melanoma model exhibited a significant weight loss compared to the control group where the weight remained consistent. The SC primary melanoma group in contrast to the IV metastatic melanoma group, illustrated an increase in weight. This reflected the increase in the primary tumor mass at the injection site in the flank.



4-1 Schematic of the experimental design of the *in vivo* subcutaneous and intravenous melanoma mice model.

Female C57BL/6 mice, split into three experimental groups. Control mice who received no intervention (n=6), Subcutaneous melanoma (SC) (n=7) group mimicking primary melanoma received a single SC B16F1 1×10^6 injection on day 1 in the flank, and an intravenous (IV) melanoma (n=7) group mimicking metastatic melanoma received a single IV B16F10 1×10^6 injection on day 1.

At end point on day 14, after schedule 1 peripheral blood was obtained to quantify mtDNA. Primary melanoma tumor mass was dissected and measured (mm). Livers were harvested, macroscopic metastatic nodules noted. Liver RNA extracted to analyse STING via qPCR. Liver immune cells were isolated and analysed through flow cytometry. F4/80 enrichment for liver resident macrophages analysed with qPCR to assess macrophage markers and STING.

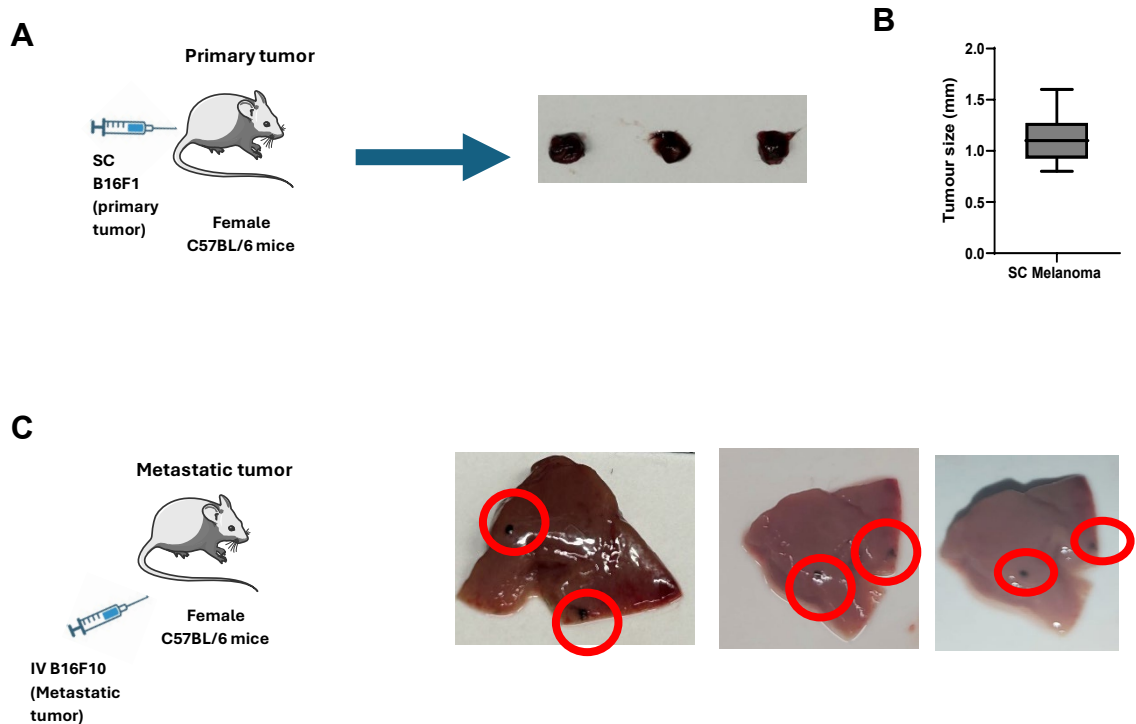


4-2 Variation in bodyweight of each experimental group

(A) Control (n=6) (B) group received subcutaneous B16F1 melanoma injection (n=7) and (C) group received IV B16F10 melanoma injection (n=7).

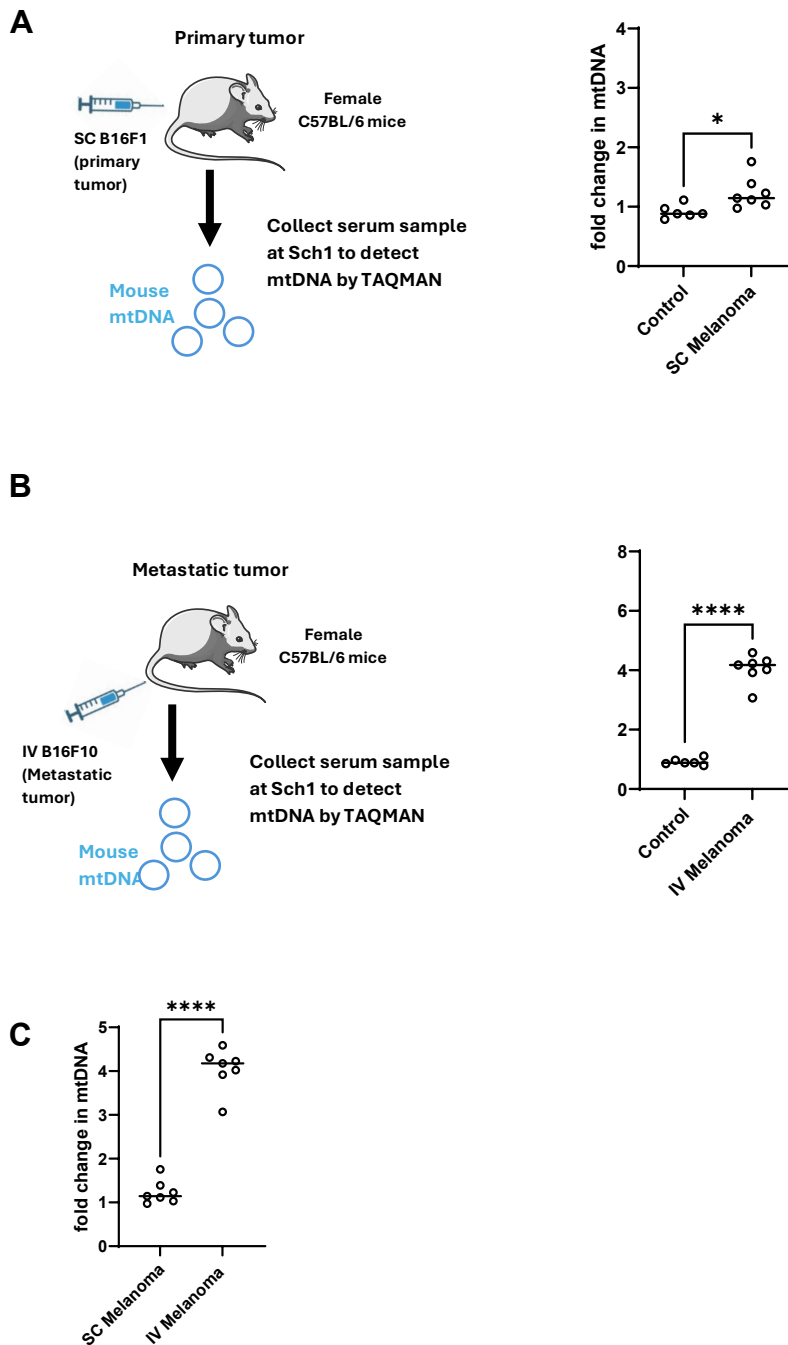
At end point, the primary tumor mass was dissected from the flank of the injection site and measured in the SC model (Figure 4.2A &B). The primary melanoma tumor mass harvested ranged between 0.9 to 1.6mm in size, with mean tumor mass size of 1.1mm. In the metastatic melanoma model, macroscopic analysis and photograph of the metastatic melanoma nodules in the liver were documented (Figure 4.3C). Peripheral blood samples were also obtained at end point on day 14 post engraftment. Serum was obtained, DNA extracted in the same manner as that utilized in the patient samples, mtDNA was subsequently quantified via qPCR.

In the SC primary melanoma model, mtDNA was found to be significantly higher than that detected in control mice who received no intervention (* $p < 0.0103$) illustrated in Figure 4.4A. Like the serum attained from patients with metastatic melanoma, serum from the metastatic melanoma mouse model was found to have significantly higher levels of circulating mtDNA compared to the control (** $p < 0.0000$), as illustrated in Figure 4.4B. Furthermore, compared to the SC primary melanoma model, the mtDNA increase observed in the IV metastatic melanoma model was markedly higher (Figure 4.4C).



4-3 The phenotype in primary and metastatic melanoma mouse model.

- (A)** Schematic of experimental design. C57BL/6 mice received a single subcutaneous dose (SC) in the flank of 1×10^6 murine B16F1 cells ($n=7$). At end point on day 14 after schedule 1, tumor mass was dissected and photographed.
- (B)** At end point on day 14, after schedule 1, the primary tumor mass that was dissected from the injected flank site was measured (mm).
- (C)** Schematic of experimental design. C57BL/6 mice received intravenous dose (IV) of 1×10^6 murine B16F1 cells ($n=7$). At end point on day 14 after schedule 1, the liver was dissected and metastatic nodules photographed.



4-4 Serum mtDNA is significantly elevated in SC and IV melanoma mouse model compared to control.

(A) At end point serum was obtained from the peripheral blood, DNA extracted. mtDNA quantified via qPCR. Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of control ($n=6$) samples subtracted from the primary melanoma ($n=7$) samples. Data indicate mean $\pm$ SD. Data was analysed using Mann-Whitney U test. $*p < 0.0103$.

(B) At end point serum was also obtained from the peripheral blood, DNA extracted. mtDNA quantified via qPCR. Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of control ($n=6$) samples subtracted from

the metastatic melanoma (n=7) samples. Data indicate mean $\pm$ SD. Data was analysed using Mann-Whitney U test. ****p < <0.000.

(C) mtDNA level in serum obtained from peripheral blood of SC primary melanoma (n=7) and IV metastatic melanoma (n=7). Fold change was calculated based on the $\Delta\Delta C_t$ value of the C_t value of control (n=6) samples subtracted from the primary melanoma (n=7) and metastatic melanoma (n=7) samples. Data indicate mean $\pm$ SD. Data was analysed using Mann-Whitney U test. ****p < <0.0001.

4.3 Summary

To investigate the impact and role of circulating mtDNA on melanoma progression, I first established melanoma mouse models mimicking primary melanoma and metastatic melanoma. Engraftment was confirmed by locating the primary tumour mass in the subcutaneous model, which mimics primary melanoma. For the metastatic melanoma IV model, metastatic melanoma nodules in the liver, a known metastatic site were documented.

Phenotypic assessment revealed distinct systemic effects, mice in the SC model exhibited progressive weight gain, reflecting the increasing size of the flank tumour mass. The mice in the IV model demonstrated weight loss, particularly at the experimental endpoint, consistent with the general decline in health associated with disseminated disease.

Following confirmation of melanoma engraftment, I next investigated the presence of circulating mtDNA. Both primary (SC) and metastatic (IV) melanoma models exhibited significantly higher levels of mtDNA compared to control mice.

5 Results – Melanoma engraftment induces a down regulation in STING pathway in liver resident macrophages which exhibit an M2 phenotype

5.1 Introduction

Most melanoma cases are identified in the early stages resulting in good patient outcomes. However, up to 5% of melanoma cases are diagnosed with metastatic melanoma at the time of presentation (309). Furthermore, up to 20% of patients will go on to eventually develop metastatic melanoma at a later stage. Metastatic melanoma has a poor prognosis (309-310). Metastatic sites include the lungs, brain, bone and liver. Metastasis to the liver, seen in 1 in 4 patients, presents a particular challenge, as currently there is no cure for it (311). Additionally, melanoma liver metastases are an indicator of treatment resistance and a predictor of poor response to systemic therapy (310-311).

As well as melanoma, metastases to the liver are observed in other cancers such as colorectal, lungs and breast for example (309). Whilst this organotropism is not fully understood at a molecular level, the Steven Pagets hypothesis of 'seed and soil' highlighted the need for a receptive microenvironment to facilitate and encourage metastases to distant organs from the primary tumor site (312).

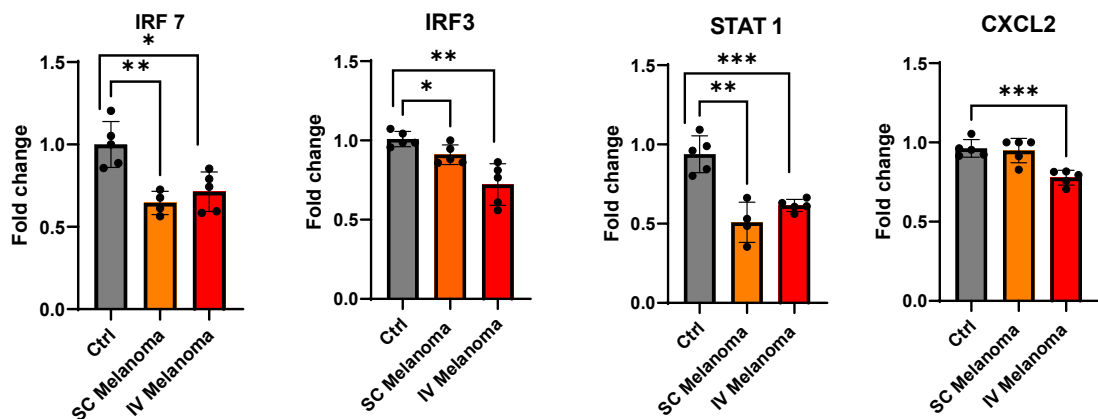
Since then, signaling pathways such as the cGAS-STING implicated in facilitating and mediating this metastatic tumor seeding to the liver have been identified (313). In the liver the cGAS-STING signaling system is expressed in non-parenchymal cells, mainly Kupffer cells, sinusoidal endothelial cells and HSCs (318). When the cGAS-STING IRF3 and NF- κ B is triggered, IFNs, Pro-inflammatory factors and chemokines are released, it can result in both anti-tumor effects initially, followed by tumor survival and immune evasion when chronically stimulated (318). The effector immune cells such as macrophages resulting from the STING signaling pathway activation, are known to play an essential role in the pre-metastatic and metastatic niche in liver metastases (319, 320, 333).

In chapter 4, having shown that both primary (SC) and metastatic (IV) melanoma engrafted mice models exhibited significantly higher levels of circulating cell-free mtDNA compared to control mice. Next, I wanted to investigate the signalling

mechanism that mediates the interaction between melanoma derived mtDNA and the liver tissue, specifically identifying the effector immune cells and signalling pathways that maybe implicated in this. Additionally, I set out to investigate how mtDNA activated liver tissue facilitates melanoma disease survival and progression. I hypothesise that elevated mtDNA in melanoma can promote pre-metastatic niche development in the liver leading to metastatic progression. In this chapter I set out to investigate the role of the STING pathway and immune cells in liver metastases, a common melanoma metastatic site, utilising both *in vivo* and *in vitro* techniques.

5.2 Melanoma engraftment results in downregulation of STING-associated gene expression in whole liver lysates

To investigate the role of the STING pathway in melanoma liver metastases, liver RNA was obtained from both the SC and IV melanoma mouse model at end point on day 14 post melanoma engraftment. STING-associated genes were found to be significantly down regulated in the SC and IV Melanoma mouse model (Figure 5.1).



5-1 Melanoma engraftment caused a downregulation of STING-associated gene expression in whole liver lysate of engrafted melanoma mice.

Whole liver RNA lysates were obtained at end point from both SC and IV melanoma mice models; liver RNA extracted; qPCR was utilised to quantify the genes. Relative gene expression between control (n=5) and melanoma engrafted mice (n=5). Fold change was calculated based on the $\Delta\Delta C_t$ value obtained from the ΔC_t value calculated from the C_t value of the housekeeping gene (GAPDH) subtracted from the C_t value of the gene of interest. Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001.

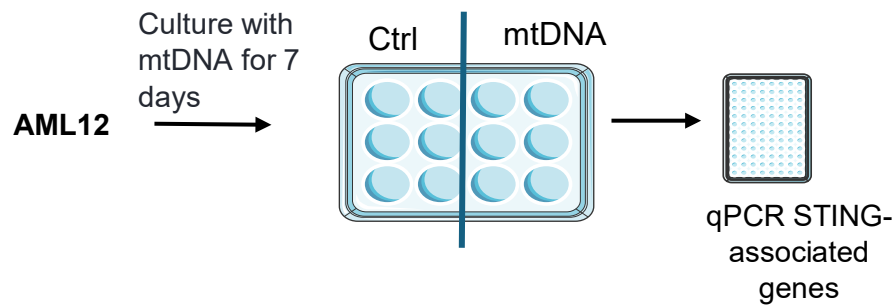
mtDNA normalisation between serum and isolated mtDNA for *in vitro* assays

To ensure that the concentration of mitochondrial DNA (mtDNA) used in the *in vitro* assays was physiologically relevant, I first averaged the mtDNA qPCR Ct values obtained from the serum of mice intravenously injected with melanoma (mean 22.515 ± 1.61). These values were then compared to the Ct values from mtDNA extracted from the melanoma cell line B16F10 (mean 17.27 ± 0.79). Using the mean, I calculated that there was a difference of 5.24 Ct difference which equals 37.7-fold more in the *in vitro* starting content. Based on this comparison, the isolated mtDNA was diluted to match the level observed *in vivo* (53 μ L of mtDNA was used in a 2ml final volume of media), thereby establishing a relevant and representative concentration for use in subsequent *in vitro* experiments.

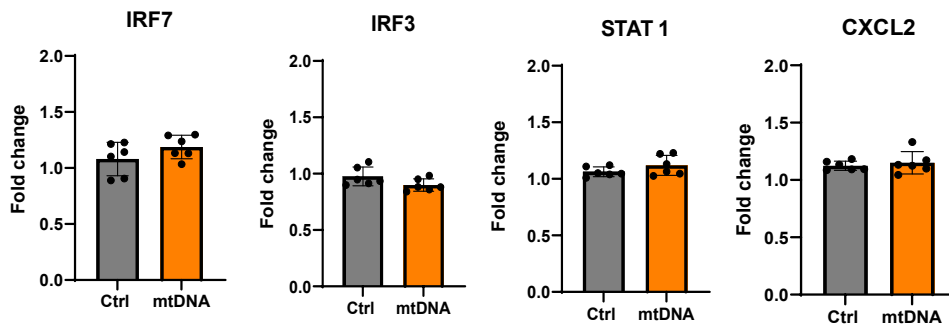
5.3 STING-associated gene expression in hepatocytes have no response to mtDNA

Given that the liver comprises both parenchymal cells (hepatocytes) and non-parenchymal cells (including resident immune cells such as Kupffer cells), next I investigated whether the downregulation of STING-associated genes observed in the liver lysates of melanoma-engrafted models occurs in parenchymal or non-parenchymal cells. To achieve this, I utilised the Alpha Mouse Liver 12 (AML12) hepatocyte cell line. AML12 cells were cultured with melanoma-derived mtDNA extracted from the B16F10 melanoma cell line, as described in the Methods section. AML12 cells were treated daily with 53 μ L of mtDNA or 53 μ L of PBS (vehicle control) for 7 days. At endpoint, RNA was extracted, and STING-associated gene expression was quantified using qPCR (Figure 5.2A). No changes in STING-associated gene expression were observed in AML12 cells challenged with melanoma-derived mtDNA (Figure 5.2B).

A



B



5-2 No change observed in STING-associated genes in AML12 hepatocytes cell line challenged with melanoma derived mtDNA.

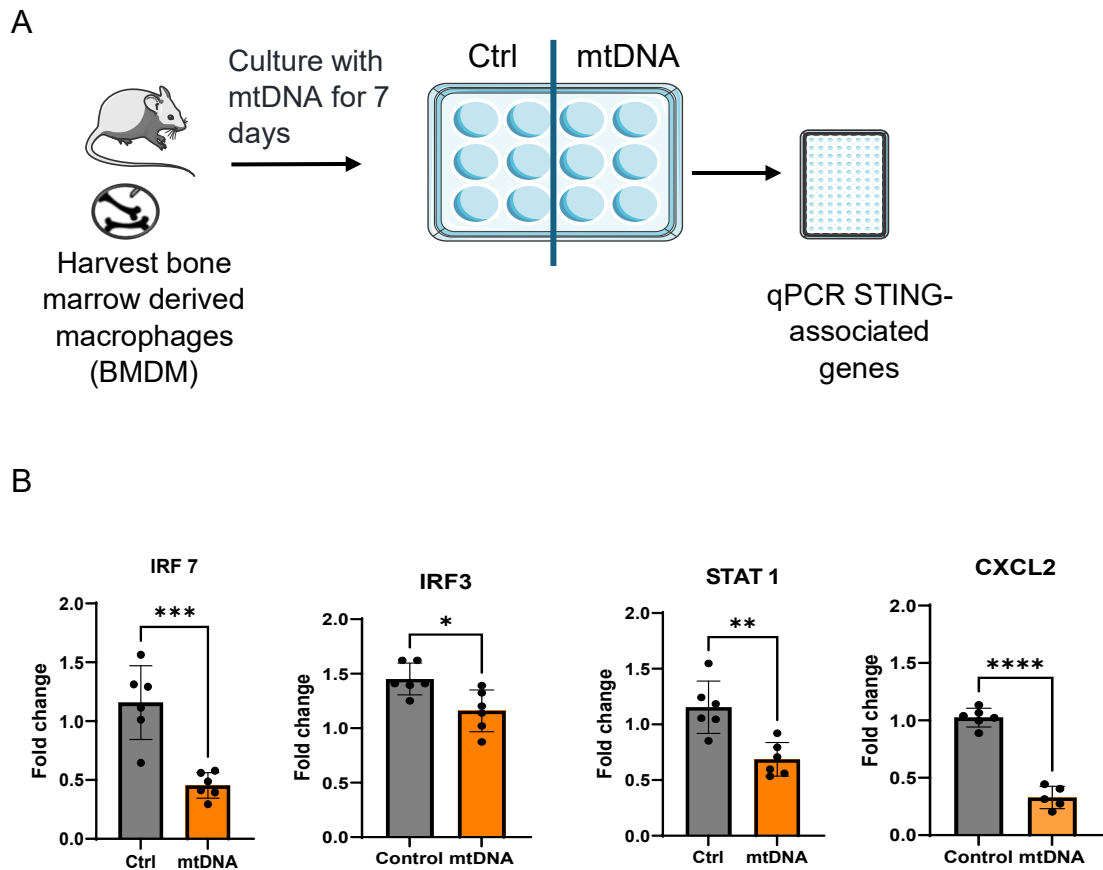
(A) *in vitro* experimental design schematic. AML12 (n=6) were challenged with 53ul of mtDNA derived from the melanoma cell line B16F10 daily for 7 days. RNA was subsequently obtained and qPCR STING-associated genes were analysed.

(B) Relative gene expression between control (n=6) and AML-12 cells treated with melanoma derived mtDNA treated cells (n=6). Fold change was calculated based on the $\Delta\Delta C_t$ value obtained from the ΔC_t value calculated from the C_t value of the housekeeping gene (GAPDH) subtracted from the C_t value of the gene of interest. Data indicate mean $\pm$ SD. The difference was not statistically significant $p > 0.05$.

5.4 STING-associated gene expression in BMDM in response to mtDNA

To investigate the role that non-parenchymal liver cells, specifically immune cells play in response to mtDNA, next I utilised BMDM given that it is a primary immune cell. To do this I utilised BMDM from wild type C57BL/6 mice. The BMDM were co-cultured and challenged with 53µl of melanoma-derived mtDNA daily for 7 days. At end point, RNA was extracted and STING-associated genes quantified utilizing qPCR (Figure 5.3A).

In contrast to the lack of response observed in AML12 hepatocytes treated with mtDNA, BMDMs exhibited a significant downregulation of STING-associated genes following mtDNA challenge (Figure 5.3B). This difference between hepatocytes and macrophages highlights the critical role of immune cells in mediating the melanoma-derived mtDNA-induced STING response within the liver microenvironment. This suggests that the downregulation of STING signalling occurs predominantly in non-parenchymal cells, such as liver-resident macrophages.



5-3 Down-regulation of STING-associated genes in wildtype BMDM challenged with melanoma derived mtDNA.

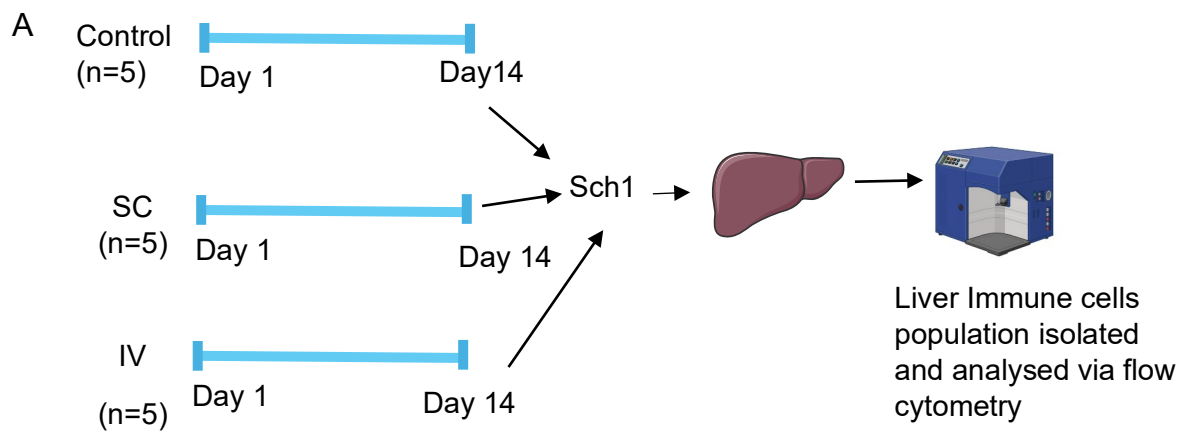
(A) *in vitro* experimental design schematic BMDM (n=6) were challenged with 53 μ l of mtDNA derived from the Melanoma cell line B16F10 daily for 7 days. RNA was subsequently obtained qPCR STING-associated genes were analysed.

(B) Relative gene expression between control (n=6) and BMDM treated with melanoma derived mtDNA treated cells (n=6). Fold change was calculated based on the $\Delta\Delta C_t$ value obtained from the ΔC_t value calculated from the C_t value of the housekeeping gene (GAPDH) subtracted from the C_t value of the gene of interest. Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001.

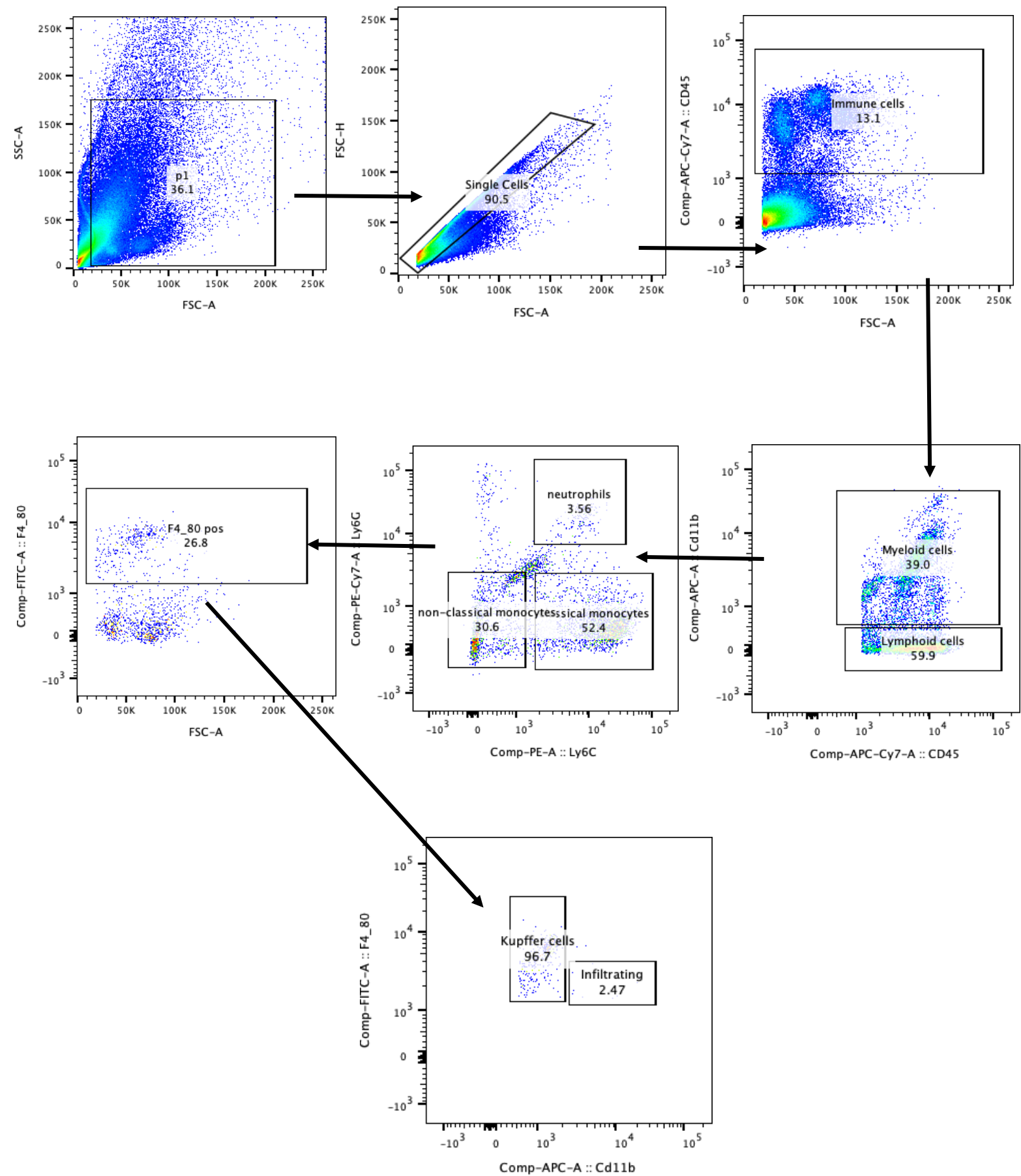
5.5 Immune Cell Profiling in Liver Following Melanoma Engraftment

To investigate the role of immune cells in response to melanoma engraftment, *in vivo* melanoma mouse models were employed. Two models were used, subcutaneous (SC) (n=5) and intravenous (IV) (n=5) melanoma (Figure 5.4A). At the experimental endpoint, liver immune cells were isolated from both models. These cells were subsequently analysed using flow cytometry to assess immune cell populations and their response to melanoma engraftment (Figure 5.4B).

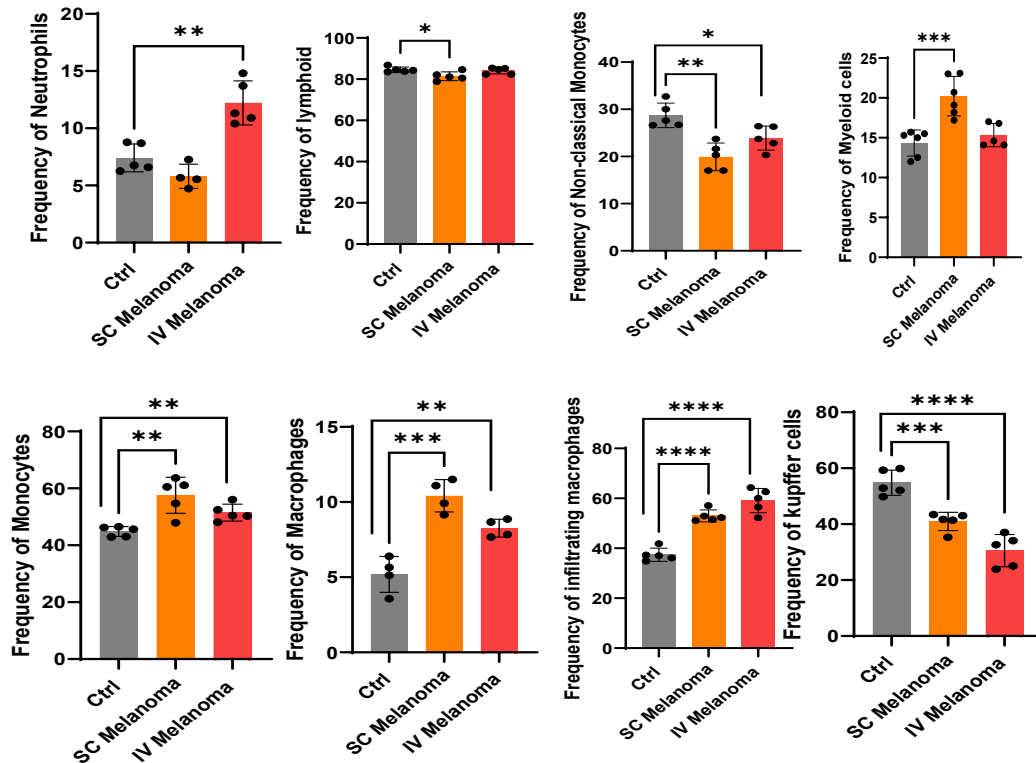
Flow cytometry analysis revealed distinct immune cell dynamics in response to melanoma engraftment. A significant decrease in the frequency of non-classical monocytes was observed in both the subcutaneous (SC) ($p=0.0010$) and intravenous (IV) ($p=0.0185$) melanoma models, suggesting a consistent systemic impact of melanoma on this monocyte subset (Figure 5.4C). A significant increase in the frequency of myeloid cells was detected only in the SC melanoma model ($p=0.0008$), indicating a model-specific expansion of myeloid populations potentially linked to localised tumour growth (Figure 5.4C). There was a significant alteration in myeloid populations in response to melanoma engraftment. In contrast to non-classical monocytes, total monocyte frequency was significantly increased in both models SC ($p=0.0025$) and IV ($p=0.0027$) indicating a shift in monocyte subset distribution (Figure 5.4C). The infiltrating macrophages were significantly increased in both SC ($p<0.0001$) and IV ($p<0.0001$) liver melanoma models, highlighting a consistent macrophage recruitment or expansion in the hepatic microenvironment following melanoma engraftment (Figure 5.4).



B



C



5-4 Increased infiltrating macrophages in the liver of both primary SC and metastatic IV melanoma mice models.

(A) Schematic of experimental design.

C57BL/6 mice received either a single SC injection of 1×10^6 murine B16F1 cells (n=5) or single IV injection of 1×10^6 murine B16F10 cells (n=5), with a group receiving no intervention making up the control cohort (n=5). Melanoma was left to engraft, before sacrifice on day 14 post injection followed by liver immune cell isolation and flow cytometry analysis

(B) Gating strategy for the liver immune cells population identified by flow cytometry analysis.

Post melanoma engraftment, immune cells isolated from the liver were stained with an antibody panel using markers for lymphoid and myeloid populations.

(C) Increased infiltrating macrophages in melanoma engrafted models.

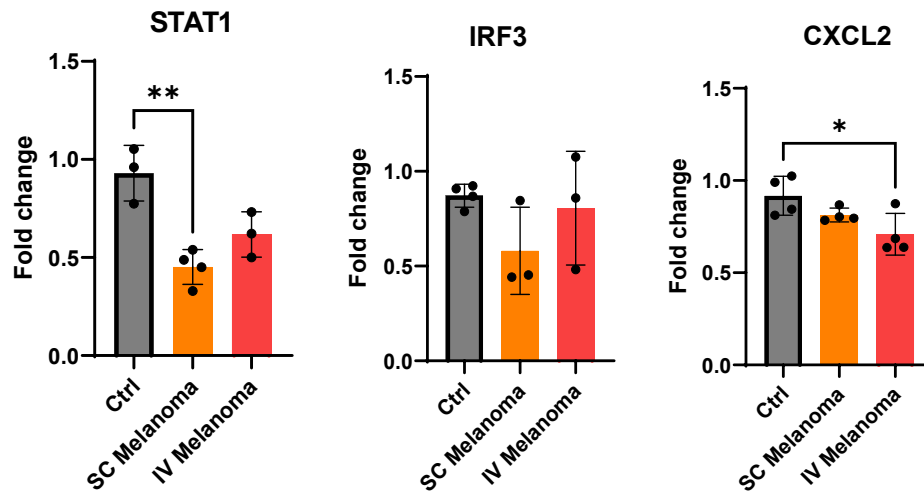
The frequency of immune cells in the liver of engrafted primary (n=5) and metastatic melanoma (n=5) models compared with control (n=5) mice.

Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

5.6 Down regulation of STING pathway in liver macrophages following melanoma engraftment

Given the observed increase in infiltrating macrophages and immune cells in the liver of melanoma-engrafted mice, I next investigated whether STING expression was altered in liver-resident macrophages. At endpoint following both SC and IV melanoma engraftment, liver immune cells were isolated. F4/80+ macrophages were then purified using magnetic bead separation from both control and melanoma-engrafted mice. Subsequent analysis of STING-associated gene expression in the isolated F4/80+ macrophages revealed a down-regulation of STING-related genes in both SC and IV melanoma models (Figure 5.5). This suggests a potential suppression of STING-mediated innate immune signalling in liver-resident macrophages in response to melanoma presence.



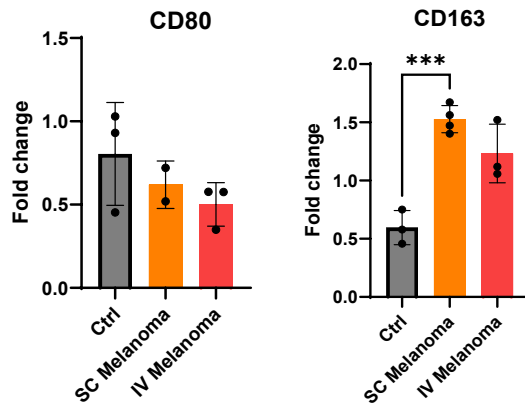
5-5 Melanoma engraftment caused a downregulation of STING-associated gene expression in liver F4/80 positive macrophages of both SC and IV melanoma engrafted models.

Relative gene expression between control (n=4) and melanoma engrafted mice, SC (n=4), IV (n=4). Fold change was calculated based on the $\Delta\Delta C_t$ value obtained from the ΔC_t value calculated from the C_t value of the housekeeping gene (GAPDH) subtracted from the C_t value of the gene of interest. Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test. *p < 0.05, **p < 0.01. Some data points were not included due to undetectable RNA levels in the test.

5.7 M2 phenotype in liver resident macrophages in melanoma engrafted mice

To characterize the phenotype of the liver-resident macrophages in melanoma-engrafted mice, expression of M1 (CD80) and M2 (CD163) markers were assessed via qPCR. F4/80+ macrophages were previously isolated from liver immune cells using magnetic bead separation.

Analysis revealed an increased expression of the M2 marker CD163, indicating a shift toward an M2-like phenotype in liver-resident macrophages in both SC and IV melanoma models (Figure 5.6). This suggests a potential immunosuppressive or tumour-promoting macrophage polarisation in response to melanoma engraftment.



5-6 Melanoma engraftment mediated an M2 phenotype in liver F4/80 positive macrophages of both SC and Iv melanoma engrafted models.

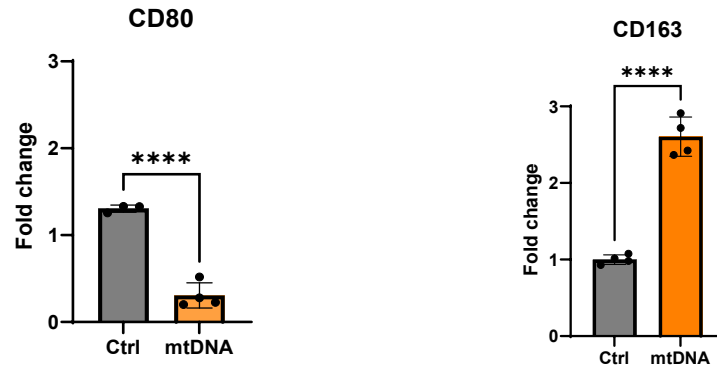
Relative gene expression between control (n=3) and melanoma engrafted mice, SC (n=4), IV (n=3). Fold change was calculated based on the $\Delta\Delta C_t$ value obtained from the ΔC_t value calculated from the C_t value of the housekeeping gene (GAPDH) subtracted from the C_t value of the gene of interest. Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test. ***p<0.001. Some data points were not included due to undetectable RNA levels in the test.

5.8 M2 phenotype observed in BMDM challenged with melanoma-derived mtDNA

To explore whether melanoma-derived mtDNA influences macrophage polarisation like what was observed *in vivo*, BMDMs were challenged with melanoma-derived mtDNA for 7 days.

As illustrated in Figure 5.7, qPCR analysis revealed a significant upregulation of the M2 marker CD163 ($p < 0.0001$), indicating a shift toward an immunosuppressive or tumour-promoting phenotype. A significant downregulation of the M1 marker CD80 ($p < 0.0001$), further supporting the polarisation toward an M2-like state.

These findings suggest that melanoma-derived mtDNA may contribute to the reprogramming of macrophages toward an M2 phenotype, potentially mirroring the tumour-associated macrophage profile observed in the liver of melanoma-engrafted mice.



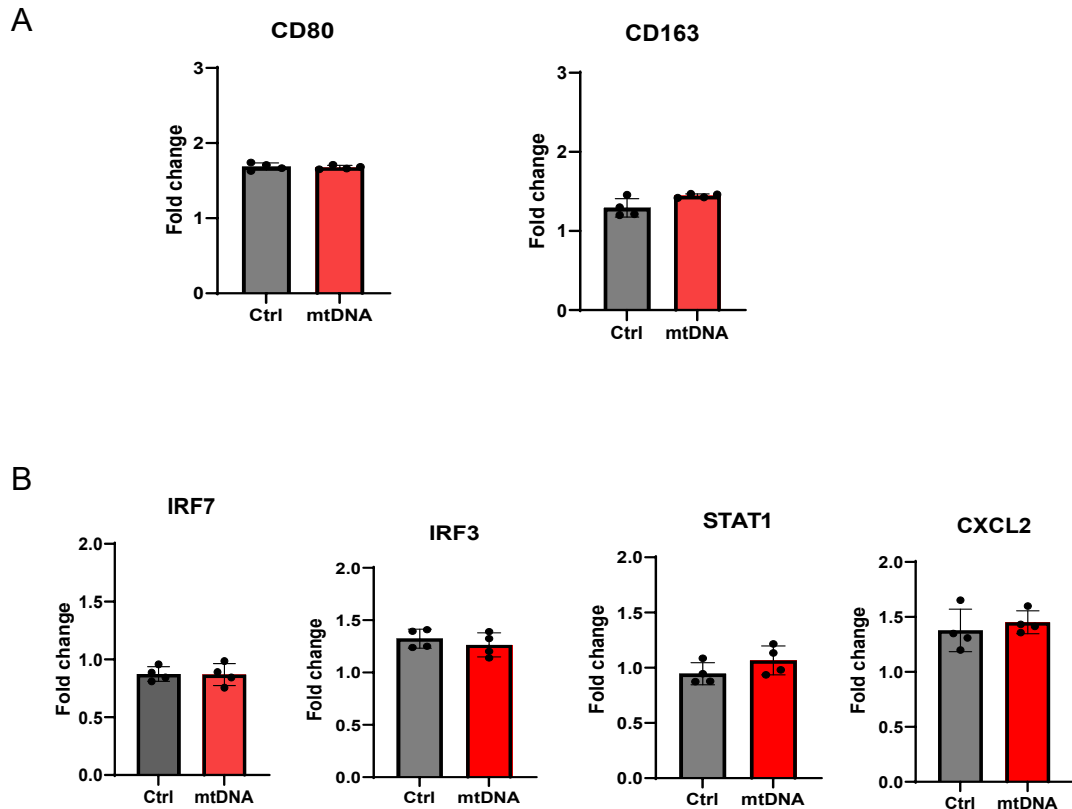
5-7 M2 phenotype observed in BMDM challenged with melanoma derived mtDNA.

BMDM were cultured with melanoma derived mtDNA for 7 days. RNA extracted and qPCR for Macrophage markers M1 (CD80) and M2 (CD 163) were analysed.

Relative gene expression between control (n=4) and mtDNA treated BMDM (n=4). Fold change was calculated based on the $\Delta\Delta\text{Ct}$ value obtained from the ΔCt value calculated from the Ct value of the housekeeping gene (GAPDH) subtracted from the Ct value of the gene of interest. Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test. ****p<0.001.

5.9 STING ^{-/-} BMDM exhibit no response to melanoma-derived mtDNA stimulation

To determine whether the observed M2 polarization in BMDMs exposed to melanoma-derived mtDNA was dependent on STING signalling, STING^{-/-} BMDMs were co-cultured with melanoma-derived mtDNA under the same conditions as wild-type (WT) BMDMs. Unlike WT BMDMs, STING^{-/-} BMDMs did not exhibit an M2 phenotype following mtDNA exposure. Specifically, the upregulation of CD163 and downregulation of CD80 observed in WT BMDMs (Figure 5.7) were absent in STING^{-/-} BMDMs (Figure 5.8A). Furthermore, the downregulation of STING-associated genes seen in WT BMDMs in response to mtDNA (Figure 5.3B) was not observed in STING^{-/-} BMDMs (Figure 5.8B). These findings suggest that STING signalling is required for the mtDNA-mediated M2 polarisation of macrophages, highlighting a potential mechanism by which melanoma may modulate the immune microenvironment.



5-8 STING ^{-/-} BMDM show no response to the stimulation with the melanoma derived mtDNA.

(A) Macrophage polarisation analysis in STING^{-/-}-BMDM treated with melanoma-derived mtDNA.

STING ^{-/-} BMDM were cultured with mtDNA for 7 days. RNA extracted and qPCR for Macrophage markers M1 (CD80) and M2 (CD163) were analysed and compared between the mtDNA treated STING ^{-/-} BMDM (n=4) and control STING^{-/-} BMDM (n=4).

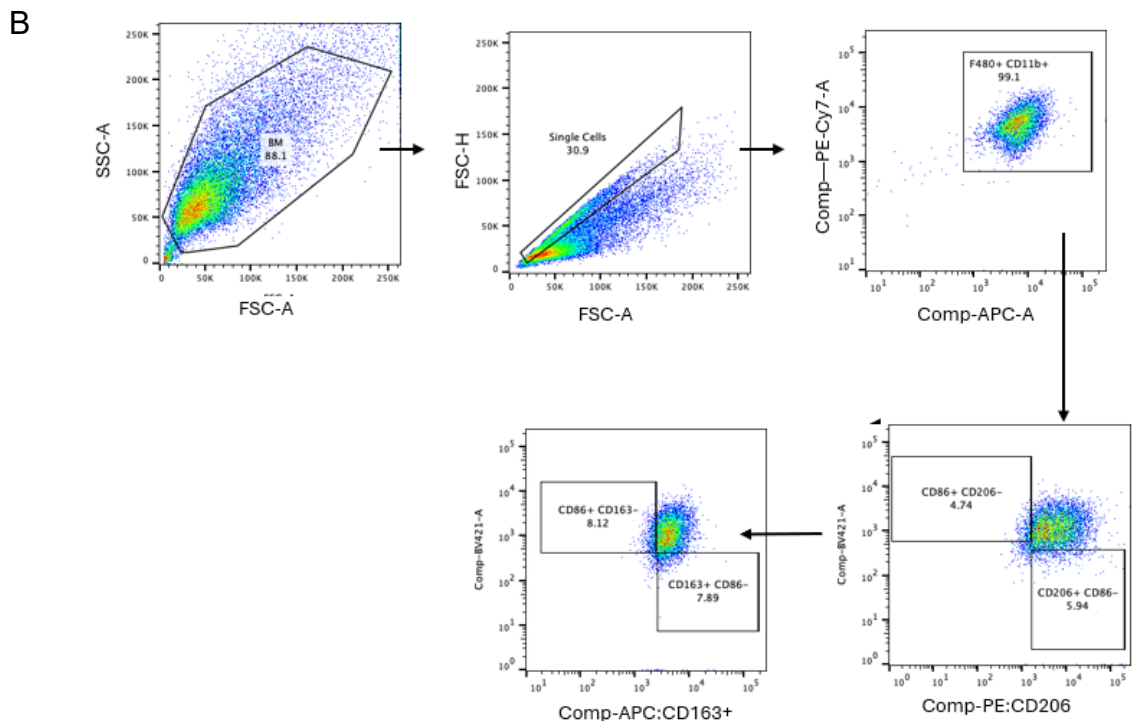
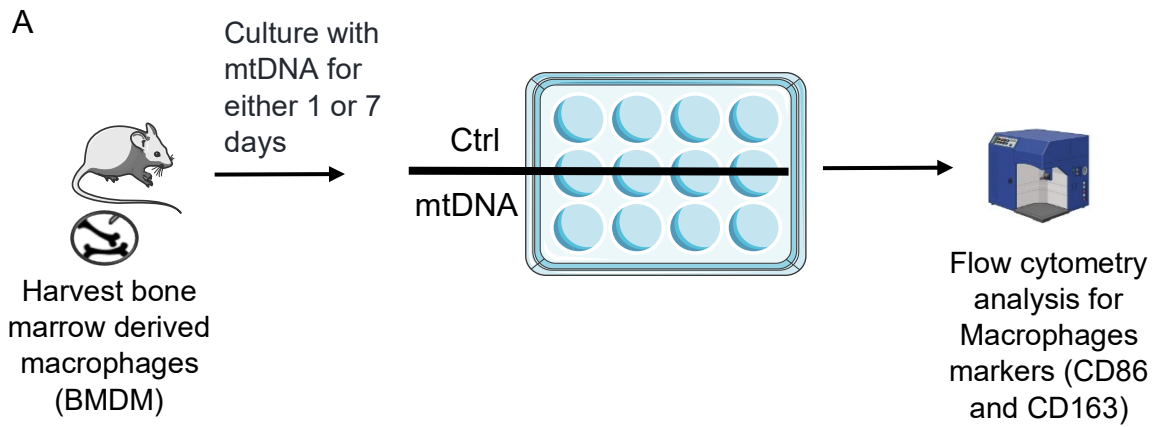
(B) STING-associated genes expression analysis in STING^{-/-} BMDM challenged with melanoma derived mtDNA.

Relative gene expression between control (n=4) and mtDNA treated STING^{-/-} BMDM (n=4). Fold change was calculated based on the $\Delta\Delta C_t$ value obtained from the ΔC_t value calculated from the C_t value of the housekeeping gene (GAPDH) subtracted from the C_t value of the gene of interest. Data indicate mean $\pm$ SD. Statistics are presented as Mann-Whitney U test.

5.10 mtDNA stimulation mediates M2 phenotype in BMDM

To investigate the difference between short and prolonged stimulation of BMDM with melanoma derived mtDNA, two time points were investigated, a 1 day and a 7-day challenge. Here BMDM were cultured with melanoma derived mtDNA for 1 day and 7 days, at end point flow cytometry analysis was carried out at the same time specifically looking at the M1 marker (CD86) and the M2 marker (CD163). CD163 was selected as the M2 macrophage marker in this study, as it is a well-validated and widely recognised scavenger receptor expressed on alternatively activated macrophages (325-328). CD163 is particularly associated with the anti-inflammatory M2c phenotype and is upregulated in response to IL-10 and glucocorticoids, reflecting an immunosuppressive and tissue-remodelling microenvironment (325-328). Its consistent and robust expression on tissue-resident and monocyte-derived M2 macrophages makes it a reliable marker for identifying M2 polarisation in histological sections, and it has been extensively used in the literature for this purpose in a variety of tissue types. Experimental schematic is illustrated in Figure 5.9.

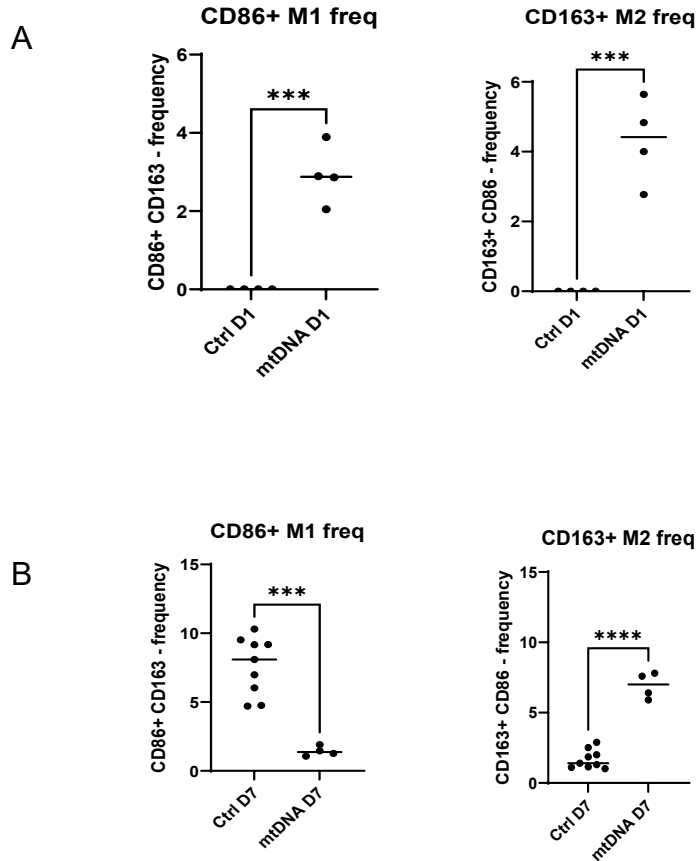
BMDM stimulated for 1 day exhibited a significant increase in both the M1 ($p=0.0002$) and M2 ($p=0.0004$) phenotype compared to control BMDM that received no stimulation (Figure 5.10A). However BMDM stimulated for 7 days with melanoma derived mtDNA, showed a significant decrease in the frequency of M1 ($p=0.0001$) and a significant increase in M2 phenotype ($p<0.0001$) (Figure 5.10B).



5-9 Macrophage markers analysis of short (1 day) vs prolonged (7 days) stimulation of BMDM with mtDNA.

(A) Schematic of experimental design. BMDM were cultured with melanoma derived mtDNA for either 1 or 7 days. At end point flow cytometry analysis was carried out.

(B) Flow cytometry gating strategy to identify macrophage polarisation, M1 (CD86) and M2 (CD163) population in BMDM cultured with mtDNA for 1 and 7 days.



5-10. 7-day mtDNA stimulation of BMDM mediated an M2 phenotype.

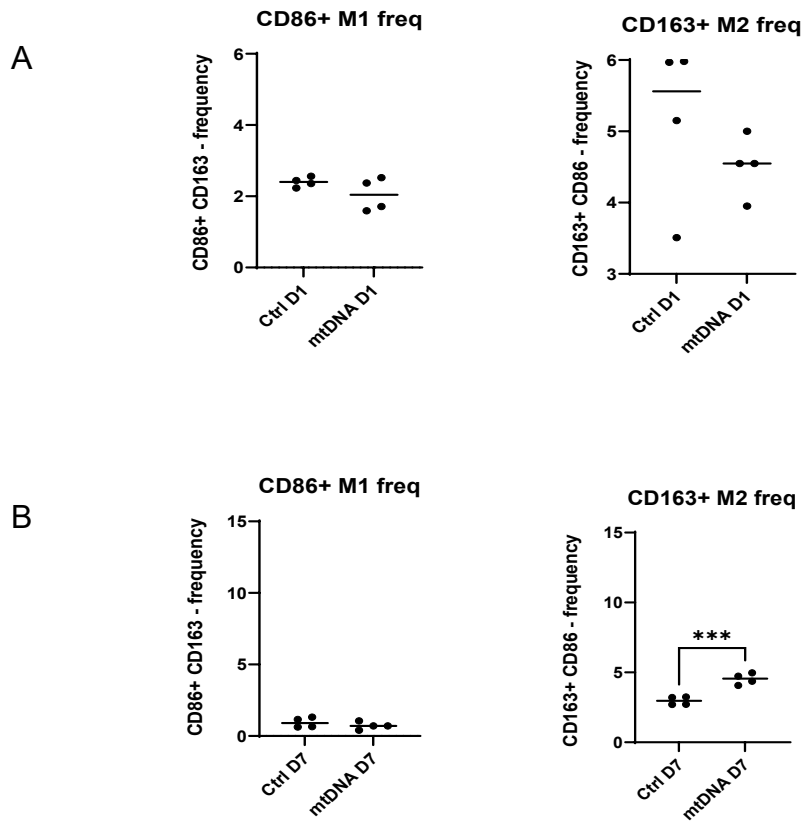
(A) BMDM cultured with mtDNA for 1 day (mtDNA D1) (n=4) analysed for macrophage polarisation of M1 like (CD86) and M2 like (CD163) phenotype compared to control BMDM (Ctrl D1) (n=4).

(B) BMDM stimulated for 7 days with melanoma-derived mtDNA (mtDNA D7) (n=4) analysed for the frequency of M1 like (CD86) and M2 like (CD163) phenotype compared to control BMDM (n=9). *Statistics are presented as Mann-Whitney U test. *** $p < 0.001$, **** $p < 0.0001$.*

5.11 Macrophage Phenotype Polarisation in WT vs STING^{-/-} BMDMs

To investigate if the macrophage polarisation in response to a 7-day stimulation observed in WT BMDM (Section 5.9) is STING related, the same experiment was conducted in STING^{-/-} BMDM.

In contrast to WT BMDMs, no macrophages polarisation was observed in STING^{-/-} BMDMs following stimulation with mtDNA. Both M1 and M2 phenotypes were assessed after one day of stimulation, with no significant differences detected (Figure 5.11A). In the cohort stimulated with mtDNA for seven days, a slight but statistically significant increase in the M2 phenotype was observed in STING^{-/-} BMDMs. Here CD163 frequency increased from a mean of 3% in control to a mean of 4.5% with mtDNA (Figure 5.11B). In the WT BMDM under the same conditions, CD163 frequency also increased from a mean of 2% in control to a mean of 6.4% with mtDNA (Figure 5.10B). This suggests that STING^{-/-} BMDMs exhibit a slight, increase in M2 polarisation following prolonged mtDNA stimulation similar to the WT BMDMs. Thus, suggesting alternative pathways to the cGAS-STING can also be implicated in mounting a response. Such pathways include the NLRP3 Inflammasome, and the TLR9–NF- κ B Axis (198, 204, 205).



5-11 melanoma-derived mtDNA stimulation of STING^{-/-} BMDM for 1 vs 7 days.

(A) Macrophages polarisation (CD86 vs CD163) in STING^{-/-} BMDM stimulated with mtDNA for 1 day (mtDNA D1) (n=4) compared to control STING^{-/-} BMDM (Ctrl D1) (n=4).

(B) Macrophages polarisation (CD86 vs CD163) in STING^{-/-} BMDM stimulated with mtDNA for 7 days (mtDNA D7) (n=4) compared to control STING^{-/-} BMDM (Ctrl D7) (n=4).

*Statistics are presented as Mann-Whitney U test. *** $p < 0.001$.*

5.12 Summary

In this chapter, I demonstrate that STING-associated gene expression is downregulated in the liver of melanoma-engrafted mice, both in whole liver lysates and specifically in F4/80⁺ liver macrophages. This downregulation coincides with a shift toward an M2 macrophage phenotype in the liver microenvironment. Similarly, *in vitro* studies revealed that BMDMs challenged with melanoma-derived mtDNA for 7 days exhibited STING downregulation and M2 polarisation, characterized by increased CD163 and decreased CD80 expression, mimicking the phenotype observed *in vivo*. In contrast, AML12 hepatocytes showed no change in STING-associated gene expression when exposed to mtDNA, highlighting the immune-specific nature of this response.

Importantly, the STING-dependent mechanism was confirmed using STING ^{-/-} BMDMs, which did not exhibit STING downregulation or M2 polarisation upon mtDNA stimulation. While prolonged mtDNA exposure in wild-type BMDMs resulted in a significant decrease in M1 and an increase in M2, STING ^{-/-} BMDMs displayed only a slight but significant increase in M2 markers, suggesting partial compensation by alternative pathways. Collectively, these findings highlight the critical role of STING signalling in mtDNA-mediated macrophage reprogramming, contributing to an immunosuppressive tumour microenvironment in melanoma.

Melanoma like many cancers influence and shapes the immunosuppressive tumour microenvironment by recruiting anti-inflammatory myeloid immune cells reprogramming monocytes into tumour associated macrophages (363). Tumour associated macrophages broadly divided into the classically activated M1-like macrophages and the alternatively activated M2 like macrophages, depending on their functional state (363). The M1 like macrophages exhibit an anti-tumour role, producing pro-inflammatory cytokines such as TNF- α , facilitating the cytotoxic T-cell response to tumour melanoma cells (363). In melanoma the M1 macrophages are typically outnumbered in the melanoma tumour microenvironment (363). The M2 like macrophages induced by TGF- β , tumour derived factor such as VEGF and interleukins 10, and 13, facilitate angiogenesis, pro-tumour activity and suppress the anti-tumour immune response (363). In metastatic melanoma M2 like macrophages dominate the tumour micro-environment promoting melanoma

immune evasion and in some cases resistance to immunotherapy (363). High M2-like macrophages infiltration is typically associated with poor prognosis (363).

It is understood that melanoma promotes the macrophages polarisation by secreting factors that induce this polarisation, such as macrophage colony-stimulating factor and VEGF driving M2 polarisation and suppressing M1 like macrophages (363). Additionally, hypoxia in melanoma cells facilitates the process of hypoxia inducible factor 1 α (HIF-1 α) M2 polarisation (363). In this thesis by showing the M2 phenotype exhibited in both the *in vivo* melanoma engrafted mice and *in vitro* mtDNA treated macrophages for 7 days, I provide further support that chronic stimulation and inflammation harness an immunosuppressive environment permissive for tumour growth and survival.

Moreover, I show that the observed immunosuppressive phenotype in the liver is mounted by primary immune cells, macrophages the most abundant immune cell and not non-immune cells, such as hepatocytes (364). I demonstrated this by showing that culturing alpha mouse liver cells with melanoma derived mtDNA had no effect on the STING expression. The difference in response between the two cell types has several explanations. Macrophages are innate immune cells with phagocytic function; hepatocytes have minimal phagocytic role. Given that macrophages are innate immune cells they have high levels of cGAS-STING compared to the much lower levels of STING in hepatocytes, hence the variation in detection and response to stimulation by the mtDNA (216). STING is not expressed in equal amounts in all cell types (216). STING is highly expressed in innate immune cells, such as macrophages including liver Kupffer cells and monocytes, whose role is to detect foreign cells and damaged DNA (216). This contrasts with cell types that have low levels of STING such as hepatocytes and neurons, cells that are inclined to avoid and minimise chronic inflammation (365-368). I show that in the alpha mouse liver cells, stimulation with mtDNA did not trigger changes in STING, levels remaining unaltered. In this thesis I show that liver macrophages are a key STING responder in metastatic melanoma. Taken together, these findings highlight the critical role of STING signalling in mtDNA-mediated macrophage reprogramming, contributing to an immunosuppressive tumour microenvironment in metastatic melanoma.

6 Discussions and Conclusions

6.1 General discussions

Melanoma is the leading cause of death from skin cancer, it affects people of working age, having physical, psychological, social and financial implications (1, 3). Better outcomes can be realised through new therapies or improved diagnostic and prognostic tools, developed from a better understanding of the biology of the disease. In melanoma current investigation tools are invasive, costly and anxiety inducing (79). The clinical utility of biomarkers in melanoma remains unclear (88, 89). Circulating cell free mtDNA has been implicated in several malignancies (168-176). However, in melanoma, studies on mtDNA as a biomarker are scarce (166, 167). Additionally, no studies have investigated the role of cell free mtDNA in metastatic melanoma and disease progression. Developing a better understanding of the functional significance of mtDNA in melanoma can potentially provide an insight into its role and implications in the disease progression of melanoma.

In this thesis I have demonstrated that circulating cell free mtDNA is elevated in human metastatic melanoma. mtDNA detected had the ability to distinguish between the two patient cohorts, the remission and relapsing groups. Thus, highlighting the potential role circulating cell free mtDNA can play as a biomarker to diagnose and monitor disease progression in melanoma.

Like the findings in human metastatic melanoma, I demonstrate that cell-free mtDNA levels are elevated in both primary and metastatic melanoma mouse models, with the levels significantly higher in metastatic melanoma compared to primary melanoma. I demonstrate an increase in infiltrating macrophages in liver of mice engrafted with melanoma. Furthermore, I demonstrated that melanoma engraftment induced a down regulation in STING pathway in both whole liver lysates and liver resident macrophages, which exhibited an immunosuppressive M2 like phenotype.

6.2 Key findings

6.2.1 Cell free circulating mtDNA is elevated in the serum of metastatic melanoma

In this thesis I have shown that patients with biopsy proven metastatic melanoma have elevated levels of circulating mtDNA in the peripheral blood serum compared to healthy controls.

Elevated levels of circulating mtDNA in cancer patients have been documented in many studies, raising the question of whether detecting mtDNA could potentially be utilised as a non-invasive biomarker (161, 369, 370). Research examining mtDNA copy number in melanoma remains limited (167). The largest study, which included 500 melanoma patients and 500 controls, found that individuals with melanoma exhibited higher mtDNA copy numbers than controls (167). Nevertheless, the study was retrospective in design and focused on a cohort of patients with low-risk melanoma. This study also used blood to measure the mtDNA copy number (167). Other studies measuring mtDNA copy number have used plasma, whole blood itself, or peripheral blood leukocytes (177, 369).

The data I present in this thesis observed mtDNA in peripheral blood serum in patients with high-risk metastatic advanced melanoma. We chose to measure mtDNA in serum as it is generally the current gold standard for use in both research and diagnosis in the clinical setting. In addition to the current non-standardised method of sample choice, currently there are variations in the way mtDNA is quantified. Most studies use qPCR, while some have utilised droplet digital PCR (369). This variation and lack of standardisation in sample choice and quantification methods of mtDNA have somewhat contributed to the variability in the current literature that exists, hindering the development of mtDNA as a biomarker for use in the clinical setting. In this thesis qPCR was utilised to assess mtDNA as it is a well-established and used method to analysing mtDNA in human, *in vivo* and *in vitro* samples.

Currently there is no biomarker in use for melanoma in clinical settings (76). All current investigations are invasive with known risks (76, 77). Additionally, all investigation tools used in melanoma currently are time consuming, anxiety inducing and costly (78, 79). Whilst other cancers such as prostate cancer have

benefited from having a biomarker in clinical use such as the prostate specific antigen (PSA), melanoma has lagged in the biomarker field (87, 90, 371).

Much of the research on biomarkers and liquid biopsy has traditionally focused on nuclear DNA as a potential biomarker (158, 159). Whilst cell free nuclear DNA tracks tumor specific mutations and alterations in the detected copy number, mitochondrial DNA has the natural advantage of been present in abundance in comparison. Nuclear DNA only has two copies compared to thousands of copies of mtDNA per cell. Thus, mtDNA has a higher sensitivity for detecting low levels of circulating cell free DNA in the blood stream, compared to cell free nuclear DNA and circulating tumour DNA. However, compared to nuclear DNA, tumour specific mutations in mtDNA are challenging to track due to the high baseline variability in mtDNA, mitochondrial heteroplasmy. Furthermore, mtDNA has a lower specificity compared to the traditional nuclear DNA as it can be altered due to other factors such as inflammation, stress, trauma and aging. Thus, the GRAIL's test approach which screens for specific methylation patterns in circulating DNA, exhibits a superiority for specific cancer screening compared to mtDNA (372). However, its detection is known to be significantly lower in the early stages of disease, which is where the mtDNA has its advantage due to its high detectability because of its higher copy number, especially when cell free DNA tumour fraction is low in the early stages. Additionally, mtDNA has simpler structure, and shorter length (161). All of which makes mtDNA easier to sample and process during analysis (161, 162).

Currently various liquid biomarkers are undergoing trials to evaluate and validate their clinical utility in assessing minimal residual disease, predicting resistance to treatment, and monitoring disease progression in solid tumours like melanoma. Examples include the tumour informed ctDNA assay utilised in Natera Signatera, which is a tumour patient specific ctDNA that can be utilised to monitor disease recurrence and molecular residual disease such as breast and colorectal cancers (373, 374). It has the ability to track up to 16 mutations specific to the patient's tumour, thus providing a highly sensitive and specific biomarker detected in the blood stream (373). For melanoma and mitochondrial DNA, the CheckMate-067 study found that inherited mitochondrial genetics was a predictor of response and efficacy to immune checkpoint inhibitors (375). In this study the association between mitochondrial genetics and efficacy to immune check point inhibitor in

1,225 metastatic melanoma patients was assessed. They found that mitochondrial genetics, specifically haplogroup T was associated with treatment resistance to the anti-programmed cell death protein-1-based immunotherapy inhibitor (375). This study therefore provided evidence to support the development of a blood-based biomarker that can be utilised to predict immunotherapy efficacy and treatment resistance in advanced melanoma patients.

Developing and integrating cell free mtDNA into a liquid biopsy tool can have several added benefits over the current liquid biomarkers such as ctDNA and cell free nuclear DNA. The difference between mtDNA and current nuclear DNA biomarkers lies in the abundance, sensitivity and specificity of each. Currently mtDNA is viewed as a potential biomarker that can complement ctDNA detection, when ctDNA analysis is uninformative or limited in the early stages of disease.

In this thesis, in addition to illustrating the high level of mtDNA detected in metastatic melanoma patients, I also show that mtDNA had the ability to distinguish remission and relapsing groups. I showed that the four patients who developed a recurrence were noted to have increased mtDNA level detected during the post operative sequential follow up blood test. This highlights that mtDNA could potentially be used to monitor disease status and progression. Currently when a recurrence is suspected clinically a spectrum of invasive investigations are triggered and conducted. Furthermore, I show that patients with a mutation of the oncogene *BRAF* who have aggressive melanoma, had higher levels of mtDNA detected. As *BRAF* positive patients go on to have targeted therapy over immunotherapy, the fact that mtDNA levels detected was higher in this subset of patients, highlights a potential role for mtDNA to be used as a biomarker to guide optimal therapy choice.

6.2.2 Establishment of *in vivo* Melanoma Mouse Models and Circulating mtDNA Levels

To allow me to investigate the significance of circulating cell-free mtDNA on melanoma disease progression, I first had to establish in house melanoma mice models. Here I set up two melanoma mice models, a primary in situ early-stage melanoma and an advanced disseminated metastatic melanoma model. For the primary melanoma model, the melanoma cell line B16F1 was utilised and administered via a subcutaneous route in a selected flank. To establish a model that mimics metastatic melanoma, here I utilised the B16F10 melanoma cell line, that was administered intravenously through the tail veins. B16F1 cells have a lower metastatic potential compared to the B16F10 which have a high metastatic potential (357). The models established and shown in this thesis exhibited the phenotype required where the subcutaneous primary melanoma model developed a viable and palpable tumor mass. The intravenous metastatic melanoma model developed metastatic nodules observed in the liver as shown in this thesis (Figure 4.3). In addition to the observed phenotype, I show that the models had higher levels of circulating cell-free mtDNA as observed in humans with metastatic melanoma. I show that the IV metastatic melanoma models had a significantly higher mtDNA level compared to the primary melanoma model. When measuring the mtDNA levels in the *in vivo* experiment, the same methodology was utilised when analysing both the human and mouse serum samples. I ensured to utilise the same extraction technique, volumes, and reagents to minimise the variation between the two systems as much as possible.

Establishing the optimal metastatic melanoma model was a key part of this thesis to allow for the subsequent experiments investigating the impact of circulating mtDNA and melanoma engraftment on disease progression. Whilst the established metastatic model I show here exhibited the required phenotype, the model does have its limitations. The tail vein intravenous route creates an experimental metastasis model, and not a spontaneous natural model. That is because melanoma metastasis typically occurs via a lymphatic spread followed by a hematogenous dissemination of the tumor from the primary tumor site. The tail vein injection intravenous model delivers the melanoma cells directly into the

blood stream, bypassing the early stages of metastasis and the immune response at the primary local tumor site. This experimental metastasis model may not fully represent metastatic melanoma in humans. Additionally, the tail vein injection intravenous model exhibits a pulmonary bias where the injected cells through the tail vein appear in the lungs as the first site. Thus, limiting representation of other known common metastatic sites such as the brain and liver.

An alternative to the tail vein intravenous model is the use of intrasplenic injection. This model is known to be reproducible and efficient for studying liver metastases (376). This was not possible for me as we do not have the license to carry out intra-splenic injections, and like the tail vein injection model it still by passes the natural early stages of metastases. To address some of the other limitations associated with tail vein intravenous injections such as operator dependence technique and variability in cell delivery several steps were implemented. The intravenous injections were prepared and administered by the same operator. The mice used were all females and aged between 8-12 weeks. Using just females is another limitation of this study as we know variations occur between sexes in melanoma (377). Using mice between 8-12 weeks is not thought to be a limitation of this study as melanoma usually occurs in young adults (377). Future studies should include both sexes and consider alternative models to replicate the findings shown in this thesis and improve translational relevance.

In this thesis I chose to study the liver as the metastatic site in melanoma. That is because the liver is not only a common metastatic site in ~15-30% in advanced melanoma, but it is known to be an indicator of treatment resistance, and a predictor of poor response to immunotherapy and targeted therapy (309, 378). Additionally, in almost most cases there is no cure for liver metastasis (310, 311). Thus, I wanted to study liver metastases in melanoma, to develop a better understanding of this and contribute to the field of potentially developing novel treatments for such patients.

6.2.3 Melanoma engraftment induces a down regulation in STING pathway in liver resident macrophages which exhibit an M2 phenotype

Metastatic melanoma is an immunogenic cancer capable of evading the immune response as well as developing resistance to established immunotherapy agents (52, 53, 55). The development and utilisation of immunotherapy have revolutionised the way metastatic melanoma is managed greatly enhancing prognosis (43-46). Given that the cGAS STING signalling pathway plays a pivotal role in mediating an innate immune response, mounting an anti-tumour response, investigating the STING pathway in response to melanoma engraftment was an appealing prospect (215). Furthermore, in the broader context of cancer immunology, it is understood that acute STING activation in the early phase mediates the suppression of tumour growth and metastases, while the chronic prolonged activation of STING can facilitate the survival and metastases of tumour (288-290). Shedding light on this dual STING role specifically in melanoma was a key aspect of this thesis.

In this thesis I show that the engraftment of primary and metastatic melanoma in mice exhibited a downregulation of STING in both whole liver lysates and F4/80+ cells isolated from the liver. This provided direct evidence that prolonged chronic STING activation led to inhibition of the STING signalling pathway which has become overwhelmed, exhausted or desensitised to the stimulus of the tumour. The presence of such direct evidence supporting this finding of chronic activation of STING, inhibits the signaling pathway is scarce. To my knowledge no published data currently demonstrates that melanoma engraftment leads to the down regulation of STING in whole liver lysate or F4/80+ liver macrophages, making our findings potentially novel.

The evidence that currently exists instead supports the phenomena that the STING pathway is inhibited in melanoma cells through mechanisms such as epigenetic silencing (379). Others have shown that restoration of STING can regain its function in melanoma cell lines mediating its downstream activities including IFN release, enhancing T cell cytotoxicity towards the melanoma cells (380). The current evidence that supports the phenomena of chronic activation and stimulation of STING resulting in immunosuppression comes mainly from the broader cancer immunology context and not melanoma as such (381). In my thesis I show a direct demonstration of prolonged chronic stimulation mediated by

the engraftment of melanoma resulting in STING downregulation. Although a different disease entity to melanoma, colorectal cancer with liver metastases was found to have a decrease in STING in the tumour associated macrophages (TAM) of the metastatic liver foci (382).

In addition to the down regulation of STING that I show in this thesis, I also show that F4/80+ cells isolated from the livers of mice engrafted with melanoma, exhibited an M2 pro-tumour immunosuppressive phenotype. Here I show that there was an increase of the M2 CD163 macrophages compared to a decrease in the M1 pro-inflammatory anti-tumour CD80 macrophages. Thus, providing evidence that STING and macrophage phenotype shape the melanoma immunosuppressive environment.

The M2 like phenotype and down regulation of STING gene expression observed in the *in vivo* experiments that I illustrated in my thesis is potentially mediated by STING. When looking *in vitro* I showed that culturing STING^{-/-} BMDM with melanoma derived mtDNA had no effect on either STING gene expression or the macrophages phenotype. This contrasts with the STING suppression and the M2 phenotype observed both *in vivo* of melanoma engrafted mice and wild type BMDM challenged with mtDNA *in vitro*.

Furthermore, I also show that macrophage polarisation occurs and transitions over time. I show that after 1 day of culture of wild type BMDMs with melanoma derived mtDNA, the macrophages exhibited a mixture of both M1 and M2 phenotype. After 7 days of co-culture with mtDNA, I show that the macrophages exhibited a polarisation towards an M2 phenotype. The polarisation of macrophages is known to play a crucial role in facilitating tumour growth and progression in cancer (363).

6.3 Limitations

The data I present in this thesis has provided evidence to support the possibility of developing mtDNA into a novel non-invasive biomarker to monitor melanoma disease progression. This adds to the field of data on clinical utility of biomarkers in melanoma instead of the currently used invasive, costly and anxiety inducing investigative tools. Moreover, this thesis provides novel evidence of direct demonstration of prolonged chronic stimulation mediated by the engraftment of melanoma resulting in the downregulation of the STING signalling pathway, first of its kind in the field of melanoma. Nevertheless, as with all studies there were several limitations. A larger sample size for the human proof of concept study measuring mtDNA would have enhanced the statistical analysis due to the heterogeneity of melanoma. The small sample size in this thesis is a limitation; a bigger sample size would support progressing mtDNA detection into early phase clinical trials.

This study did not investigate the presence of mutations in mtDNA which is known to have implication on the immune response (369). Thus, looking at mtDNA mutation status could be important and may contribute to the ability to stratify melanoma patients for immunotherapy.

Similarly for the *in vivo* and *in vitro* data, the number of mice in each group was kept feasibly low, applying the 3Rs (Replacement, Reduction, and Refinement) of animal research. Having a greater number in each group would have allowed for greater statistical significance to be detected when comparing data sets. The variability in the melanoma engraftment due to missed intravenous injections or poor cell viability could have occurred. To address some of the limitations associated with tail vein intravenous injections such as operator dependence technique and variability in cell delivery, the intravenous injections were administered by the same operator. The presence of engraftment was confirmed by the subcutaneous mass in the primary melanoma group and metastatic nodules in the liver for the metastatic melanoma group. The *in vivo* finding of STING downregulation in F4/80+ cells and whole liver lysate, could have been further supported and enhanced by utilising cell sorting to isolate macrophages and hepatocytes from the *in vivo* melanoma engrafted mice. Furthermore, to further strengthen my findings, obtaining data from human samples such as

biopsies from melanoma liver metastases could hold merit. These samples can be used to investigate STING expression to confirm the presence or absence of STING down regulation in human melanoma liver metastases as I observed *in vivo*.

6.4 Future work

In this thesis I have shown that circulating mtDNA could be developed into a non-invasive biomarker to monitor disease progression and response to treatment in melanoma. The patient cohort in this thesis are patients with advanced metastatic melanoma. It would be interesting to expand this to include patients of different melanoma stages, stage III with lymph node involvement to investigate if mtDNA can be utilised to identify patients prior to dissemination of melanoma to the most aggressive stage, metastatic melanoma stage IV. This could potentially allow us to stratify what levels of circulating mtDNA are significant prognostically.

I have shown that circulating mtDNA is elevated in metastatic melanoma, though the source of this has not been investigated in this thesis. Investigating the source of this mtDNA whether it originates from the tumor or the tumor microenvironment would be the next natural thing to investigate here.

To determine if melanoma is directly excreting mtDNA into the plasma, xenograft melanoma models could be utilised. Xenograft model in which a panel of human melanoma cell lines from primary and metastatic tumours is injected into immunocompromised (NSG) mice via two routes, SC mimicking primary melanoma, and IV mimicking metastatic melanoma. Peripheral blood samples can then be obtained pre and post melanoma injection at a set timeline interval. qPCR can then be utilised to determine the presence (or not) of cell free human mitochondrial DNA and mouse mtDNA. If human mtDNA is present in the serum, it would suggest that the tumour is releasing mtDNA into the serum. If no human mtDNA is observed with increased mouse mtDNA, then this would suggest that the tumour is causing release of mtDNA from support cells or the tumour microenvironment. These data will determine if melanoma cells are the source of increased levels of mtDNA or if the tumour microenvironment is the source. Additionally, the mechanisms and reasons for the release of this circulating mtDNA could be investigated. Developing an understanding of this could provide new areas for targeted therapy.

In this thesis I show that mtDNA had no effect on STING -/- bone marrow derived macrophages on the STING expression and macrophage polarisation. To expand on this finding the use of a STING agonist and antagonist could be utilised. My hypothesis would be that a STING agonist could re-activate the STING pathway

in wild type bone marrow derived macrophages or mice, to promote M1 like macrophage polarisation. A STING antagonist may suppress the mtDNA effect observed, halting the development of the M2 like macrophage phenotype. However, these are my hypotheses and need to be investigated further. Additionally, to investigate the impact of STING agonist and antagonist on melanoma progression *in vivo*, live *in vivo* imaging could be utilised to track luciferase tagged melanoma cells. At end point, metastatic melanoma progression can be assessed by liver autopsy, and macrophages isolated from liver to assess STING gene expression.

6.5 Conclusions

To summarise in this thesis, I demonstrate that circulating cell free mtDNA is elevated in metastatic melanoma in both human and melanoma engrafted mice. The circulating cell free mtDNA level distinguished between the patient group who remained in remission compared to the group who developed a relapse post tumour resection. This highlights the potential role mtDNA can play as non-invasive biomarker in advanced melanoma.

I demonstrated that melanoma engraftment mediated a downregulating of STING in the livers from melanoma mice models. Furthermore, the liver macrophages in melanoma engrafted mice exhibited an M2 like phenotype. To our knowledge this is the first data that has demonstrated that melanoma engraftment in mice resulted in the downregulation of STING in liver-resident macrophages, making this a novel finding. Overall, the data presented provides an insight into the functional role that melanoma derived mtDNA plays in harnessing an immunosuppressive microenvironment in melanoma. Therefore, facilitating its ability to evade an immune response, and promote melanoma disease progression.

7 References

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