Chapter 1

Introduction

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1. Introduction

Cancer is one of the world's most significant health problem. It is considered to be one of the most lethal of diseases. The National Cancer Institute defines cancer as a group of disorders in which aberrant cells have the ability to divide and spread to surrounding tissue [1]. Cancers can develop in any part of the body and spread occasionally through the blood and lymphatic systems to other parts of the body. In 2020, there were identified 18.1 million new cases of cancer worldwide. 9.3 million of these instances involved men, while 8.8 million involved women [2]. According to WHO, an estimated 9.6 million deaths, or one in every six deaths, were attributed to cancer in 2018, making it the second highest cause of deaths worldwide (cardiovascular diseases being the highest). With this growing global burden, prevention and treatment of cancer is one of the most significant public health challenges of the 21st century.

1.1 Reservations with respect to conventional therapy

Though being an extremely difficult disease to control or manage, the advancement in medical science over the years has enabled scientist to formulate therapeutic endeavours for almost all cancer types. Of these therapeutic processes, chemotherapy, radiotherapy and immunotherapy are widely used across the world. These treatments have proven to be very effective to treat many types of cancer. Nevertheless, the use of chemotherapy and immunotherapy as antitumour medicines, have a number of important limitations. The first problem is a purported "clinical cure" in which the tumour cells are mostly eradicated, leaving the tumour clinically undetectable, despite the fact that minute quantities of tumour cells are still present and flare up much later after the initial therapy is over, thus resulting in tumour recurrence. The second factor is the presence of cancer stem cells, which, despite initially constituting a very small cell population, continue to grow because they are significantly less

sensitive to treatment drugs, thereby leading to resistant tumour relapse [3]. Another issue is the inability of the physician to administer therapeutic agents extensively since doing so results in considerable normal tissue damage and unacceptable side effects that make it impossible to provide additional curative therapy. The tissues damage caused by antitumour entities can be quantify by the toxicity cost function *J*, which depends on the second-order of the drug-dosage [4], can be represented by:

$$J(t) = \tfrac{1}{2}\,(F_1(t)d_1^2 + F_2(t)d_2^2 + F_3(t)d_3^2 + \ldots\ldots)$$

where $d_1, d_2, d_3, \ldots$ are the different levels of the antitumour entities, and $F_1, F_2, F_3, \ldots$ are the weighting factors of the different antitumour entities at each instant. We use this toxicity cost function principle to manage the temporal schedule of the antitumour entities for the toxicity minimisation.

These disadvantages need to be well addressed, even though it is also known that occasionally there is permanent elimination of some types tumour by therapeutic agents. Some recent examples of such therapy-induced regression of tumours are multimodal chemotherapy and immunotherapy combined using drugs (like alkylators as dacarbazine or temozolomide), antitumour lymphocyte therapy, along with interleukin [5, 6]. Nevertheless, despite therapeutic advances, very large number of cancer patients succumb to the disease.

1.2 Spontaneous cancer regression

Although a malignant tumour is typically encountered by the physician during its progression phase, permanent spontaneous regression of malignant tumours which is episodic but a well-documented biological process. Everson and Cole defined in 1966 that the spontaneous regression and remission of cancer as "the partial or complete disappearance of a malignant tumour in the absence of all treatment, or in the presence of therapy which is considered inadequate to exert significant influence on neoplastic disease"

and in many cases the tumour's disappearance is complete and permanent [7]. A long-known phenomenon, spontaneous regression was initially identified in the context of acute infection [8]. In the past, it was known as the St. Peregrine tumour. It was named after Peregrine Laziosi, a young priest of 12th century who suffered from a tibial tumour with a terrible infection that spread to his skin, caused a fracture, and necessitated an amputation. While being closely monitored by a doctor, the tumour in the reported case was seen to dissolve suddenly without leaving any scars [9]. The process of permanent spontaneous regression of malignant breast tumours is occurring subclinically across human populations at 22–46% rate, as per the Scandinavian and Wisconsin Screening Registries which have tracked a population of 0.33 million and 2.95 million individuals respectively [10, 11]. Furthermore, it is evident in autopsy studies that about half of general individuals have malignant focus in uterine cervix or prostate, with confirmation of permanent containment, and moreover, malignant neuroblastoma fully regresses from larger-sized tumours [7, 10]. As per PubMed, there are about 14,000 titles of papers dealing with spontaneous cancer regression, covering virtually all types of malignant diseases such as sarcomas, carcinomas, lymphomas, melanomas and so on [12]. Though the regression process eliminates malignant cells, it does not damage the normal tissue, i.e. normal cells are protected overall. Typically, the duration of a tumour's regression generally occurs across a period of months, generally 1 to 2 months. Such spontaneous (endogenously-initiated) regression of malignancy also occurs in animals, including worms and molluscs and even took place in dinosaurs which are now extinct [13]. As a matter of fact, there are numerous species of animals known to completely regress malignant lesions, which are usually fatal in man, such as melanoma. Indeed, investigators have earlier elucidated the energetics and biothermodynamic basis of spontaneous tumour regression [14, 15]. Actually, the investigation of the spontaneous regression process may help the scientist to formulate

incisive pointers on inducing permanent regression process on human malignancies. To paraphrase, tumour reduction on regression can be of two type: (i) spontaneous tumour regression, which is induced by the body's own protective immune system is thus this regression processes internally generated (endogenous); (ii) treatment-induced tumour regression, which is induced by therapeutic agents, like radiotherapy, hormones or chemotherapy administered by physician, this regression processes externally generated (exogenous).

1.3 Quantitative systems biology formulation

Spontaneous cancer regression depends on various factors like the amount of the tumour load, the invasiveness of the disease, and the robustness of the patient's immune response. Mathematical modelling can be a potentially vital tool for understanding the spontaneous regression process, just as modelling has been a well-known approach for optimizing better treatment strategies for cancer patients. Over the years, researchers have used various models to address the biological process of tumour growth and of anticancer entities [16–19]. In this thesis we have considerably modified our previously developed model [20], in which tumour cell kinetics, chemotherapy dynamics, dynamics of immune system (NK cell, circulating lymphocyte and cytotoxic T-cell), and immunomodulation/immunotherapy dynamics, are represented by a system of six differential equations. Our present novel mathematical model of this thesis is incisively based on experimentally observed biological processes, specifically the lethal effect on the tumour cell, as induced by the immune cells viz. cytotoxic T-cells ($CD8^+$), natural killer (NK) cells, and the body's self-generated antitumour biochemical agent, interleukin-2.

Here, we attempt to quantitatively formulate the general methodology of complete tumour regression and discern the unitary principles that enable this regression. Various

differential equation-based quantitative models are available in the literature to replicate the dynamics of the biological process of cancer regression, and some of these models can be delineated now. For instance, Perry [14] has used the laws of mass action and first-order dynamics to characterize the reaction kinetics of tumour cell lysis during tumour regression brought on by therapeutic agents such as chemotherapy medications that cause DNA damage in the malignant lesion. As a result, the tumour cell population declines exponentially [14]. However, a residual tumour cell population asymptotically exists under the exponentially-decreasing trajectory and this population of residual cells is frequently a factor in tumour recurrence and incurability.

It is well-known that three complementary processes can reduce tumour cell population:

(i) Decrease of the proliferation of tumour cells: Here, chemical alkylation or chemomodulation of DNA are two methods for reducing cell proliferation, these methods lead to DNA damage [21, 22],

(ii) Increase of tumour cell lysis: This occurs through the medium of antitumour lymphocytes [23–25],

(iii) Further enhancement of tumour cell lysis: The activation of the antitumour lymphocytes can be boosted by cytokines (for example, immunomodulation by interleukin-2) [26].

1.4 Mechanism of complete tumour regression negative using biasing

The mathematical framework of these aforesaid three processes have been developed by de Pillis et al. [27], Kuznetsov et al. [28] , and Kirschner et al. [29] based on experimental data, and the predictions of the modelling have also been empirically validated [30]. These models effectively describe the computational dynamics of antitumour activity by DNA damage and immunological action. However, in all these models, the tumour cell

population follows first-order biochemical kinetics and exponential asymptotic decay of tumour population with some residual malignant cells always remaining under the asymptotic tail of the decay curve, and complete eradication of all tumour cell fails to occur, and thus future relapse of the cancer lesion happens [**Figure 1.1(a)**]. As already delineated, the tumour cells population M decays exponentially with time *t*, which is governed by first-order chemical reaction rate kinetics during the interaction between the cell and the drug [14]----this process can be represented as

$$M = M_0 \, exp \, (-\kappa_M t)$$

Now, after differentiating *M* with time, we will get

$$M' = M_0 \, exp \, (-\kappa_M t) \, (-\kappa_M)$$

i.e $$M' = M \, (-\kappa_M)$$

i.e $$M' + \kappa_M M = 0 \qquad (1)$$

where M_0 is the initial tumour cell population, M' is the time derivative of *M,* and κ_M is the decay rate constant of the drug's cytotoxicity (Figure 1.1 (a)).

In this thesis, we aim to improve the aforesaid models using our procedure of expressing a negative bias in the asymptotic decay curve. This methodology used here can remove the aforesaid asymptotic cancer cell population, and enable eradication of all malignant cells, with permanent elimination of the tumour without any future recurrence. Here the tumour cell trajectory exponentially approaches a negative value M*, indicating that the tumour cell population trajectory becomes zero at time t_F (Figure 1.1(b)). This indicates that $M = [(M_0+M^*)\exp(-\kappa_M t)]-M^*$, so that the negative bias $M^* = -M_0\exp(-\kappa_M t_F)/(1- \exp(-\kappa_M t_F))$. Here, the tumour population curve goes on diminishing and definitively reaches the value of zero at F (i.e. tumour cell population becomes extinct), and then the

curve becomes horizontal, proceeding along the t-axis to Y and follows the horizontal axis onwards. It may be noted that the tumour cell population cannot decrease below zero towards a negative value because it would be biologically impossible. In addition, the subject's benign condition (i.e., the absence of any tumour cells) can be treated as the desired baseline condition, and thus the undesirable tumour cell population state M can be taken to be a tumour system deviation, i.e. error E_M, from the benign condition. Thereby, from Figure 1.1 (b) we can delineate that $E_M = (M - M^*)$, whereby the error time derivative becomes $E_{M'} = (M' - M^{*\prime})$. Therefore, the error equation of eq. (1) becomes,

$$E_{M'} + \kappa_M E_M = 0,$$

Now, by substituting the values of E_M and E'_M, we can get

$$(M' - M^{*\prime}) + \kappa_M(M - M^*) = 0 \qquad (2)$$

The term $M^{*\prime}$, or the temporal rate of change of M^*, in equation (2) is equal to zero since the value of M^* is a pre-determined constant (as desired by the researcher). As a result, eliminating the term $M^{*\prime}$, from equation (2) provides the necessary tumour cell reduction rate:

$$M' = -\kappa_M(M - M^*) \qquad (3)$$

Eq. (3) is the condition for complete tumour regression. Finally, we furnished the translational aspects and validation of our approach using experimental findings, which enables the formulation of a guided controller-based treatment planning system governing the infusion of chemotherapy, interleukin, and antitumour T-cell immunotherapy, for complete extinction of the malignant lesion, along with cancer stem cell elimination and normal tissue protection.

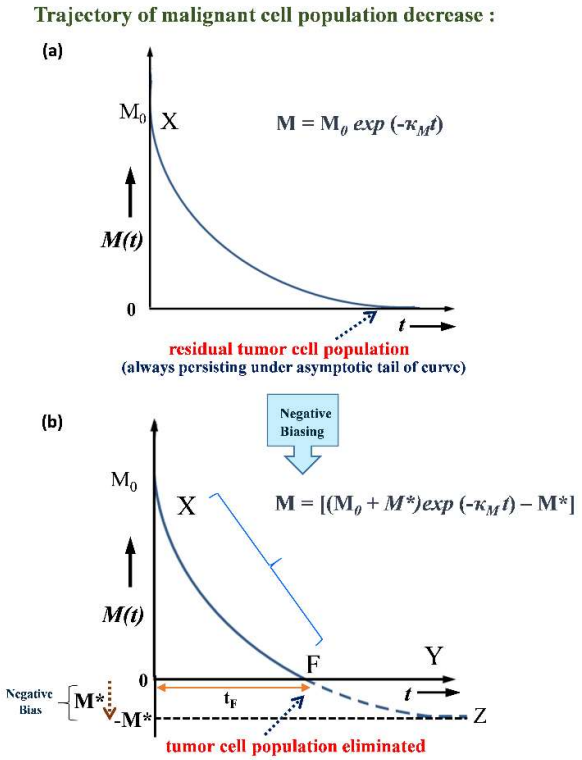


Figure 1.1 Permanent Tumour Elimination Process by First order kinetics.

(a) In customary therapy, the elimination of the tumour cell population M(t) follows the exponentially-decreasing curve, with the vast majority of tumours eliminated, there is the persistence of residual tumour cells asymptotically under the graph, which might produce tumour recurrence after the end of therapy duration.

(b) A Negative bias shift process added to the exponentially decreasing trajectory enables the residual tumour cell population to become zero at a definitive finite time t_F. This curve M(t) also decreases exponentially by the first-order kinetics process and approaches the negative bias value (-M*). Hence, at F the tumour cell population undergoes extinction, and there are no more tumour cells to reproduce, i.e., complete regression of tumour occurs, eliminating the malignant growth.

1.5 Melanoma cancer: as a case study of spontaneous regression process

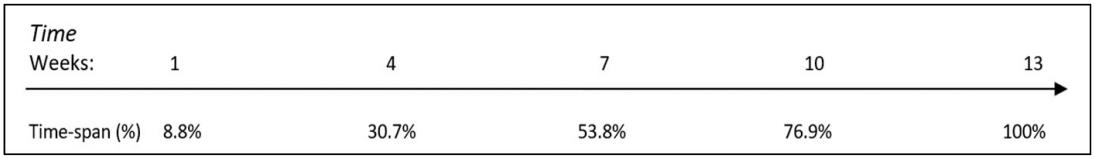
In this malignancy, the melanocytes (the cells that give the skin its tan or brown color) start to continuously proliferate without retardation. Thereby, melanoma, a specific type of

skin cancer occurs. In comparison to several other types of skin cancer, melanoma is much more aggressive. Melanoma is very lethal because it is able to spread to other body regions unhindered. Of all cancers, skin cancer often more prevalent regions inhabited as in parts of Australia, United states, South Africa, Brazil etc. Melanoma is the leading cause of death from skin cancer. According to the American Cancer Society's projections in Unites States for year 2023, there will be an estimated about 97,610 new melanomas cases diagnosed, out of which about 58,120 will be in men and 39,490 in women. Over the past few decades, melanoma rates have been significantly increasing[31]. Efficient treatment of this lethal tumour is a considerable challenge in oncology.

Like other solid tumours, melanoma may spontaneously regress from an immunological perspective, disappearing partially or completely[32]. This regression in melanoma is a typical phenomenon to be observed[33]. This process describes the disappearance or elimination of melanoma, the regression presumed to result from an immune system reaction against the tumour cells in the host. Regression has been shown in up to 58% of thinner melanomas, where it is a relatively frequent occurrence with an incidence range of 10% to 35%[34]. In fact, an examination of 10,098 patients with melanoma regression revealed that these patients could have strong clinical correlations[35]. Understanding the mechanism behind spontaneous melanoma regression is crucial for its clinical applicability [36]. We need to first comprehend the signaling pathways involved in the progression of melanoma, and then delve further into the process of melanoma regression.

The mathematical model that we develop is validated with immunohistochemical experimental findings and microarray assay results of a preclinical model of permanent spontaneous tumour regression of melanoma. Here, numerous tumours appear in pigs, the tumour grow rapidly for the first 1½ months, and then spontaneously regress completely

by 3–4 months in half of the animals, but in the other half, the tumours spread and kill the animals [37]. In the study, six pigs who went into the regression mode, were investigated, the tumours had biopsy under anesthesia at three weekly intervals across three months, at these five different time points as follows, t_0 = day-of-birth (d) + 8 days after birth (i.e., d + 8), thence t_1 = d + 28 days; t_2 = d + 49; t_3 = d + 70, and t_4 = d + 91 (Schema-1). At each time point 5–6 tumour-masses were biopsied, and subjected to microarray investigation using Ingenuity algorithm. The data thus obtained we used to validate our system biology model and mathematical approach.



Schema-1. Temporal sequence of tumour biopsy analysis across the sponteneous tumour regression process.

1.6 Melanoma signalling pathway

We also study the tumour reversion process, namely the process by which the tumour in the expending progression phase undergoes arrest, and then become a tumour in the diminishing regressing phase. To understand the tumour reversion phenomenon, we investigated the tumour progression process comparing it with the tumour regression process. For this we proceed towards a systems of the progression phase.

The genetic alteration in mitogen activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) signaling pathways are the main cause of melanoma progression. MAPK and PI3K signaling pathway helps melanoma in proliferation, invasion, survival and angiogenesis[38], both pathways are activated by Ras protein mutation. The v-Raf murine sarcoma viral oncogene homolog B (BRAF) is by far the most crucial mutated oncogene in melanoma, with the BRAF V600E mutation being the most significant mutation, accounting for 40–50% of all mutated melanomas and 80% of BRAF-

mutated tumours. On the other hand, the neuroblastoma RAS viral oncogene homolog (NRAS), which is mutated in over 30% of all melanomas, is the second most significant mutant oncogene [39]. The dynamics of the MAPK signalling system and the PI3K signalling system are consolidated as below.

1.6.1 MAP Kinase-ERK dependent pathway

The above mentioned NRAS and BRAF proteins are part of the mitogen-activated protein kinase (MAPK) signal transduction system, which controls cell growth, survival, and proliferation and mediates the response of cells to mitotic external stimuli for cell division. Three tissue-specific isoforms of the small proteins produced by the RAS gene family—HRAS, KRAS, and NRAS—are linked to the cytoplasmic membrane. Most NRAS mutations have been found in melanomas [40, 41]. RAF and phosphatidylinositol 3 kinase (PI3K) are two downstream cytoplasmic proteins that can be activated by NRAS. The three members of the RAF kinase family—ARAF, BRAF, and CRAF—are proteins whose activation depends on the formation of complexes by these various isoforms [42]. All three proteins contribute to the MAPK pathway's signal transduction. BRAF causes the activation of MEK kinase in melanocytes, which then triggers the activation of ERK, which is the last enzyme in the MAPK cascade. In 40–60% of melanoma cases, the BRAF gene is altered; the most frequent mutation (occurring in around 90% of cases) is represented by the substitution of valine for glutamic acid at codon 600 (BRAFV600E) [43]. The BRAFV600E variation provides ongoing stimulation of cell proliferation and tumour formation through activating phosphorylation of ERK, as do the remaining mutations in the BRAF kinase domain. However, the finding that BRAF is even mutated in common nevi implies that melanoma development requires the activation of BRAF's oncogenic pathway but is not a prerequisite for it [44].

1.6.2 PI3Kinase-AKT dependent pathway

The second system that uses RAS to regulate cell proliferation is made up of the signal transduction pathway: PTEN-PI3K-AKT [45]. The activation of PIK3 and the activity of the phosphatase PTEN protein both affect intracellular levels of PIP2 and PIP3 phosphoinositols in physiological settings [46]. High PIP3 levels modulate the synthesis of proteins essential in cell growth and survival, as well as apoptosis, by progressively activating downstream AKT (mostly AKT3 in melanoma) and its substrate mTOR. The activation of AKT leads to the inhibition of apoptosis by inactivating numerous pro-apoptotic proteins, such as BAD (BCL-2 antagonist of cell death) and MDM2 (which results in the degradation of p53), as well as promoting cell proliferation through the induction and stabilization of CCND1; and inhibiting apoptosis [40]. In conclusion, PTEN inactivation and PI3K-AKT stimulation result in the abnormal proliferation of cancer cells and development of apoptosis resistance. Around 70% of all melanomas have dysregulated activation of the AKT pathway, which is caused by AKT3 amplification and PTEN loss due to epigenetic silencing or deletion, as originally described [47, 48].

We analyse the aforesaid two tumour progression phase (MAPK and PI3K subsystems) in comparison to the tumour regression phase, and thereby obtain appreciable molecular biological understanding of tumour dynamics behaviour as well as relevant molecular targets and possible drugs, after therapeutic implications.

1.7 Research objective

The objective of the present thesis is to formulate a quantitative systems biology approach that can give incisive mechanistic insights into the process of complete permanent tumour regression, which often occurs naturally in the form of endogenous spontaneous regression as noted in cancer registries of general populations. Furthermore, molecular biology-based pathway analysis of spontaneous complete regression of different

types of tumours would give an understanding on what possible pharmacological agents have the potential of actuating these signalling pathways and thus duplicate the process of complete regression on a patient's tumour as a therapeutic approach. For this, detailed endeavour has also been made to undertake melanoma microarray data-analysis to reveal the genes and signalling pathway mechanism involved and to identifying candidate molecules for therapeutic utility.

1.8 Scope of the thesis

The present thesis deals with the formulation of a robust quantitative systems biology approach to obtain incisive mechanistic insights into the process of complete permanent tumour regression, which often occurs naturally in the form of endogenous spontaneous remission. The thesis consists of six chapters including the present introductory chapter. The contents of the remaining five chapters are briefly described in the following:

Chapter-2 reports a novel computational systems biology model that has been developed with the help of the negative biasing technique for elucidating this unique permanent spontaneous tumour regression phenomenon, so as to furnish insight into the possibility of therapeutically replicating such regression processes on tumours clinically, without toxic side effects. An oncological informatics approach using cell-kinetics based coupled differential equations was formulated for tumour cell elimination along with ensuring protection of normal tissue. Here, the three main tumour-lysis components were investigated: (i) DNA blockade factors, (ii) Interleukin-2 (IL-2), and (iii) Cytotoxic T-cells (CD8+ T). Also, the temporal variations of the mentioned three factors were validated, utilizing preclinical experimental investigations on malignant tumours, using mammalian melanoma microarray assessment and histiocytoma immunochemical evolution. The study found that permanent tumour regression can occur by: (1) Negative-Bias shift in population

trajectory of tumour cells, eradicating them under first-order asymptotic kinetics, and (2) Temporal alteration in the three antitumour components (DNA replication-blockade, Antitumour T-lymphocyte, IL-2), these three components having characterized by the following patterns: (a) Unimodal Inverted-U function, (b) Bimodal M-function, (c) Stationary-step function. These provide a time-wise orchestrated tri-phasic cytotoxic profile.

Chapter-3 find out the gene-expression levels corresponding to the above three components: (i) DNA-damage G2/M checkpoint regulation [genes: CDC2-CHEK], (ii) Chemokine signaling: IL-2/15 [genes: IL2RG-IKT3], (iii) T-lymphocyte signaling (genes: TRGV5-CD28). Further, this study shown that the genes CASP7-GZMB are signatures of the Negative-bias dynamics, enabling eradication of the residual tumour.

Chapter-4 reports a systems biological formulation of the regression process with experimental verification, and also identifies the relevant candidate biomolecules for therapeutic utility. As a case study, the time-wise biopsy and microarrays of spontaneously regressing melanoma and fibrosarcoma tumour in mammalian/human hosts were analysed. The differentially-expressed genes (DEGs), signaling pathways, and bioinformatics framework of regression were delineated. Additionally, prospective biomolecules that could induce a complete tumour regression were investigated. Downregulation of TOP2A gene was found to be pivotal for melanoma regression, and that this gene is highly upregulated in melanoma tissues in clinical patients. The bioinformatics and docking study elucidated two classes of drugs (podophyllin derivative and anthracycline derivative) that blocks the TOP2A receptors, and could be possible therapeutic agents in melanoma patients, and would have the that have potential to duplicate the process of tumour regression in the clinical context.

Chapter-5 mainly focuses on to target two main signaling pathways of melanoma progression i.e. MAPK and PI3K/AKT signaling pathways, which we approach by inhibiting two melanoma actuating genes (BRAF and NRAS), thereby halting the melanoma progression process, and initiating the melanoma regression process. We analysed the differentially-expressed genes (DEGs), and performed Gene-ontology (GO) investigation, and bioinformatics network pharmacology for this melanoma regression process. To further elucidate the molecular mechanism, molecular docking was performed to confirm that inhibition of BRAF and NRAS oncogene can initiate spontaneous tumour regression. We identified 3 small candidate molecules (Alpalisib, Cituximab, and Obatoclax) which targets both these oncogenes, and none of these molecules have been earlier used therapeutically on melanoma. It may be mentioned that the current investigations point out that Alpalisib and Cituximab can be potential repurposed drugs which target both the pathways together instead of targeting one gene at a time, and this conjoint activity may enable therapeutically duplicating the process of spontaneous regression phenomenon on malignant melanoma patients.

Finally, **Chapter-6** summarises the main objectives and draws a line under the main conclusions of the current thesis. This chapter also highlights some of the future scope of the work related to the thesis.