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VASCULAR RELATED PATHOLOGIES IN CARDIOVASCULAR DISEASE AND CANCER

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Vascular related pathologies in cardiovascular disease and cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cardiovascular disease (CVD) and cancer are the leading causes of death worldwide. A damaged endothelium is one of the factors contributing towards these diseases. This thesis focused on understanding the implications of alterations to the physiological endothelium resulting in pathologies related to vascular disease and cancer metastasis.

Functional healing response occurs in the diseased vessel wall aimed at restoring the vessel after an injury. Existing studies state that vascular progenitor cells contribute to the injured vasculature and aid in the repair process. Yet the mechanisms underlying the amalgamation of the cells to the endothelium, their origin and functions have not been clear. Through **Study 1** using animal models of arterial injury, we examined the role of bone marrow derived cells in arterial repair and the mechanisms behind it. We observed that bone marrow-derived cells, helped in the initial stages of arterial injury and were subsequently eliminated from the artery wall. They localized in the arterial intima and most of them were of endothelial phenotype. Additionally, bone marrow-derived cells did not fuse with the intima but could differentiate into vascular cells. This helped them adjust in the vessel wall and meet the needs of their new microenvironment. Fascinatingly, local delivery of bone marrow-derived endothelial cells to the sites of arterial injury caused a 1.4-fold decrease of the intimal lesion area. These results define the role of BM derived endothelial cells in the development of intimal lesions post vascular injury and this information contributes to the existing understanding of the pathogenesis of intimal hyperplasia.

Hemodynamic forces are a cause of a dysfunctional endothelium. A turbulent blood flow could result in vascular disease. Studies have shown that red blood cell distribution (RDW) width as a risk factor for death in cancer and CVD. RDW is one of the haematological parameters commonly reported as part of a complete blood count. An RDW higher than normal is termed as anisocytosis. Anisocytosis has been traditionally used, in combination with the red blood cell corpuscular volume, to diagnose chronic inflammatory status in the body. It has been never studied before if anisocytosis is just a factor that reflects chronic inflammation in the body, or is a factor that directly affects it. Hence in **Study 2**, we hypothesized that anisocytosis leads to changes in blood flow affecting interaction between blood and vascular endothelium at the bifurcation of arteries. We found that a high RDW is a predictive factor for the interaction between cellular components of blood and vascular wall. These interactions can lead to increased inflammation in the vessels and initiation of thrombosis. Put together, we suggest that anisocytosis measured by RDW is a predictive factor of vascular diseases.

Cancer metastasis is one of the major causes of mortality and arises also due to a damaged endothelium. In **Study 3** we investigated the role of murine cytomegalovirus (MCMV) in colon cancer progression using MCMV infected and non-infected animal models. Our results indicate that MCMV did not affect tumor growth but increases the incidence of metastasis to the lungs. Additionally, using microarray analysis we found cytokeratins 1, 2 and 14 to be upregulated 100 times in the infected models compared to the non-infected. We speculate that in our case metastasis is mediated possibly through a cytokerin mediated pathway. The mechanism for dissemination is under investigation.

In **Study 4**, we investigated the effect of C/EBP β on metastasis and the relationship between C/EBP β expression and overall survival of breast cancer patients. We found that decrease in C/EBP β expression was related to shorter overall survival of breast cancer patients. Loss of C/EBP β also, affected tumor growth, morphology and lung metastasis in murine 4T1 breast cancer model. Furthermore, inhibition of C/EBP β resulted in an augmented expression of MHCII and CD45+, CD3+ and CD4+ lymphocytes accumulation in the tumors. Additional experiments established the role of inflammation in C/EBP β -mediated metastasis formation.

LIST OF SCIENTIFIC PAPERS

- I. **Ananthaseshan S**, Grudzinska MK, Bojakowski K, Kurzejamska E, Gaciong Z, Naucler CS, Religa P **Local transplanted CD34+ bone marrow derived cells contribute to vascular healing after vascular injury. *Accepted for publication in Transplantation Proceedings***
- II. **Ananthaseshan S**, Bojakowski K, Prah L, Skolumowska A, Menkens H, Gaciong Z, Wojtkowski M, Lundel F, Religa D, Religa P **Anisocytosis measured by red distribution width is associated with increased interactions of blood cells with vascular wall. *Manuscript***
- III. **Ananthaseshan S**, Kurzejamska E, Bojakowski K, Lazarczyk M, Prakash V, Davoudi B, Sacharczuk M, Fuxe J, Rahbar A, Menkens H, Naucler CS, Landázuri N, Religa P **Murine cytomegalovirus enhances the metastatic potential of colon cancer. *Manuscript***
- IV. Kurzejamska E, Johansson J, Jirström K, Prakash V, **Ananthaseshan S**, Boon L, Fuxe J and Religa P **C/EBP β expression is an independent predictor of overall survival in breast cancer patients by MHCII/CD4-dependent mechanism of metastasis formation. *Oncogenesis (2014) 3, e125; doi:10.1038/oncsis.2014.38***

CONTENTS

1	General Introduction	1
1.1	Vascular Ageing	1
1.1.1	Causes	2
1.2	Blood and Blood vessels	5
1.2.1	Blood	5
1.2.2	Blood vessels	6
1.3	Vascular Remodeling	8
1.3.1	Vascular injury	10
1.3.2	Intimal hyperplasia	11
1.4	Cancer	12
1.4.1	Normal and Tumor Vasculature	13
1.4.2	Angiogenesis in cancer	14
1.4.3	Colon cancer	15
1.4.4	Metastasis	16
1.4.5	Cytomegalovirus and cancer	19
1.5	Cancer Immunology	24
1.5.1	Immune surveillance	25
1.5.2	Immune evasion mechanism	27
1.6	C/EBP β	29
1.6.1	Development of mammary gland	31
1.7	Cancer progression and EMT	31
1.8	REd blood cell distribution width (RDW)- a prognostic factor for patients with cardiac diseases and cancer	32
1.8.1	Red blood cell distribution width and CVD	32
1.8.2	Red blood cell distribution width and cancer- A risk factor for death	33
1.9	CVD co-morbidity in cancer	33
1.10	CVD and cancer induced thrombosis	35
2	Aims of the thesis	37
3	Results and discussion	39
4	Conclusions	51
5	References	57

LIST OF ABBREVIATIONS

BM	Bone Marrow
CVD	Cardiovascular Disease
CMV	Cytomegalovirus
CTC	Circulating Tumor Cell
DTC	Disseminated Tumor Cell
EC	Endothelial Cells
EPC	Endothelial Progenitor Cells
ECM	Extra Cellular Matrix
EMT	Epithelial to Mesenchymal transition
FGF	Fibroblast Growth Factor
HIF	Hypoxia Inducible Factor
HSC	Hematopoietic Stem Cell
ICAM	Intercellular Adhesion Molecule
MCP-1	Monocyte Chemoattractant Protein-1
MMP	Matrix Metalloproteinases
NO	Nitric Oxide
PDGF	Platelet Derived Growth Factor
RDW	Red Blood Cell Distribution Width
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor

1 GENERAL INTRODUCTION

Cancer and Cardiovascular diseases are the two most prominent causes of death worldwide. [1] Emerging evidence indicates shared risk factors and a common biology between these diseases. For instance, chronic inflammation has a significant role in contributing to both diseases. [2, 3] An alteration of the vasculature and the endothelial cells plays a key role in pathogenesis of CVD and cancer. [4] The widespread overlap regarding disease prevention and risk factors for these diseases suggest a common mechanism in terms of molecular pathways. [5]

1.1 VASCULAR AGEING

An important factor that contributes to vascular dysfunction is ageing related injury of normal endothelial functioning. This is responsible for numerous age-related illnesses of the vascular system and other organs. All organs undergo a progressive decline of function and structure over time due to ageing. Endothelial cells undergo senescence during this process and display substantial changes in their properties ensuing damage to the vascular functionality and neo-angiogenic capability. Thus, changes to mechanical and structural properties of the vascular wall result in damage of arterial elasticity and reduced arterial compliance[1].

Evidence suggests that different disease state like diabetes, hypertension and end stage renal failure show a reduced arterial compliance. These changes could also be present before the manifestation of CVD. Vascular ageing contributes to the ageing dependent growth in atherosclerotic disease and hypertension. More than traditional risk factors like lipid levels, smoking, diabetes, and sedentary lifestyle, ageing is a factor that deliberates a greater risk for the disease. Mitochondrial dysfunction, microRNAs and micro environmental stressors like hypoxia, Mechanisms like mitochondrial dysfunction and micro environmental stressors, are defined to be involved in ageing-related endothelial cell senescence control.[2, 3]

The process of ageing is characterized by a functional decline in the cells and tissues and the diminished ability to suitably respond to environmental distress, including metabolic stress and reduced oxygen supply. This results in deterioration of the total fitness of the organism, which is often associated with the individual's life style.[4-6] As the vasculature is the major source of oxygen and nutrient supply in the body, endothelial cells are extremely susceptible to deviation in oxygen pressure. The age-related injury of the response to oxygen and nutrients level variation is considered a key factor contributing to arterial dysfunction that leads to age related vascular diseases, for example, atherosclerosis.[7, 8]

1.1.1 Causes

1.1.1.1 General Mechanisms

Many years ago, atherosclerosis was thought to be a lipid storage disease. Lipid deposits were formed on the artery's surfaces and grew until they became large enough to hinder blood flow and eventually result in a cardiac incident such as a myocardial infarction or a stroke.[9] Current mechanisms involve inflammation, which play a key role in atherosclerosis formation, right from its initiation and progress to its endpoint-thrombotic complications. [10]ECs, which form the innermost layer of the arterial wall, normally resist the attachment of leukocytes such as macrophages and T lymphocytes from binding to its wall. But triggers like consuming high saturated fat diet, hypertension or smoking can initiate this binding. One factor is vascular endothelial growth factor-1 (VCAM-1).[11]

Lesions also develop due to type of blood flow they experience. Shear stress occurs due to laminar blood flow and this results in several atheroprotective mechanisms.[11, 12] For example, it produces an antioxidant enzyme, superoxide dismutase or an increased expression of nitric oxide synthase. This limit VCAM-1 expression by inhibiting nuclear factor kappa beta(nF-Kbeta) production and thus combat platelet clumping.[13]

Once adhered, the monocytes penetrate the endothelium and infiltrate the intima by diapedesis a process that requires a chemoattractant gradient such as monocyte chemoattractant protein -1(MCP-1).[14] Within the intima, monocytes change into macrophages, and express scavenger receptors and inundate lipid particles, thus transforming into foam cells typical of atherosclerotic lesions.[15, 16] In the intima through lesion evolution the T lymphocytes join macrophages and secrete cytokines and growth factors that may nurture the migration and proliferation of smooth muscle cells. T lymphocytes also excite macrophages to produce collagen-degrading enzymes and secrete cytokines and growth factors that may promote the migration and proliferation of smooth muscle cells. This results in the fibrous cap protecting the blood from the thrombogenic core of the plaque, to weaken leading to rupturing of the plaque and eventually resulting in thrombosis which is the complication in most atherosclerotic cases.[17, 18]

1.1.1.2 Dysfunctional Endothelium

A normal vasculature has an organized network of blood vessels maintained by a balance between pro and anti-angiogenic factors.

A healthy tissue also displays a systematic network of lymphatic vessels that allows for transport and draining of blood and metabolic waste from the interstitium. This intricate architecture consisting of mature vessels make up the normal vasculature. These vessels allow the adequate transport of nutrients, oxygen and blood supply required to sustain the vasculature.[19, 20] Alterations to the vasculature plays a significant role in the development of numerous diseases.[21]

The endothelium plays a significant role in governing the circulation as a physical barrier and as a variety of different regulatory substances. For example, the endothelium derived prostacyclin and nitric oxide inhibit platelet function and induce vascular relaxation when they are released in response to physical stimuli, platelet derived substances or hormones. The endothelium is also a good source of heparins, heparin sulphates, thrombospondins and platelet derived growth factor. Also, several vasoactive substances produced by the endothelium such as nitric oxide, angiotensin- 2 and endothelin-1 might play a role in vascular tension. Dysfunction of these endothelium dependent factors could lead to CVD such as atherosclerosis and hypertension and is thought to be involved in stroke, tumor angiogenesis, vascular leakage and infectious diseases[22]

Endothelial dysfunction occurs when the endothelium shifts to a pro inflammatory, reduced vasodilatory and pro thrombotic state. This state is associated with most forms of cardiovascular diseases. When free radicals disrupt the balance of NO in the body, damage to the endothelium occurs leaving them excessively permeable allowing for toxins to pass into the tissues.[23] When the action of NO is inhibited, endothelial signaling is weakened resulting in numerous diseases, since in the human body, the endothelium actively maintains, around 60,000 miles of blood vessels. A normally functioning endothelium also supports the body's immune response, helps regulate blood clotting, controls the volume of fluid and the number of electrolytes and other substances passing from blood into the tissues, and produces dilation or constriction of the blood vessels.[24]

1.1.1.3 Hemodynamics

The dynamics of blood flow is called hemodynamics.[25] Blood, being a non-Newtonian fluid, is best studied with rheology.[26]

Normal blood flow ensures the transportation of nutrients, oxygen, CO₂, metabolic waste throughout the body thus enabling regulation of different functions such as maintaining cell metabolism, pH and osmotic pressure and temperature regulation along with protecting the body from harmful stimuli.

These are key factors that helps the body adapt as per the environment.[25] A laminar blood flow thus ensures proper functioning of the body while a turbulent flow and local hemodynamic factors like flow disturbances at bends and bifurcations contribute to the formation of atherosclerosis or other pathological conditions. A laminar flow occurs when the vessel wall is smooth whilst a turbulent flow occurs when there is a decrease in wall smoothness. This is due to fatty deposits on the vessel wall.[27]

The vascular ECs form the innermost layer of the vessel wall with direct contact to blood flow and are involved with vital homeostatic functions to various mechanical and chemical stimuli.[28] [29-33] Not only do they provide a selective barrier for permeability but the EC's also influence hemostasis and thrombosis through secretion of pro and anti-coagulants, fibrinolytic agents and mediate inflammatory responses via the release of cytokines and chemokines. The EC's also regulate smooth muscle migration through the release of vasodilators and constrictors and influence vascular remodeling using growth promoters and inhibitors. [34]Hence hemodynamic forces are needed for normal physiological functioning of the EC's while some forces induce a dysfunction of the endothelium by modulating EC gene expression and signaling leading to the development of pathological states, which contribute to the formation of atherosclerosis, thrombosis and its complications.[35-38]

The role of hemodynamic forces in endothelial dysfunction was first proposed when observations of initial atherosclerotic lesions were credited to a nonrandom pattern of development. These were observed typically at arterial bends and branches with a disrupted flow. This flow pattern included recirculation eddies and change in direction with respect to space (reattachment and flow separation) and time (reciprocating flow).[39-42]

Recent research show that this kind of flow and the associated reciprocating and low shear stress bring about a constant activation of several atherogenic genes in ECs. for instance, the monocyte chemoattractant protein (MCP-1) that induces monocyte infiltration into arterial wall, and platelet derived growth factor (PDGF) that augment EC turnover and SMC migration, into the subintimal space.[28, 34, 43-45]

On the contrary, the straight portion of the artery, usually safe from the atherosclerotic lesions, is exposed to constant laminar blood flow and high shear stress, all with a definite direction of flow and the associated downregulation of atherogenic genes and upregulation of growth arrest genes and anti-oxidants in EC's.[28, 43, 46-48] Hence these findings propose that laminar and turbulent flow patterns might induce a variety of molecular responses in ECs, which result in sparing of the straight parts of the arteries and formation of lesions at the curvatures.[49, 50]

1.2 BLOOD AND BLOOD VESSELS

1.2.1 Blood

Normal tissue functioning requires an adequate supply of oxygen, nutrients and blood vessels to facilitate their transport. Virchow, around a hundred and fifty years ago, explained vascular diseases in terms of cellular mechanisms, most of which stand valid till date.[51, 52] However, more recently the vessel wall has been visualized as a channel containing blood, which is provided by the heart pumping around blood in a circuit that is optimized to exchange and distribute oxygen and nutrients.

Blood is a body fluid that delivers oxygen and the essential nutrients to cells and tissues.[53] In vertebrates, blood is comprised of blood cells suspended in blood plasma. Blood has various functions such as, supplying oxygen to different tissues, supply of nutrients like amino acids, fatty acids and glucose, waste removal (CO₂, urea) etc. Blood cells constitute the following components; erythrocytes, leukocytes, platelets. The most abundant are the erythrocytes, which has the iron containing protein, the hemoglobin, which facilitates transport of oxygen [54-56].

The cellular components of blood are formed through a process known as hematopoiesis, which is a process that occurs during embryonic development and throughout adulthood. All the cells are derived from the hematopoietic stem cells [57]. In a healthy adult, around 10^{11} - 10^{12} new cells are formed every day [58]. The process occurs in two waves. The primitive wave consists of an erythroid progenitor, which appears in blood islands, giving rise to macrophages and erythrocytes during initial stages of embryonic development [59] [60]. The main purpose of this stage is to provide tissue oxygenation since the embryo grows rapidly. This wave is transitional. In humans, fetal haematopoiesis begins in the yolk sac and shifts to the liver briefly before establishing in the bone marrow and thymus when the baby is born.[61, 62] The definitive wave follows and includes haematopoietic stem cells. They are multipotent which can produce all blood lineages of the adult organism.[63].

Bone marrow is the tissue encompassing the center and the epiphysis of bones.[64] It is also the place for production and maturation of B cells. It is an organ composed of trabecular and cortical bone, cartilage, hemopoietic and connective tissues. The trabecular bone is composed of a framework of fine bone plates filled with hematopoietic marrow, fat containing marrow or blood vessels.[65] The bone marrow consists a vascular component (stroma) and a hematopoietic component (parenchyma) The parenchyma includes hematopoietic stem cells (HSCs) and hematopoietic progenitor cells, localized near the endosteum of the bone and more around blood vessels.

The stroma comprises multipotential non-hematopoietic progenitor cells as well which can differentiate into various tissues of mesenchymal origin, including, endothelial cells, osteoblasts, reticular cells, adipocytes and fibroblasts. The stromal cells including ECs deliver signals for migration of specific leukocytes into and out of the bone marrow, including in rolling/extravasations along the vascular endothelium.[65, 66]

1.2.2 Blood vessels

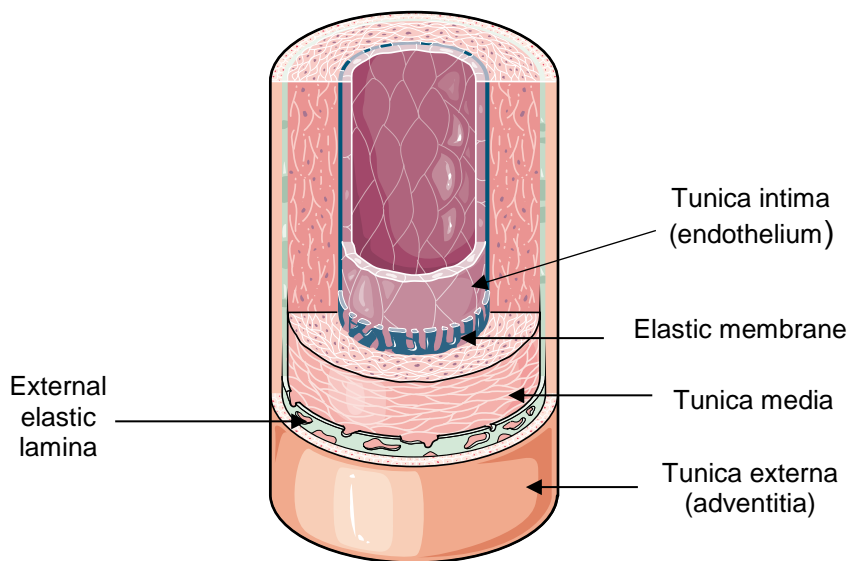


Fig 1. Structure of the vessel wall

Angiogenesis is a process through which fresh blood vessels are formed from existing vessels. It is a normal and key process that occurs throughout life and is involved in development and growth, disease as well as wound healing. Oxygen plays a critical role in this process along with hemodynamic factors that are essential for the survival of vascular networks and for structural adaptations of vessel walls. [67-70]

The blood vessels originate through a process beginning with the mesodermal layer. The first system to develop in the embryo is the cardiovascular system. [71]The luminal surface of this system that is in close contact to the blood is made up of a single layer of endothelial cells derived from the mesoderm. It is formed by the following processes. Hematopoietic stem cells and angioblasts arise from the differentiation of hemangioblasts followed by vasculogenesis which is the de novo formation of blood cells from angioblasts.

It is an active process that involves cell to cell interactions along with cell to extracellular matrix interactions. This process is directed spatially and temporally by morphogens and growth factors. Vasculogenesis includes differentiation of mesoderm stem cells into angioblasts followed by migration of angioblasts to form blood islands where they give rise to endothelial cells.[72, 73]

There are two types, the sprouting and intussusceptive angiogenesis. Sprouting angiogenesis occurs when sprouts of endothelial cells migrate towards a stimulus, in this case VEGF-A. It thus adds vessels to parts of tissues that previously lacked blood supply. Intussusceptive, on the other hand forms blood vessels by splitting vessels due to which interstitial fluids invade an existing blood vessel. [74, 75]

The steps involved in sprouting angiogenesis process are: Basement membrane degradation followed by endothelial cell (EC) proliferation, focused migration of the cells, formation of new vessels (tubulogenesis), fusion of the vessels, vessel pruning and pericyte stabilization. The process begins in response to a hypoxic environment. VEGF-A initiates the process.[76-78] An endothelial tip cell responds to this stimulant by guiding the sprouting EC through the ECM towards VEGF-A.[79] The ECs migrate through the ECM via filopodia, which are long, thin projections which extend from the migrating tip of the cells.

These secrete proteolytic enzymes which degrade the basement membrane. When enough filopodia have anchored to the substratum, the tip cell is aligned with the VEGF-A receptor through contractions of actin filaments within the tip cell. Meanwhile, the capillary sprout elongates due to endothelial stalk cell proliferation, since they follow the tip cell causing the elongation process. A lumen is formed within a series of stalk cells due to vacuoles developing and coalescing. These stalk cells form the trunk of the recently formed capillary. When the tip cells of two or more capillary sprouts encounter at the origin of the VEGF-A secretion, the tip cells merge together forming a continuous lumen via which oxygenated blood can flow. VEGF-A levels return to near normal, when the local tissues obtain suitable amounts of oxygen. Pericyte recruitment induces maturation and stabilization of the capillary.[80-82]

Intussusceptive angiogenesis also called splitting angiogenesis, is a process where a single vessel splits in two due to an extension of the vessel wall into the lumen. This type is quick and effective compared to sprouting angiogenesis since, initially, it only requires restructuring of existing ECs and does not require immediate EC proliferation or migration. Intussusceptive angiogenesis occurs lifelong but plays an important role in vascular development in embryos where development is quick and resources are limited. Intussusception mainly causes new capillaries to develop from existing capillaries.[83-85]

All blood vessels except for capillaries are made up of three layers, tunica intima, tunica media and the adventitia. The tunica intima, the thinnest layer is comprised of a monolayer of endothelium cells that line the vessel wall, surrounded by an elastic connective tissue called the internal elastic lamina.[86] [87], whose function is to related to elastic resilience to sustain blood pressure The tunica media is made up of numerous layers of elastic lamina and smooth muscle cells. This is the thickest layer. The SMC's present in the medial layer influence blood pressure through the production of multiple ECM components (elastin, collagen).[88] The adventitia is comprised of loose connective tissues made up, primarily of collagens and elastic fibers, fibroblasts and macrophages. The fibroblasts play an important role in fibrogenesis. The adventitia also plays a significant role in vascular remodeling and development of vascular diseases such as transplant vasculopathy, atherosclerosis, hypertension and restenosis. [89, 90]

1.3 VASCULAR REMODELING

Vascular remodeling is an intricate process of structural changes that involve at least four different process – cell growth and death, cell migration and degradation of extra cellular matrix (ECM). The process is dependent on an interplay between locally generated growth factors, hemodynamic stimuli and vasoactive substances.

Generally remodeling is an adaptive process which occurs in response to long term changes in the hemodynamic conditions, but it might eventually contribute to pathophysiology of circulatory disorders and vascular diseases. [91, 92] For instance, physiologically, vascular remodeling occurs during pregnancy. Adequate uteroplacental blood flow is needed for a normal pregnancy outcome. This process takes place with the growth and remodeling of the uterine circulatory system along with growth of a new organ, the placenta.

Vascular remodeling involves various patterns and the nomenclature used to denote these patterns is circumferential remodeling. It is normally called inward or outward to signify tapering vs widening of the vessel wall. The term expansive remodeling is used to denote increase in circumference and it is employed as a substitute for outward These terms were

primarily proposed by Mulvany.[93] Also, taken into consideration is the wall mass, which can increase (hypertrophy), decrease (hypotrophy) or remains unchanged (eutrophy). For example, the remodeling of vessel to larger lumen with the same wall thickness occurs during pregnancy in the uterine circulation. This is called outward hypertrophic because the cross-sectional area is increased. This is due to the diameter of the chief uterine artery almost doubling in magnitude in humans during pregnancy and the enlargement occurs with or without the thickening of the vascular wall. Another study on human myometrial radial arteries from preeclamptic women the pattern of remodeling was inward eutrophic since there was no change in cross-sectional area indicating a rearrangement of existing wall elements around a smaller lumen.[94, 95]

Several factors contribute to vascular remodeling resulting in pathological implications. For example, when pulsatile blood flow and pressure induce various kinds of hemodynamic forces such as shear stress, hydrostatic pressure, constantly on blood vessels. Since the ECs are exposed to flowing blood, they bear most of the shear stress because of frictional forces rising from blood flow corresponding to the vessel luminal surface. [30, 34, 96] This increase or decrease in shear stress plays a key role in vascular remodeling and homeostasis. [97-100] During the process of remodeling, for a compensatory arterial response towards changes to occur, a functional and intact endothelium is required to undergo adjustments in function and structure in response to alterations in shear stress.[98]

Vasoactive factors also play an important role in vascular structure determination. They have an acute effect on vascular muscle tone and they may also impact matrix production and migration of cells. Angiotensin 2, for example, induces growth of vascular smooth muscles cells through PDGF AA and TGF beta 1.[101-103] They are also involved in activation of EC through receptors coupled to ion channels which in turn modulate intracellular calcium concentration.[104]

Vascular remodeling can also occur in response to increased arterial pressure, in which case, the structure of the vessel wall is modified such that the ratio of the width of the wall to the width of the lumen is raised either, by an increase in muscle mass or rearrangement of cellular and non-cellular elements. This results in heightened peripheral resistance typical for hypertension. [105-107]

Another form of remodeling includes reduction in lumen diameter and is related to other diseases such as cardiac allograft vasculopathy and restenosis post percutaneous intervention.[108] Alternatively, remodeling could also lead to higher lumen diameter and compensate for increased plaque load. [109] Vascular remodeling also involves changes

primarily in lumen dimensions due to active reorganization of wall components. This type is associated with constant high blood flow, observed in patients -an arteriovenous fistula and was shown in animal models.[110, 111]

Remodeling of vessel wall also occurs in response to vascular injury. A neointima forms in a sequential process as part of a reparative answer to injury. This involves thrombosis, migration and proliferation of vascular cells, matrix production, and inflammatory-cell infiltration. Mechanical injury results in vessel constriction and smaller vascular lumen in response to the scarring in the outer vessel layer. Key features of transplant vasculopathy include inflammation and formation of intimal hyperplasia.[108, 112, 113]

1.3.1 Vascular injury

Vascular trauma is the injury caused to a blood vessel- the artery, that carries blood to an organ, vein, which returns blood back to the heart.[114] Hemodynamics, hypoxia, ischemia and endothelial dysfunction are factors that contribute to vascular injury. Vascular injury is brought about by mechanical injury due to surgical manipulations and from tissue ischemia due to obstruction of the vasa vasorum. The level of the resulting neointimal lesion is related to the extent of vascular injury.[115, 116]

Distressed flow patterns contribute to pathogenesis of various clinical disorders such as atherosclerosis, arterial aneurysm, post-surgical intimal hyperplasia, ischemic/reperfusion injury. This is due to disturbances of blood flow in arteries generated due to surgical interventions like end to end anastomosis in bypass graft or stent insertion in balloon angioplasty. [117]Additional flow disturbances that facilitate endothelial dysfunction is the termination or inactive flow and its recovery later in clinical conditions related with ischemia/reperfusion and hypoxia injury. Hence myocardial recovery following acute infarction becomes complex and the outcome could be cell damage, arrhythmia and death.[118] Tissue injuries induced by solid organ transplantation, tissue resuscitation and key vascular surgical intrusions could also result in a dysfunctional endothelium. This results in inflammatory responses with recruitment of WBCs from circulation.[119-121]

Another mode of injury occurs through ischemia/hypoxia. Hypoxia is a vital component of an ischemic result. Hypoxia is the reduction of oxygen while ischemia is the lack of perfusion. Ischemia is a process, which occurs when the tissue's demand for energy substrates is not met with supply.[122, 123] Reperfusion injury or ischemic/reperfusion injury occurs when blood flow and oxygen resume to a tissue after a hypoxic/ischemic event. HIF 1 alpha is stabilized in response to limited oxygen. This is worsened by modification of locally released vasoactive

mediator such as NO and its action. Sudden re-oxygenation post hypoxia activates the release of free radicals, like reactive oxygen species (ROS) and reactive nitrogen species (RNS), which modify EC homeostasis and results in swelling and tapering of the blood vessel lumen along with a host of other damaging effects. ROS and RNS are highly reactive molecules and include hydrogen peroxide, peroxynitrite, hydroxyl radical, and superoxide resulting in oxidative stress, disparity between the production of reactive species and antioxidant defences that causes tissue damage. Tissue injury is characterized by a loss of tight junctions, leading to augmented permeability, separation from the basement membrane, and, often, EC apoptosis or necrosis. Eventually these structural changes contribute towards microvascular perfusion impairment.[124, 125]

1.3.2 Intimal hyperplasia

Intimal hyperplasia is the universal response of a vessel to an injury. It is the thickening of the tunica intima.[126] It is connected to increased cell number and the amount of ECM in the intimal layer of the vessel.[127] Physiologically, it occurs in the involution of the uterus, during closure of the ductal arteriosus post birth (DA) In the foetus, the process of intimal thickening starts in the second trimester of pregnancy with the build-up of glycosaminoglycans in the sub endothelial region (SER). This is followed by separation of ECs from the internal elastic lamina, followed by migration of SMCs into the sub endothelial region. This phenomenon was also observed in the mature DA in the neonate, indicating that this is a constant process. [127] [128]

Pathologically, it occurs post balloon angioplasty[129], transplantation[130], artery bypass conduits[131], in pulmonary hypertension[132] and pre-atherosclerotic lesions.[133]

Intimal hyperplasia occurs in a few stages. The key stimuli are inflammation, injury and enhanced vessel wall stress. [134]For instance, balloon injury to the rat carotid artery does not incite a marked inflammatory response but nonetheless generates intimal hyperplasia. Inflammation is, nevertheless, a confounding feature of most other models of vascular injury and therefore may contribute to the extent of intimal hyperplasia. [135]Direct effects of inflammatory mediators on early transduction events, mainly the NF- κ B pathway, have been implicated. Synergistic communication between growth factors and inflammatory cytokines to cause MMP induction and activation is another probable mechanism. In addition, proteases and growth factors directly derived from macrophages possibly play key roles.[135-138]

Two situations which evidently demonstrate a relationship between increased mean wall stress and intimal hyperplasia are vein grafting and pulmonary hypertension.[134] The mediators

involved remain largely undefined but increased MMP and PDGF expression has been observed in experimental (pig) vein grafts and was reversed in parallel with intimal hyperplasia when the grafts were supported by an external stent.[139-141]

The injured artery recruits inflammatory cells such as macrophages and leukocytes and mobilizes vascular progenitor cells from their niches. Another crucial factor in the neointima formation, platelet-derived growth factor (PDGF), FGF along with additional factors, is produced by platelets, smooth muscle cells, endothelial cells and macrophage foam cells. [86]

These factors along with PDGF act as chemo attractants, which promote cell migration of the SMC's into the neointima from the media. Also, PDGF promotes production of collagens and proteoglycans. In parallel, the ECM is remodelled by MMP's, which further promote SMC migration. Thus, the neointima grows in response to cell proliferation, increased ECM synthesis, apoptosis and fibrosis.[86, 142]

Various sources of cells contribute to neointima formation. They can originate from the adventitia from cells such as pericytes, fibroblasts and vascular progenitor cells or from circulating progenitor cells, for example- endothelial progenitor cells, smooth muscle progenitor cells or bone marrow derived cells.[143, 144]

In this thesis, through study 1, we investigated the role of intimal hyperplasia in vascular remodeling post balloon injury and carotid ligation in a mouse model.

1.4 CANCER

Cancer is a disease involving abnormal cell growth, which arises when cells undergo uncontrolled proliferation and lose their ability to control apoptosis. More than two hundred diverse types of cancer exist, and their etiology remains uncertain. Current hypothesis state that cancer is a genetic disease and numerous mutations in various genes are considered as cancer promoting genes. Additional factors such as lifestyle, obesity, tobacco, alcohol consumption and virus infections constitute risk factors for cancer development [145, 146]

Dysfunctional endothelium is a hallmark of many diseases like diabetes mellitus, atherosclerosis and cancer.[147] Endothelial cell migration is a vital component of angiogenesis and requires a tight regulation of the contractile and noncontractile conditions of the cell. These processes require the combination of signals elicited by hepatotactic, chemotactic and mechanotactic stimuli. This movement is in turn, related to the activation of intracellular pathways that congregate on cytoskeleton remodelling.[148, 149]

There are several types of malignancies that affect humans and they are classified based on the type of cell that the tumor cells resemble. These include carcinomas representing a group of cancers originating from epithelial cells, including the most common cancers breast, colon, lung and prostate cancer, - Sarcomas a neoplasm derived from connective tissue (e.g. bone, cartilage, fat), originating from mesenchymal cells outside the bone marrow and lymphomas and leukaemia's: neoplasm arising from blood cells that leave the bone marrow and mature in lymph nodes and blood.[150]

1.4.1 Normal and Tumor Vasculature

There are fundamental differences between the normal and tumor vasculature. The normal vasculature has an organized network of blood vessels maintained by a balance between pro and anti-angiogenic factors. A healthy tissue displays a systematic network of lymphatic vessels that allows for transport and draining of blood and metabolic waste from the interstitium. This intricate architecture consisting of mature vessels make up the normal vasculature. These vessels allow the adequate transport of nutrients, oxygen and blood supply required to sustain the vasculature. [20, 151]

The tumor microenvironment consists of different cell types such as endothelial cells, pericytes, and fibroblasts. These cells contribute through the rearrangement of the ECM and secretion of various growth factors and cytokines to tumor progression.

In tumors, aggressive neoplastic growth is accompanied by an over expression of pro angiogenic factors which lead to the formation of aberrant blood vessels. These blood vessels are immature and highly permeable and give rise to a disorganized vascular structure comprised of irregular vessels with different shapes and diameters. This is due to the scarcity in smooth muscle cells and possible discontinuous endothelial cell lining with an abnormal basement membrane. [152, 153] Augmented vessel permeability leads to deviation in osmotic forces that results in buildup of vascular contents and high interstitial fluid. Blood flow is hindered due to resistance caused by the defective shape of the blood vessels. This, in turn, leads to insufficient oxygen supply with a localized hypoxia.[154, 155]

Other aberrant characteristics of the tumor vasculature include arteriole-venous shunts abnormal bulges and plasma channels lacking red blood cells. The typical blood vessel arrangement found in the healthy tissue (consisting of arterioles, arteries and venules) sometimes cannot be identified. The vessel endothelial cells are dysfunctional and lose their expression of endothelial markers. The lymphatic vessels in tumors are also leaky, unstable and dilated. Due to this type of arrangement, various functional processes within the tumors

are drastically different to those of the healthy tissue. For instance, the normal processes of nutrient delivery through these dysfunctional blood vessels and of metabolic waste removal via the lymphatic system are greatly reduced. [20, 153]

1.4.2 Angiogenesis in cancer

Angiogenesis is a process through which new blood vessels form from existing vessels. It is a normal and key process involved in development and growth, as well as in wound healing. It is however, also involved in cancer, since it is the fundamental step for a tumor to grow in size and metastasize. Tumor angiogenesis was first reported in 1971 by Judah Folkman. He suggested that tumor growth is dependent on angiogenesis.[67-70]

For a tumor to grow (more than 1-2 mm in diameter), it needs an independent blood supply, hence angiogenesis is a necessary step. The tumor secretes growth factors that recruit new blood vessels. The process continues after a tumor matures and is a vital step in sustained tumor growth and for tumor metastasis.[156-158]

The newly formed vessels provide an exit route for tumors cells, through which cells may detach from the primary tumor and enter the blood stream. The angiogenesis process is regulated by the production of various angiogenic stimulators. This includes members of the VEGF and FGF families along with regulating angiogenic inhibiting factors like angiostatin and endostatin. The latter regulate and modulate the process both at the primary and metastatic sites. Vascular density plays an important role as a prognostic factor for many tumors. Highly vascular tumors are more metastatic.[159, 160]

Regulation of angiogenesis occurs through hypoxia or ischemia. Many proangiogenic factors and their receptors maybe modulated by this process. For example, factors like VEGF, FGF, and TGF-beta, PIGF and angiopoietins and HIF. Oxygen deficiency stimulates and regulates HIF which in turn triggers genes for VEGF- A and VEGFR 1. The VEGF family is the most important player in angiogenesis. [161-167]

VEGF induces vascular endothelial cell proliferation and growth and survival. The VEGF family consists of various receptors, namely VEGF- A, VEGF-B, VEGF-C and VEGF-D with their receptors VEGF -1, VEGF-2 and VEGF-3. VEGF-A along with the receptor VEGF -2 are important regulators of angiogenesis.

VEGF initiates angiogenesis by binding to specific receptors. When a tumor needs to grow, it releases growth factors, VEGF which binds to the extracellular receptor on the endothelial cell on the blood vessel. VEGF A, B and PIGF bind to VEGF 1, while VEGF A, C and D to VEGF

-2. VEGF C and D bind to VEGF 3 on the endothelial cell of the lymph vessel thereby stimulating lymph angiogenesis. [157, 168]

Once VEGF binds, dimerization of the receptors takes place which activates intracellular tyrosine kinase domain (ITK) thereby inducing auto phosphorylation. This further activates downstream signals required for the angiogenesis process.

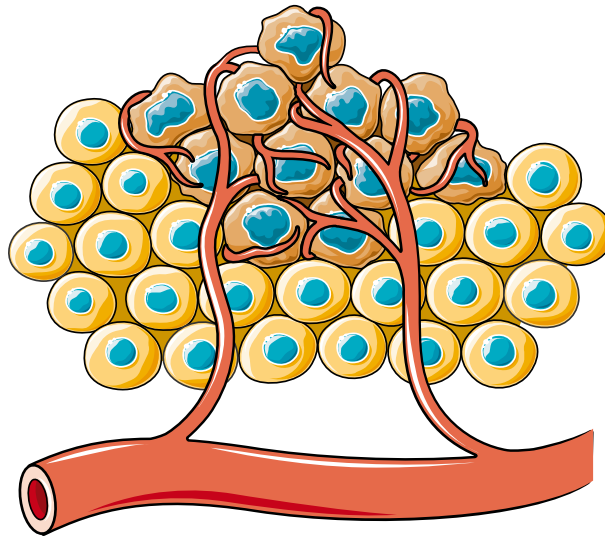


Fig 2. Angiogenesis process

1.4.3 Colon cancer

Colon cancer is one of the foremost causes of cancer-related deaths worldwide. The chief cause of mortality in colon cancer patients is liver metastasis, either present already at cancer diagnosis stage, or developing after primary tumor resection. Survival rates of patients continue to increase with time, mainly because of improved diagnostics and treatment. [169] Colonic epithelium consists of roughly ten million invaginations, called crypts whose base of contain multiplying daughter cells and dividing stem cells and form the starting point for the cell migration towards the epithelium surface. Here the cells die and become replaced by continuously streaming new cells.[170]

One of the factors or an amalgamation of chromosomal instability(CIN), CpG island methylator (CIMP) phenotype, microsatellite instability(MSI) contributes to colon cancer progression. Genetic variability is usually caused by loss of heterozygosity and aneuploidy. Alternatively, mutations in the cell cycle genes or tumor suppressor gene may also lead to cellular transformation. Similarly, microsatellite instability and mutator phenotype are caused due to epigenetic and/or genetic modifications resulting in impaired cellular pathways, such as

DNA repair mechanism. Non-coding RNAs, more prominently microRNAs and long non-coding RNAs have also been implicated at numerous CRC stages.[171]

The acquisition of mutations in the adenomatous polyposis coli (*APC*), followed by the mutational activation of oncogene *KRAS* and the inactivation of the tumor suppressor gene, *TP53* signals the beginning of the CIN pathway. [172] The major players in CIN tumors are loss of heterozygosity (LOH) and aneuploidy. This not only constitutes most of the sporadic tumors (85%) but also involves familial adenomatous polyposis cases linked with germline mutations in the *APC* gene.[173] Promoter hypermethylation of several tumor suppressor genes is a characteristic feature of CIMP pathway most importantly *MGMT* and *MLH1*. This is often associated with *BRAF* mutation and microsatellite instability. [174]Inactivation of genetic alterations in short repeated sequences contributes to the MSI pathway. Also, hypermethylation of DNA mismatch repair gene might lead to MSI.[175]

Both genetic and environmental factors contribute to histopathological changes. Key environmental factors involve toxins, pathogen invasion, polyamines, ROS (reactive oxygen species) production and stress. [176]

The growth of adenomas is triggered by adverse conditions like bacterial or viral invasions, subsequently causing mutation in the APC (Adenomatous Polyposis Coli) regulatory pathway usually affecting either APC or β -catenin. APC represses β -catenin, which diminishes the tendency to abnormal tissue expansion by augmenting protein expressions that promote and affect cell division and cell adhesion. As cells migrate from base crypts towards the epithelium surface, APC expression increases and hinders β -catenin. This in turn promotes apoptosis at the surface and provides optimal balance in production from the crypt base in parallel.[177]

Transformation of dysplastic epithelium (stage I) to early adenoma phase (stage II) takes place due to COX2 mutations and appear in most human colorectal adenocarcinomas. This is followed by mutations in RAS genes, with K-RAS being the most common gene and less H-RAS the least. K-RAS mutations, next to common mutations in DCC, MLH1 and MSH2 facilitate the transition from early to late adenoma (stage III). Lastly, progression to tumor metastasis (stage IV), involves genes such as BAX, E2F4, MSH3, MSH6, TGF- β R2, BAX and MMPs, p53 and SMAD4 affecting liver, lungs, bone and brain.[178]

1.4.4 Metastasis

Metastasis is a multi-step process involving a modulation of phenotype of cancer cells, invasion of cells to enter circulation and form distal metastasis. It is one amongst the

hallmarks of cancer with circulating tumor cells being the prime cause in the formation of distal metastasis. Metastasis is the key factor for cancer related deaths. Thus, understanding the mechanism of tumor dissemination is the central factor of cancer research. In cancer patients, the disseminated tumor cells are detectable in the peripheral blood as circulating tumor cells (CTC's) while in the lymph nodes they are detected as disseminated blood cells (DTC's). Hence the identification and characterization of these cells have resulted in perceptions of the molecular mechanisms of metastasis.[179]

Metastatic disease accounts for majority of cancer related deaths. Metastases are believed to develop from dormant circulating tumor cells that are seeded into various organs. The process of metastasis formation involves an invasion-metastasis cascade of events. A stepwise progression is proposed. First, the normal cells undergo genetic alterations leading to the formation of pre-malignant lesions.[180] These steps are clinically recognized. Several types of pre-malignant lesions such as hyperplasia or dysplasia, can be detected in different organs prior to the formation of a malignant tumor. The lesions are caused either by genetic alterations or by an external factor (for example, a virus infection); the former causes a monoclonal expansion of cells, whereas the latter causes a polyclonal expansion of cells. Unknown factors precipitate pre-malignant lesions to develop into cancer. Further progression leads to invasive cancer, with a substantial risk of metastases. [197-198]

A metastatic tumor is composed of cells that are phenotypically different and heterogeneous, when compared to a noninvasive tumor.

They are believed to originate from circulating tumor cells that have left the primary tumor and can reach distal organs through the lymphatic system or via the blood circulation. Once there, they extravasate and invade the parenchyma and form micro-metastasis that can later develop into macroscopic metastasis. Loss of endothelial cell integrity and a selective permeability of the endothelium provides for the transmigration process.[181-184] Several factors including an inflammatory environment are believed to aid in the survival of tumor cells in other organs.

Angiogenesis also provides an exit route for metastatic cells. This is due to the nature of permeable and immature vessels formed within the tumor leading to cells detaching and entering circulation. Thus, a highly vascularized tumor gives rise to more metastasis compared to less vascularized tumors. Butler et al showed that about 2×10^6 mammary carcinoma cells are shed from the primary tumor each day, some of these cells may be capable of giving rise to metastasis. [185]

The most important correlation between angiogenesis and metastasis are the studies on vascular density of the tumor and patient survival. A study by Weinder et. al. proposed a direct link between tumor vascularization and metastasis in breast cancer and suggested that this information can function as an independent prognostic factor for patient outcome. This study was repeated by others and the findings were confirmed and are not limited to breast cancer. Thus, these studies show the importance of vascular density in tumor aggressiveness[184, 186]

The blood system is considered as the main mode for metastatic spread, but increasing evidence shows that the lymphatic system could play an important role in metastasis as well. [187]

The lymphatic capillary is a thin walled structure comprising a single layer of endothelial cells lacking inter-endothelial tight junctions. They do not have smooth muscle cells and a basement membrane as the blood capillaries. The key function of the lymphatic vasculature is to regulate of tissue fluid homeostasis, antigen collection from peripheral tissues, and to mediate immune cells such as antigen-presenting dendritic cells to move from the periphery to lymph nodes. It also provides a one-way transport system that relies on skeletal muscle contraction for the transport of lymph.

Lymphangiogenesis is the process of formation of new lymphatic vessels from preexisting vessels. It plays a significant role in tissue homeostasis. An impaired vessel or excessive formation of the vessel may promote the metastatic process. Generally, lymphatic vessels were thought to be indirect participants in tumor metastasis by providing conduits for tumor cells to transfer into draining lymph nodes, but recently the discovery of numerous key lymphatic-specific molecular markers and an augmented accessibility of *in vitro* and *in vivo* experimental systems to study lymphatic biology have pointed towards a more dynamic role for the

This is a process which occurs in adults only during pathological conditions such as inflammation, tissue repair or tumor growth. Many molecular factors that contribute to lymphangiogenesis have been studied recently, among which VEGF C and D bind to the VEGF-3 receptor expressed on the endothelial cells of the lymphatic vessels and promote this process. [188-190]

More recently, numerous factors with pro lymphangiogenic activity have been recognized. These comprise of hepatocyte growth factor (HGF), which binds to the c-met receptor, angiopoietin-1 together with its endothelial cell-specific receptor Tie-2, FGF1 and -2, PDGF, insulin-like growth factor-1 and -2, and endothelin-1. [191-195]

Tumor-induced lymphangiogenesis is mediated by lymphangiogenic growth factor produced and secreted by the tumors. The role of VEGF-C and VEGF-D in cancer progression has been extensively studied. The overexpression of either of the two factors in tumors results in a significantly amplified tumor-associated lymphatic vessel growth (mainly at the tumor margin) and increased occurrence of lymph node metastasis. The lymphangiogenic growth factors along with increasing vessel density, also enlarge and dilate vessel size. VEGF 2 receptor is important in this process, while VEGF 3 receptor is involved in endothelial cell sprouting.[195-200]

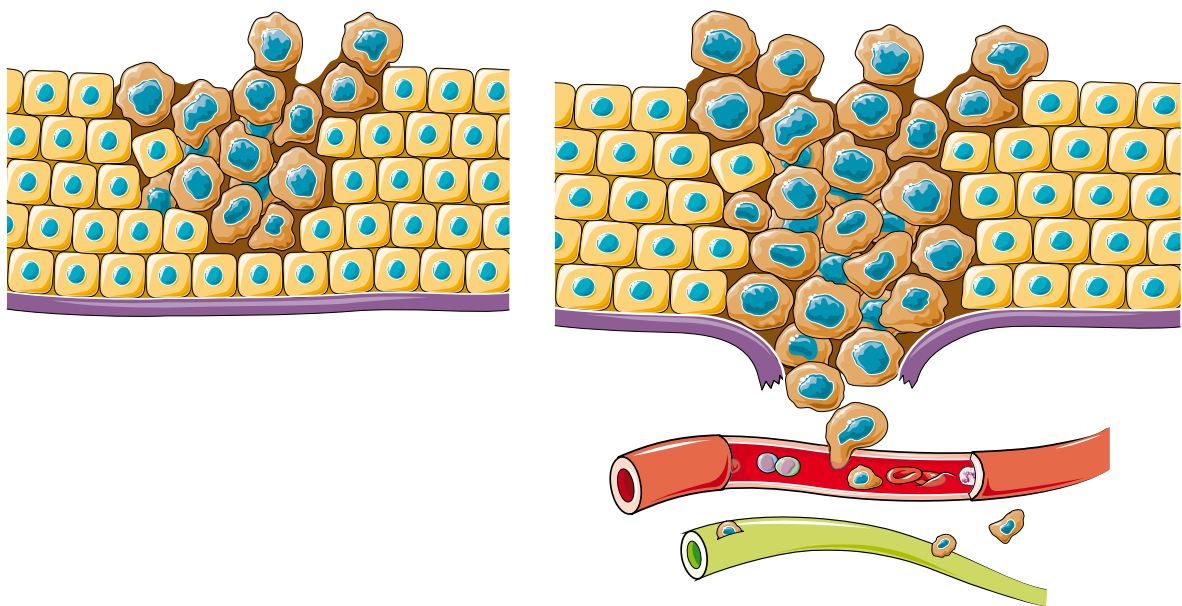


Fig 2. In situ and invasive cancer

1.4.5 Cytomegalovirus and cancer

Emerging evidence indicate that cytomegalovirus (CMV), which is not considered as an oncogenic virus, is highly present in several types of cancer. CMV belongs to the family *Herpesviridae* and the subclass *Betaherpesviridae*. CMV remains latent in the body for the life time of its host after a primary infection without causing any clinical disease in healthy individuals. It can reactivate from time to time, but is kept under control by the immune system. However, in immunocompromised individuals CMV infection may be life threatening; it causes major morbidity and mortality in stem cell and organ transplant patients as well as in AIDS patients. It is also a common cause of birth defects in children who suffer from a congenital infection.

Emerging evidence suggest that the virus is detected in high prevalence in tumors of different origin. Over 90% of glioblastoma, neuroblastoma, medullblastoma, colon, breast and prostate cancers are positive for CMV proteins and nucleic acids. The virus is also detected in lymph node and brain metastases of colon and breast cancer patients. However, it is rarely found in healthy tissue surrounding the primary tumor.

Although HCMV is found in lymphnode and distant metastases, the potential virus related mechanisms of metastasis promotion are not understood [201, 202]

1.4.5.1 HCMV Structure

HCMV is the largest and the most complex amongst the herpesviruses. Its genome is approximately 235 kbp and consists of 252 open reading frames which encodes for 180 proteins. Until recently, it was believed that CMV encodes about 180 proteins which are involved in various biological events that affect the infected cells physiological function. A recent study proposes that this number may surpass 750 proteins, most of which are not vital for virus replication and alternately find implications in different pathologies. Only 50 proteins are essential for viral replication. This indicates that HCMV could in fact be much more complex than previously believed.[203-205]

It consists of an inner core of double stranded DNA surrounded by a nucleocapsid along with a thick layer of tegument protein surrounded a lipid bilayer. HCMV has a typical virion structure of about 200-300 nm in size. The nucleocapsid 125 nm in diameter, which is surrounded by a tegument layer and a lipid envelope. The lipid bilayer consists several viral glycoproteins and glycoprotein complexes that serve important functions in viral entry. The capsid is comprised of five core proteins, namely major capsid protein, minor capsid protein, triplexes, smallest capsid protein and portal protein and minor capsid binding protein. [205]

During the capsid assembly process during viral replication, three diverse types of capsids are observed; - A capsids with only a capsid shell, B capsids with a capsid shell and assembled proteins and and a mature C capsid with a capsid shell containing the viral DNA genome. The third type is the only form that has completed maturation. The first two types of capsids do not contain viral DNA, and hence fail to form mature infectious virions. Alternatively, A or B capsids proceed to envelopment and form a type of virus particles called defective virus particle like or noninfectious virus particles. CMV infected cells also produce dense bodies that do not contain capsids, they only contain proteins, [206]

The nucleocapsid is covered by a thick layer of viral derived proteins called the tegument. The tegument also contains a selection of viral and cellular RNAs. Most of the tegument proteins are phosphorylated, which possibly helps facilitate stable association and incorporation into the virion compartment.[207] The most abundant HCMV tegument proteins are pp65, pp71 and pp150 and they play important roles in un-coating the particle during entry and during virion assembly.

In addition to this they also play important functional roles through modulation of the host immune response to infection, thus providing a favorable intracellular environment for viral replication.[208-210]

Every herpesvirus expresses gB and gH/gL proteins that are essential for viral entry. Studies show that herpesvirus gB molecules are fusion proteins and they are associated with liposomes. Like for other herpesviruses, HCMV gB and gH/gL are important for virus entry. Along with the above proteins, the phospholipid envelope also contains the UL128-131 complex, referred to as the pentameric complex. These glycoproteins, together, play important roles in virus entry into host cells, cell-to-cell spread, and virion maturation [211-215].

1.4.5.2 Entry and Assembly

Different steps are required for initiation of viral entry. and are differently utilized in different cell types. HCMV initiates infection by binding to cell surface heparan sulfate proteoglycans (HSPGs) and this binding is one of the relatively conserved features of herpesvirus entry pathways. HCMV binding to HSPGs is implied to play a vital role in initial stage of entry. This is thought to enhance the attachment to subsequent receptors in a cascade that ultimately leads to fusion [216, 217]. In fibroblasts, the virus attaches to specific cellular receptors and subsequently the viral envelope fuses with the cell membrane followed by the release of nucleocapsids into the cytoplasm. Alternatively, the virus is taken up by receptor mediated endocytosis and fusion of the virus envelope with the endosomal membrane occurs in the cytoplasm.

HCMV can adhere to and penetrate both permissive and non-permissive cell types, but fruitful replication is detected in a very limited range of human cells. This shows that viral gene expression is restricted due to a post penetration block in non-permissive cells [218]

Once the virus fuses with the cell, the nucleocapsid is released in the cytoplasm. After the nucleocapsid is translocated into the nucleus an interaction with nuclear pores occur followed by release of the viral genome into the nucleus.

Following insertion, the viral genes are expressed in a thorough manner. Three classes of proteins are expressed; the Immediate Early (IE), followed by Early (E) and Late (LA) proteins. The IE proteins are the first to be expressed (within 3-4 hours) followed by the other E and L proteins. Once the IE proteins are expressed, these proteins help in regulating the expression of the other genes, acting either as auto stimulators or transactivators. The E and L proteins are required for setting up the structure of the virus particle along with coding for other proteins that modulate host cell functions.[208] The whole infection cycle takes 48-72 hours.[208]

Two major gene products, IE-72 and IE-86, are responsible for regulating the expression of most the HCMV genes. IE-72 is unessential for virus growth whereas IE-86 is required for regulating its DNA replication. Both have an effect on cell cycle regulation through interactions with tumor suppressor proteins, promotion of cell-cycle progression, initiation of DNA synthesis, telomerase activity and apoptosis inhibition [219]

Post replication, the viral DNA is packaged into the synthesized capsid and exported through various cellular compartments. Here it acquires its remaining structural components (the tegument and envelope). Virus budding takes place in Golgi-derived intracellular vacuoles.[220] The mature virions are consequently transported to the plasma membrane, where they are released following fusion of the transport vesicle and the plasma membrane.

1.4.5.3 HCMV latency and reactivation

A property common to all herpesviruses is their ability to establish lifelong persistence in their host following primary infection, but the exact sites and the mechanisms controlling this process is not clearly understood. Analysis of the peripheral blood show that monocytes and CD34 + progenitor cells are the sites that carry the latent virus *in vivo*. [221] In these cells, due to the combined effect of a lack of viral activators, the presence of latency-associated repressors and the dominance of cellular transcriptional repressors of the major immediate early promoter gene (MIEP), the chromatin around the viral MIEP becomes heavily repressive thus suppressing lytic transcription and maintaining latent infection. Another crucial factor playing a role during latency is GATA-2. This transcription factor aids in latency persistence of HCMV in myeloid cells. Subsequently, a pathway of interactions seems to occur during latency. This results in the targeting of cellular hsa-miR92a which in turn, up-regulates cellular GATA-2 expression [222] leading to a downstream influence on latency-associated LUNA gene expression [223] eventually warranting UL138 expression[224]. Studies have shown that expression of the viral transcript UL138 is required for viral latency establishment and

maintenance. This occurs through down regulation of multidrug resistance–associated protein- (MRP1) reduced cellular leukotriene C4 export, which possibly inhibits the migration of infected DCs to draining lymph nodes thus impairing HCMV-specific immune response generation. [225] The mechanism of HCMV reactivation is poorly understood but is known to be triggered by inflammatory cytokines and differentiation of monocytes into macrophages or dendritic cells [226]

Efforts have been made to detect specific latency-associated transcripts in experimental models. It is known that HCMV encodes a viral interleukin (IL)-10 homologue (UL111a; cmvIL-10) in HCMV infected cells which is a well-known immunosuppressive cytokine. During latency, this protein's transcript undergoes alternative splicing, resulting in the expression of latency-associated cmvIL-10. This helps the virus to avoid recognition by immune system and clearance during latency.[227]

1.4.5.4 CMV and angiogenesis

HCMV has been shown to induce angiogenesis, which could be significant for HCMV-enhanced tumor formation. HCMV infects different cell types that are involved in angiogenesis, such as endothelial cells, fibroblasts macrophages and smooth muscle cells. It also promotes angiogenesis through direct and indirect mechanisms. Additionally, infection at sites close to the vessel might promote angiogenesis by releasing angiogenic factors. CMV infection of fibroblasts and EC promotes the synthesis and release of several angiogenic factors that stimulate several stages of angiogenesis. It is known that VEGF is essential for angiogenesis to occur. HCMV drives the production of VEGF through US28 induced COX-2 expression and NF-kB activation. When an angiogenic stimulus occurs, EC's respond by attracting and binding leukocytes and platelets resulting in production and release of various pro-angiogenic factors. It has been shown that CMV infection modifies the types and quantities of these factors. It is called the HCMV secretome. The main components of this secretome are chemokines and cytokines such as CCL 3, 5 7 and CCL 20, CXCL 5 and 16, IL 6, receptors ICAM 1. Also, growth factors such as TGF beta, HGF and ECM modifiers like MMP's are involved.[228-232]

Infection is initiated when HCMV binds to the cell through entry receptors and then enters the cells through fusion at the plasma membrane or via receptor mediated endocytosis (see above). Viral proteins that are present in the envelope can remain on the cell surface after entry and activate the cell. For example, US28 remains on the cell surface after entry and G protein mediated signaling pathways. This further activates transcription factors NF-kB, NFAT, TCF

and LEF. The activity of US28 can further be modulated by chemokines such as CCL2, 5 and CX3CL1. Once the transcription factors are activated, they promote cell proliferation through cyclin D1 angiogenesis through VEGF.[233] US28 induces secretion of cytokines and growth factors such as VEGF , IL-6, which in turn act on their related receptors and induce and autocrine cellular response and activation of STAT-3. This also increases cellular responses of the neighboring cells.

For example, US28 induces prostaglandin production and activates cognate receptors to induce production of VEGF, thus promoting angiogenesis. [234, 235]

1.4.5.5 Tumor dissemination

HCMV is suggested to play a role in tumor dissemination. For example, HCMV infection of neuroblastoma cells leads to enhanced tumor cell adhesion to endothelial cells resulting in the disruption of EC monolayer integrity. This leads to enhanced neuroblastoma invasiveness and higher trans-endothelial migration.in *in vitro* models[236] [237]. Studies also show the role of HCMV in EMT related to metastasis. A study by Shimamura et al examined the role of HCMV in the induction of TGF- β and its role in EMT. Upon infecting human renal tubular epithelial cells *invitro* it was observed that these cells undergo morphological and transcriptional changes closely related to EMT. Also, TGF- β and MMP-2 expression was induced and could further fuel EMT process. HCMV IE proteins was suggested to control this process as their overexpression mimic these effects and as targeting late gene expression did not inhibit these changes.

Several factors involved in EMT are similarly modulated by HCMV. This includes induced production of growth factors and induced signaling pathways. Further studies are required to comprehend the role of HCMV infection in EMT transition and metastasis formation.

In this thesis through study 3 we investigated the role of murine cmv in colon cancer metastasis.

1.5 CANCER IMMUNOLOGY

Cancer cells that exist in the primary site of a tumor are immune protected, cells which exit this site and enter circulation are compromised and vulnerable to immune surveillance, hence the survival of these cells are essential for metastatic spread. Thus, immune escape mechanisms are required for cell survival. [179]

Immune escape mechanisms or immunoediting are important aspects for cancer cell survival. Immunoediting is a process, that comprises of immune surveillance and tumor progression.

It consists of three phases leading to cancer progression. The first phase is called elimination, when neoplastic cells are contained and destroyed by innate and adaptive immune cells.

The second phase equilibrium is attained following an escape of the neoplastic cells from elimination and their interactions with immune cells reach an equilibrium with the immune system exerting a constant pressure. Hence even though the immune system wants to prevent the cells from progressing, it unwillingly contributes to tumor cell survival by selective clonal selection. This leads to the third stage, the escape phase, where the colonies that survived the previous phases, gain the ability to grow in an immune competent environment.[238-240]

Tumors that escape immune evasion acquire resistance to immune factors, for example interferon gamma. Insensitivity to interferon gamma, enhances tumor resistance to immune attacks. Also, tumors evade immune cells either by shedding or restricting presentation of their ligands for recognition by NK cells and cytotoxic T lymphocytes.

The tumors also downregulate other factors that elicit a tumor-immune response, for example pro inflammatory cytokines and chemokines. [241-246]

1.5.1 Immune surveillance

NK cells and macrophages are the most well studied cells with respect to tumor suppression and surveillance. NK cells are an important part of the innate immune system and they play a major role in defense against tumors and viruses. The interactions of these cells with tumors are mediated by a network of receptors and ligands including the major histocompatibility complex (MHC) class 1- related inhibitory molecules. Inhibition of NK cell signaling leads to tumor lysis through cytolytic granules release and apoptosis induction. These effects of the NK cells are decreased in CTC positive patients with metastatic colon and breast cancer compared to CTC negative patients. [246-249]

Macrophages, along with NK cells play an important role in controlling metastatic progression. Macrophages are vital for antibody dependent phagocytosis of tumors. This process is mostly governed by macrophages in the liver. A study by Denève *et al* showed comparisons of CTC counts between peripheral and mesenteric blood samples in patients with colorectal cancer confirmed that a considerable proportion of the viable CTC population seem to be filtered and trapped in the liver. Thus, these findings highlight the importance of the liver microenvironment in mediating the outcome of interactions between tumor cells and immune cells. This often promotes tumor-cell death and sometimes facilitates disseminated tumor cells (DTC) survival and growth.[250-252]

1.5.1.1 Immunoediting

The immunological elimination of tumor antigen is driven by T- cell recognition. Studies have validated this. A study on genetic mapping using a mouse model of a highly immunogenic and unedited sarcoma to determine its mutational landscape demonstrated that cancer immunoediting is the result of a T-cell-dependent immunoselection process which leads to extension of tumor cell clones that lack immunodominant rejection antigens displaying reduced immunogenicity. A study by DuPage et al reached a similar conclusion. Studies also show the involvement of innate immunity in the immunoediting process. NK cells apparently activated by local amplification of endogenous IL-12 can produce IFN- γ that in succession induces activation of M1 macrophages. These act as significant effectors of cancer immunoediting. Thus, these results show that the host's immunocompetence level plays a vital role in the extent to which a tumor undergoes immunoediting.[253, 254]

1.5.1.2 Elimination

Many reviews have described and summarized the mechanisms that take place in the elimination phase. The role of host recognition molecules such as NKG2D; IFN- γ , perforin, effector molecules like Fas/FasL, and TRAIL; and an intact lymphocyte compartment in protective anti-tumor immunity, are well known. Both type I (IFN- α/β) and type II interferons (IFN- γ) are essential for the growth of anti-tumor immune responses but play diverse role in cancer immunoediting processes. While IFN- γ targets both tumor cells and hematopoietic tumor cells, IFN- α/β acts primarily on host cells. Recently, two studies showed that type I IFNs are mandatory for initiating early anti-tumor response and act on CD8 α /CD103⁺ DCs to augment cross-presentation of tumor antigens to CD8⁺ T cells. Type I IFN sensitivity in macrophages, NK cells and granulocytes, all of which express type I IFN receptors was not essential for tumor rejection.[255, 256]

1.5.1.3 Equilibrium

Adaptive Th1-like immunity also plays an important role in the equilibrium phase and studies have described this process. For instance, a study using an immune-mediated dormancy model of fibrosarcoma and another study using the same mouse model of MCA- induced fibrosarcoma and p53 mutant tumors showed that immune-mediated tumor latency may be a very lengthy process and related to the balance of IL-12 promoting elimination, and IL-23 (sharing the common subunit IL-12 (p40)) sustains tumors in equilibrium and promotes persistence. Apart from a trivial tumor-promoting role for IL-10 various other pathways (e.g. IL-4, IL-17A, TNF, IFN- $\alpha\beta$) were shown to be nonessential for this phase.[242, 257]

Tumors that escape go on to metastasize. Specific mechanisms involved in this process are discussed below.

1.5.2 Immune evasion mechanism

As mentioned before, CTCs leaving the immunosuppressive primary tumor microenvironment are exposed to the active immune surveillance mechanisms. In addition, the possibility that CTCs will be lysed by tumor-specific immune cells increases significantly outside the immunosuppressive reserve of the tumor since peripheral immune cells are more abundant than CTCs. Hence the circulatory system is considered a hostile environment for cancer cells. Primary tumors are predicted to shed thousands of cells into the bloodstream every day, but evidently only a very small percentage develop the ability to grow into distant metastases which supports this assumption.

However, studies have identified new pathways through which CTCs might evade or survive encounters with immune cells. The most established mechanism of tumor evasion includes the ones previously described (NK cells and macrophages), CD 47 signaling, FAS/FASL signaling and hypoxia induced apoptosis.[258]

1.5.2.1 MHC molecules and NK -cell ligands

MHC I molecules that expressed on the surface of basically all nucleated cells present peptide epitopes that are processed from intracellular proteins for examination by T cells. Thus, presentation of tumor-associated antigens (TAAs) to T-cell receptors (TCR) in the context of MHC I molecules is crucial for initiation of an adaptive CD8⁺ CTL response. Hence, downregulation or entire loss of MHC I expression at the cell surface is a mechanism used by tumor cells to 'hide' from CTLs and thus avoid death. As a standby to counteract this mechanism, NK cells become activated when MHC I molecules are under expressed or absent and they are activated by the missing 'self-hypothesis'. Therefore, to escape both NK-mediated and CTL-mediated cytotoxicity, CTCs have to find a way to present MHC I molecules without presenting TAAs. or avoid NK cell activation [259-262]

Cytokeratin 8 (CK8), along with its heterodimeric partners CK18 and CK19, have been shown to inhibit MHC I interactions with TCRs on CD8⁺ CTLs. Overexpression of these cytokeratins has long been detected in malignant tissues. This mechanism demonstrates how cancer cells develop new methods of immune evasion, and that interfering with MHC-mediated antigen presentation seem like a critical approach to escape immune recognition.[263, 264]

1.5.2.2 FAS/FASL induced apoptosis

This apoptotic pathway is highly important to immune evasion. The transmembrane receptor FAS can initiate apoptosis, and activation of this receptor on T cells via binding to FASL is a suggested mechanism of tumor-mediated immunosuppression in several malignancies. For example, histopathological analyses have shown that FASL is upregulated in metastases compared to the primary tumor in patients with melanoma or colorectal cancer. [286-287] Also, in patients with breast cancer, upregulation of FASL has been associated with increased apoptosis of T cells. Hence FASL expression on tumor cells may actively induce apoptosis in immune cells. Vice versa, tumor cells that express FAS will most likely be vulnerable to apoptosis evoked by tumor-specific immune cells, which can also express FASL. Hence, instantaneous loss or downregulation of FAS and upregulation of FASL on tumor cells might add to tumor evasion strategies of immune-mediated cytotoxicity and increase the probability for metastatic progression.[265-269]

1.5.2.3 CD47-mediated signaling

Several studies by Irving Weissman and colleagues have emphasized the role of the leukocyte surface antigen CD47 in cancer, mostly, in cancer-cell evasion of phagocytic clearance. CD47 binds to its ligand signal-regulatory protein α (SIRP α , also known as macrophage fusion receptor), expressed on macrophages and dendritic cells, subsequently inhibiting phagocytosis by these cell types. Therefore, upregulation of CD47, an antiphagocytic 'don't eat me' signal, might confer CTCs with a non-immunogenic profile by allowing them to escape the consequences of cell-damage-induced upregulation of pro-phagocytic signals and, thus, the immune sequelae evoked after CTC recognition in the context of adaptive immunity. Steinert *et al* have assessed the gene-expression profiles of primary tumors and CTCs from patients with colorectal cancer. Notably, CD47 was the only gene upregulated in CTCs versus the matched primary tumors, signifying a survival advantage conferred by CD47 expression for peripheral blood CTCs. Therefore, these findings along with others propose that CD47 is part of a potential metastasis-initiator cell signature, but functional analysis is required to describe the exact role of CD47 expression on CTCs.[270-275]

1.5.2.4 Hypoxia mediated immune escape

Various genes are expressed during EMT, in cancer-stem cells, or in response to hypoxia. They have been shown to be upregulated in CTCs, and many of the encoded proteins can control the immune response. Specific metabolic and molecular changes enable CTCs to adapt to and survive in a microenvironment with a lower oxygen concentration. Evidence investigating HIF-1 α expression in CTC's and functional studies of CTC's in the bone marrow of patients with

lung, breast and prostate cancer show that many CTCs and DTCs exhibit a hypoxia-associated phenotype, and that these cells can adapt well to hypoxic condition. For example, upon hypoxic stress, glucose-regulated protein 78 (Grp78) is upregulated in cell lines established from the bone marrow of patients with cancer, and expression of Grp78 is linked with mesenchymal characteristics and poorly differentiated primary breast and lung tumors. Ongoing studies are investigating if adaptation to hypoxia also promotes CTC and/or DTC evasion of immune cells. Nonetheless, the hypoxia-resistant phenotype of DTCs has implications for immunotherapeutic strategies.[276-278]

1.5.2.5 Metastasis promotion by immune cells

Studies show that metastasis can be supported by immune cells. Immune cells can, hence, be regarded as both protagonists and antagonists in the metastatic process.[279-281]

1.5.2.6 Promotion of CTC seeding

Preliminary studies of CTCs have established a positive correlation between an acute inflammatory condition and formation of metastasis in the target organ of metastatic spread.

For instance, Taranova *et al* using an allergic pulmonary inflammation model indicated that for CTCs to extravasate and form tumor filiae. They take advantage of the augmented vascular permeability and expression of adhesion molecules at the site of metastasis formation. The study also demonstrated that for CTC's to metastasize to the lung, they required the presence of CD4⁺ T cells at the site of metastasis. Along these lines, data from a murine colorectal cancer model indicated a positive correlation between CTCs and serum levels of IL-17A, a proinflammatory cytokine. In addition, the presence of IL-17A augmented tumor-cell motility. This occurred by triggering MMP-9 expression in CTCs, hence possibly supporting CTC mobilization and extravasation.[282, 283]

Put together, escape from and variations in peripheral immune responses outside the local tumor milieu, are critical steps in metastases development.

1.6 C/EBPB

C/EBP β is a protein belonging to the C/EBP family. The family overall, consists of six transcription factors from C/EBP α to C/EBP ζ and is characterized by a basic leucine zipper at the C-terminus required for binding and dimerization. The family of proteins regulate different gene expressions taking part in cell differentiation, proliferation inflammation and metabolism.

C/EBP β can form heterodimers with members of the C/EBP family, such as C/EBP α , C/EBP γ and C/EBP δ , along with other transcription factors such as Sp1 or CREB1. C/EBP β can also bind as a homodimer to some DNA regulatory regions thereby controlling the expressions of various target genes.[284, 285]

The C/EBP β gene encodes three different protein isoforms: LAP1, LAP2 (liver- enriched activator proteins) and LIP (liver-enriched inhibitor protein), which are expressed depending on differential use of specific in-frame translation sites. [286]LAP1 and LAP2 are transcriptional activators, and LIP is their inhibitor. The functions of all C/EBP β isoforms are still under investigation and largely dependent on the cellular context.[287]

The mammalian target of rapamycin (mTOR) pathway, tightly regulates the production of C/EBP β isoform. In this pathway, mTOR activation stimulates LIP expression and its inhibition enhances LAP production. Moreover, the truncated LIP isoform forms by partial proteolysis of longer C/EBP β isoforms.[288] [289] All the isoforms contain C-terminal bZIP domain and LAP1 and LAP2 maintain N-terminal transactivation and chromatin remodelling domains. The ratio of C/EBP β isoforms is believed to determine cell fate.[290, 291]

As a transcription factor, C/EBP β interacts with several target genes and is required for a number of biological processes, such as, granulopoiesis, adipogenesis, muscle repair, embryogenesis, and osteoporosis. [292]It is involved in controlling autophagy, cell growth and antibacterial defence, along with regulating insulin level and insulin receptors expression [293-295]C/EBP β is also regulates multiple genes responsible for immune and inflammatory response. Evidence show its binding to cytokine coding genes such as IL-4, IL-6, IL-5 and TNF α . It is also responsible for activation and terminal differentiation of macrophages.[296, 297] Since C/EBP β expression plays a role in astrocyte inflammatory response, its expression is altered in various neurological disorders including Alzheimer's disease, Parkinson's disease and HIV-1-associated dementia[298, 299]

Our studies emphasize the involvement of C/EBP β in breast cancer. In breast cancer, the C/EBP β gene is usually not mutated. A few rare mutations that have been found are questioned regarding their contribution to epithelial tumors. However, C/EBP β expression might be augmented in a small subgroup of breast neoplasia, described as lobular carcinoma in situ. Elevated levels of C/EBP β mRNA are however also linked to metastatic breast cancer, higher tumor grade, and overall worse prognosis.[300-304]

1.6.1 Development of mammary gland

Both LAP1 and LAP2 are expressed in non-malignant, human mammary epithelial cells, as well as in breast tumors. LAP1 is expressed in normal mammary cells, while LAP2 is found only in dividing cells, normal and neoplastic. *In vitro* and *in vivo* studies have showed potential and substantial role of C/EBP β isoforms in mammary gland development and breast cancer.[305-307] For example, *in vitro* studies show that LIP overexpression in mouse fibroblasts or mammary epithelial cells causes increased proliferation, foci formation and lack of contact inhibition. Moreover, transgenic mice with LIP overexpression in the mammary gland develop alveolar hyperplasia, invasive and noninvasive carcinomas and high grade mammary intraepithelial neoplasias. Other studies, using C/EBP β ^{-/-} mice, revealed that C/EBP β is vital for ductal morphogenesis, functional differentiation in the murine mammary gland and epithelial cell proliferation.[308-310]

In this thesis, through study 4, we investigated the role of C/EBP β in breast cancer metastasis.

1.7 CANCER PROGRESSION AND EMT

Epithelial-Mesenchymal transition (EMT) is a process by which epithelial cells transform into mesenchymal cells. This occurs due to the loss of cell polarity and cell-to-cell adhesion molecules. This process is important in wound healing and embryogenesis. The reverse process, mesenchymal epithelial transition (MET) is also essential for various organ developments. It is also known to be involved in cancer progression and metastasis formation. Epithelial cells are single or multilayer cells with various functions.

They depict apical-basal polarity and through specialized intracellular junctions, adhere and communicate with adjacent cells. Their position and interaction of the basement membrane proteins with integrins help define their physiology. The transition of the cells follows certain hallmarks and patterns. The plasticity of the epithelial phenotype enables cell transition through multiple EMT and MET rounds.

EMT increases the invasive phenotype of the cancer cells. They lose their expression cell-cell adhesion molecule, E-cadherin and the attachment to the basement membrane. TGF-beta is a major factor that induces this property in tumor cells when it acts on activated RAS- expressing cells, leading to EMT and inhibition of apoptosis.[311] Evidence suggests that activated platelets have a direct contribution to the invasive phenotype of the cancer cells at the primary tumor site. In breast carcinoma, higher levels of TGF beta 1 and TBR11 can be found [312] The expression of TGF- beta varies with different cell types, thus understanding and

quantifying the process is difficult.[311, 313, 314] In this context it is interesting to note that CMV induces production of TGF-beta and an EMT like process [315]

There are three types of EMT based on the physiological context. Type 1 EMT is the differentiation of epithelial cells to mesenchymal cells with no prior history of transition. Type 2 EMT is a process where cells have already undergone transition followed by reversion and initiation of a new EMT. Type 3 EMT leads to cancer progression and cancer stem cell properties. Similarly, following dissemination, cancer cells revert to epithelial cells through MET and secondary carcinomas are generated having similar phenotypes. A pro invasive function of cancer cells is attributed to the expression of $\alpha\beta3$ integrin that is increased due to EMT.[316, 317]

Various transcription factors like SNAIL, TWIST and ZEB have prominent roles in EMT and cancer progression. They have different profiles and functions based on the cell type in which they are expressed. TWIST 1 down regulates epithelial gene expression and enhances expression of mesenchymal genes. SNAIL 1 and 2 has a similar role. Other transcription factors such as forkhead box (FOX) and GATA family and growth factors such as VEGF and FGF are also involved in EMT regulation.[311, 313]

1.8 RED BLOOD CELL DISTRIBUTION WIDTH (RDW)- A PROGNOSTIC FACTOR FOR PATIENTS WITH CARDIAC DISEASES AND CANCER

Red blood cell distribution width is the uneven size of the red blood cells with a higher RDW than normal. It is usually denoted in combination with red blood cell corpuscular volume in diagnosis of chronic inflammatory status in the body. Anisocytosis is a RDW higher than the normal range and is commonly found in anemia and other blood disorders.

1.8.1 Red blood cell distribution width and CVD

Besides many blood disorders, RWD levels are associated with acute and chronic cardiovascular diseases such as peripheral artery disease, acute coronary syndrome and ischemic cerebrovascular disease.[318] Many studies have shown an interesting relationship between carotid atherosclerosis or stroke[319, 320]. Wen et al. observed an intricate connection between high RDW and advanced subclinical atherosclerosis such as an upsurge in intimal - medial thickness and evidence of carotid plaques.[321] Sánchez-Chaparro *et al.* showed that high RDW is associated with metabolic syndrome (MetS) a condition that encompasses various risk factors for cardiovascular diseases.[322]

A reason for a consistent increase in RDW in CVD is attributed to active stimulation of erythropoiesis by erythropoietin (EPO) a hormone, secreted during hypoxic conditions. This promotes the release of enflamed RBCs from the bone marrow. Another hypothesis for high RBC could be due to a minor reduction in RBC turnover. Since the size of RBCs gradually decreases with cell ageing, a diminished rate of RBC turnover would allow minor cells to continue longer in circulation.[323]

1.8.2 Red blood cell distribution width and cancer- A risk factor for death

Recent studies indicate that anisocytosis is a prognostic indicator of death in various atherosclerotic related diseases. It is considered as a marker for inflammation in various diseases including cancer. Research shows that cancer anemia and high RDW have a connection, both are dependent on VEGF A. [324-327]

Cancer is attributed to increased inflammation and studies show that RDW is increased in damaged cardio metabolic functions and active inflammation. [327-328] Very limited studies show that solid tumors activate inflammation.

Two studies reported the use of RDW as an added parameter in detection of anemia in colon cancer patients. Spell et al showed that the value of RDW could be a useful marker with 14, 8% cut-off value, 72% sensitivity and 69% specificity to differentiate between malignant and benign reasons leading biliary obstruction. Similarly, Ays et al reported that RDW increased in malignant lesions compared with benign breast tissue but no cut off value was suggested. Their study concluded that RDW is an interesting parameter and increases in colon cancer before anemia develops. [324-327] In this thesis through study 2 we investigated the role of anisocytosis measured by red distribution width

1.9 CVD CO-MORBIDITY IN CANCER

Post-secondary malignancies, CVD is the foremost cause of morbidity and mortality among cancer survivors[328]. CVD risk factors are predominant in cancer patients. A study by Mertens et al showed that among childhood cancer survivors, cardiovascular events are the principal non-malignant cause of death. It is responsible for a higher risk of death, about 7-fold high, among these patients when compared to their controls. Side effects of cardiotoxic cancer therapy is thought to be the fundamental cause for this observation. [329]Alternatively, a study by Enright and Krzyzanowska reiterated the necessity for precise individualized cardiovascular disease prevention program for cancer survivors. They showed that the subpar control provided

for traditional measure of risk factors like cholesterol monitoring and blood pressure among survivors. [330]

Recent developments indicate an increasing interest in a theory for development of CVD in cancer patients. This is the multiple-hit hypothesis. Jones et al. first defined this in 2007. He proposed that CVD development in cancer patients takes place when they are exposed to a series of chronological or simultaneous events that together make them more susceptible to cardiovascular reserves and ultimately result in death. Another risk factor for development of CVD is psychological distress in non-cancer populations. Put together the multiple-hit hypothesis has been well conceived.[253]

Cancer treatments include chemo, radiation, immuno or hormone targeted therapies or a combination of these. Some amongst them are cardiotoxic. For instance, for lymphoma or breast cancer treatments, chemotherapy with anthracyclines as well as radiation therapy to the chest are cardiotoxic. These can lead to a reduction in cardiovascular reserves and eventually different sets of CVDs ranging from benign to possibly fatal conditions. [331]CVDs associated with cancer treatment can occur within a few days, months or years. They include arrhythmias, myocardial infarction, thrombosis, congestive heart failure and cardiomyopathy.[332, 333]

Lifestyle factors also contribute to process. Due to cancer treatments patients might develop an unhealthy life style which includes physical inactivity and weight loss. This might lead to a reduction of CV reserves and augments CVD risk and death.

Psychological distress another important risk factor, whose presence is a bad for health outcome of patients. Independent of traditional biomedical risk factors, depression symptoms, fatigue and anxiety have shown to forecast CVD onset and prognosis in patients with established CVD. [334, 335]This was validated based on a meta-analysis of 20 studies, which showed the value of anxiety prediction for coronary heart disease occurrence in formerly healthy individuals. It showed that there is 26% higher risk of coronary heart disease development and a 48% increase in risk of cardiac death among anxious individuals.[336]

Thus, research on an adapted multiple-hit hypothesis for CVD development among cancer patients could contribute to advances regarding their care. An immediate necessity for CVD preventive procedures to reduce the delayed adverse effects of cancer therapies such as radiation and chemotherapy and early intervention could possibly help improve CVD's risk profiles.[337]

1.10 CVD AND CANCER INDUCED THROMBOSIS

The endothelium plays an essential role in the hemostatic system. Depending on precise tissue requirements and local stresses, endothelial cells can induce either antithrombotic or pro-thrombotic events. An efficient endothelium is crucial to maintain hemostasis and avoid thrombosis. Normal endothelial cells express antiplatelet and anticoagulant agents. They avert platelet aggregation and fibrin formation, respectively by releasing pro-fibrinolytic agents that initiate fibrinolysis to destroy the clot. During endothelial dysfunction, endothelial cells activate fibrin formation, along with platelet adhesion and aggregation. Thrombosis is the formation of a clot within a blood vessel resulting in a reduction of blood flow to distal tissue and organs. This limits the delivery of nutrients and oxygen and results in localized tissue and organ necrosis. Large occlusive clots (thrombi) break off and embolize forming secondary thrombi in distal locations. This is known as thromboembolism and can lead to various local or chronic disorders. For instance, acute arterial thrombosis is activated when an atherosclerotic plaque ruptures, and is the major cause of myocardial infarction and stroke[338]. Likewise, venous thromboembolism can be activated by distressed blood flow, endothelial activation, hypercoagulable conditions, such as procoagulant changes in the blood. Similarly, the process is also induced in cancer. A surface receptor thrombomodulin, is known to be involved in cancer development, coagulation and inflammation. The receptor is present plentifully in several types of endothelial cells but is poorly expressed or absent in brain microvascular and liver sinusoidal endothelial cells. Studies have also shown that the expression of tissue factor induces tumor angiogenesis in colon and breast cancer models through TF-fVIIa-dependent PAR-2 activation. This induces the expression of VEGF, IL-8, MMP-7, and CXCL-1.[339]

In this thesis through study 2 we investigated the role anisocytosis in determining blood flow along the vessel wall, its interactions, culminating in thrombosis in different vascular diseases and cancer.

2 AIMS OF THE THESIS

The general aim of the thesis was to study the implications of a damaged endothelium in pathologies related to vascular disease and cancer metastases

The specific aims were:

- To identify subtypes of bone marrow cells contributing towards intimal hyperplasia and tissue repair.
- To investigate the role of high RDW modulation of blood fluid dynamics related to CVD.
- To study the role of MCMV in colon cancer development and progression.
- To investigate C/EBP β 's role in metastasis formation of breast cancer and its effect on vascular morphology.

3 RESULTS AND DISCUSSION

Study 1:

Intimal hyperplasia is the response to an injured endothelium. It is also the reason for lumen narrowing. This process occurs in response to transplantation, artery bypass conduits and post balloon angioplasty. The aim of our study was to examine the contribution of various sub populations of BMCs contributing towards intima hyperplasia. We also examined if these cells play a key role in the process and constitute a developed intimal lesion or if they are involved only at the initial stages in arterial remodelling. We introduced carotid artery ligation in a chimeric mouse model involving bone marrow from GFP transgenic mice. Arterial injury was carried out in chimeric mice (C57BL/6 mice with BM from GFP transgenic mice) to examine if BM-derived cells migrated towards sites of arterial injury. This allowed for investigating the existence of green GFP-positive BM-derived cells in the injured arterial wall using confocal microscopy. Briefly, the left common carotid artery was exposed through a small midline incision in the neck. The artery was ligated close to the carotid bifurcation to interrupt blood flow. One-week post injury a 5-mm segment of the left carotid close to the suture was removed for further studies.

One-week post ligation, we observed GFP + BMCs at the site of the arterial injury. We also employed a mouse model of balloon angioplasty with a consequent delivery of BMCs with their distinct phenotypes to the site of injury. Here we observed BMCs at initial stages of the intimal formation but their number was continuously decreasing over time, they were 10 folds lower at day 14 compared to day 1. This indicated that the BM derived cells were involved at the early stages of arterial healing and disappeared later potentially due to apoptosis or low survival rate of these cells in the vessel wall. By confocal microscopy we also showed that BMCs migrated to the sites of vascular injury and they inhibited the initial stages of intimal hyperplasia by 1.4-fold.

Intriguingly, these cells expressed EC and SMC markers both *in vitro* in the BMC cell cultures and *in vivo* in the intima. This suggests that the BMCs are involved only in the initial stages and suggest that other cellular components apart from BMCs contribute towards the development of intimal lesions.

Asahara et al first showed EPC's involvement in neo angiogenesis. New blood vessel formation in injured areas was induced by Sca-1 and CD34 positive cell populations and led to ischemic tissue recovery in mice models of ischemic injury. Studies also showed a

reendothelialization of injured vessels in a rat balloon injury model. Furthermore, it has also been shown that EPCs derived from the host reconstructed the endothelial layer in a model of vessel graft atherosclerosis.[340, 341] Put together these data support the present hypothesis that EPCs reside in tissue resident niches as well as in the bone marrow [342-344] and that they are able to reconstitute the damaged endothelium. Endothelial progenitor cells are cells with an exclusive ability to differentiate into functional endothelial cells. Generally circulating EPCs have been characterized by the expression of CD34, CD133 and the vascular endothelial growth factor receptor-2 (VEGFR2). Recent studies have also shown some CD14⁺/low myeloid subsets as functional endothelial precursors. These have additionally been shown as functional angiogenic cells, which contribute to endothelial repair and ischemic or tumor angiogenesis [341, 345, 346]

In this thesis, we investigated the role of the endothelial constituent in BMCs contributing to arterial remodeling post injury. The first step was to find out which populations of BM derived cells were important for hyperplastic intimal formation. For this purpose, we isolated BMCs derived from GFP transgenic mice for diverse populations of, monocytic/macrophage origin (CD14), and endothelial origin (CD34, VEGFR- 2) and lymphocytic origin (CD3). These cells were then delivered locally at the site of an injured artery. Briefly, the right femoral artery was exposed up till the bifurcation through a transabdominal incision followed by placement of micro clamps on the lower aorta, left iliac artery, and the distal part of the right femoral artery. We then introduced a 2-F Fogarty balloon catheter (Baxter, Deerfield, IL) into the right femoral artery, which we inflated, and withdrew three times with rotation. Inflation was performed through the cannula and 1 ml of Ringer's solution with free discharge through a micro incision. BMCs from GFP mice in RPMI medium were imbued and incubated in the freshly injured arterial wall of C57BL/6 or C57BL/6 Nude mice for 15 to 20 minutes and blood flow was reestablished upon removal of the micro clamps. Arteries were collected at 1, 2, 7 and 14 days' post-surgery for further investigations.

From this process, we observed that the main phenotype of BM cells contributing to initial stage intimal formation are cells expressing markers for ECs (CD34 and VEGFR-2).

Additionally, our results for the first time indicate that BMCs did not fuse with vascular SMCs. Also, BM derived cells weren't equally distributed in the intima, but rather concentrated in thicker areas of the intima where they mainly expressed SMC markers. To show this, we cultured adventitial cells co-expressing Sca-1/CCR2 or c-kit/CCR2 in the presence of PDGF-BB for 7 days and then stained them for SM- α -actin. Remarkably, both cell phenotypes could differentiate into cells of SMC phenotype in the presence of

stimulating factors, such as PDGF-BB. Thus, as mentioned before, BM-derived cells are not a major cellular constituent of developed intimal lesion and are rather involved in early stage restoration and protective functions of intimal formation.

Concluding, we propose that BM-derived cells play role in healing and are of high importance in the initial stages of intimal formation. We also suggest that other sources of vascular progenitor cells apart from bone marrow are likely the major composition of the developed intimal lesions. Additional studies are needed to better define the phenotype of BM-derived cells and their progenitors and characterize the specific function in the injured vasculature.

Study 2:

Red blood cell distribution width (RDW) refers to the unequal size of red blood cells. RDW reflects the variation in size of red blood cells. RDW is generally reported as part of a complete blood count. [389]. An RDW higher than the normal range is termed anisocytosis. Anisocytosis has traditionally been used in combination with the RBC corpuscular volume, to diagnose chronic inflammatory status in the body [390] and is commonly related to chronic inflammatory diseases such as rheumatic arthritis, inflammatory bowel diseases, and cancer.

Recent studies have demonstrated that cancer anaemia and high RDW are related and dependent on VEGF A [391]. Studies have also shown that high RDW is a predictor of death in various CVDs such as peripheral artery disease, acute coronary syndrome and ischemic cerebrovascular disease. A study on a large cohort of patients with stroke, myocardial infarction, and peripheral vascular disease and confirmed that high RDW is a predictor for mortality amongst these patients. [392] Also, anisocytosis has been described as a strong predictor for adverse events related to diabetes, stroke, peripheral vascular disease, and myocardial infarction and post clinical intervention. In our study, we hypothesized that RDW is not just a factor predicting for rapid progression of cardiovascular disease but rather a factor that directly affects blood flow and its interaction with the vessel wall. In healthy individuals, erythrocytes are of the same size and shape and in flowing blood there is a lack of direct interaction between the blood cells and vessel wall. We suggest that anisocytosis leads to changes in blood flow and affects the interaction between blood cells and the vascular endothelium. Specifically, we hypothesized that anisocytosis is directly connected to thrombotic episodes and to rapid development of atherosclerosis with secondary development of cardiac infarction and stroke due to changes in blood flow.

To study this in more depth, we first determined the transcriptomic background of atherosclerosis and its associated pathways using microarray analysis of 12 carotid plaques derived from 6 patients with low / normal RDW value of < 16 and from 6 patients with abnormal parameter value of >16 . We selected and focused on 39 characteristic genes with a 2-fold increase or decrease related to atherosclerosis. These genes were also involved in cytokine/chemokine, FGF or EGF-mediated signaling pathways and connected to angiogenesis, blood coagulation, inflammation and cell adhesion. Our results also showed a positive correlation between elevated expression of FGF1, GAB1, SERPINE1, PLAT, PDGFD, PDGFC and high RDW value. Inversely, these genes were downregulated in plaques from patients with low RDW value. We also observed a decrease in the expression of some genes such as VCAM1, EIF5A, ICAM1, ICAM2, PPARG, which were associated with low RDW as compared to the high-RDW group of patients with increased expression of these genes. These differences were confirmed on protein level by immunohistochemistry.

Studies show that RBCs tend to concentrate at the core during the blood flow in a vessel. In our study, we observed a similar distribution along the vessel radius, obtained through simulations using the Lattice Boltzmann method which was employed to solve for fluid flow consisting of 5-15 % haematocrit [393]. The haematocrit played a vital role in determining to what extent the RBCs migrated towards the centre leaving a region close to the channel wall free from RBCs, the RBC-depleted region. The haematocrit declines with increasing volume fraction of RBC and appears to be less dependent on bulk flow velocities. Thrombocytes on the other hand, behave contrary to the RBCs. They tend to aggregate at the vessel wall in the RBC depleted region due to radial migration because of RBC induced transport due to shear stress. Thrombocyte morphology is another factor that influences the rate at which thrombocytes migrate towards the vessel. [394, 395]

Anisocytosis, however has never been considered a factor for the above phenomenon. One study pointed out that thrombocyte migration could be related to the volume excluded in the bulk flow for the platelets to be located in. This is because when the space between two RBCs are smaller than the size of the thrombocytes, the thrombocytes cannot reside in this volume. Accordingly, anisocytosis will influence the motion and migration of RBCs as well as platelets as different shapes and sizes of RBCs will lead to a different dynamic behaviour in the bulk flow. The flow is also affected by the size of the RBC-depleted region along with the frequency of collisions between RBCs and thrombocytes in the near-wall region. [396] [397] [398,399]

We next assessed the blood flow at the carotid artery and its bifurcation using mice models. We used 6 different models where high RDW was induced through different conditions. We triggered inflammation-dependent anisocytosis in mice through injection of CT 26 colon tumor cells that resulted in high RDW within 2 weeks. The second group of mice received erythropoietin intravenously for 4 subsequent days. The third group of mice were subjected to a low iron diet for 4 weeks and had 0.6 mL of blood drawn once a week, for a period of 3 weeks. This triggered anaemia and induced inflammation-independent anisocytosis. The other experimental groups comprised of the transgenic mice commercially acquired from Jackson laboratory that exhibited abnormalities related to red blood cell production and RDW. Finally, the control mice constituted healthy, untreated animals. The data was acquired using a Varian 9.4T MRI scanner with a 30cm bore. We used digital image processing techniques to isolate blood flow information by applying an integral analysis on time varying flow field. Our results showed that the healthy mice have a higher volumetric blood flow rate in the external arteries compared to transgenic, tumorous, anaemic, and EPO- treated mice. The flow field of healthy mice was parabolic in shape, indicating that turbulence is likely of secondary importance and the shape of the flow field in the external arteries for EPO treated mice was found to vary the least over the cardiac cycle(s), indicating a change in blood rheology. This could largely be because of blood cell concentration, shape and distribution close to the artery wall and far away[347]. Due to the complex nature of blood and the diversity in the cases considered in our study, it was difficult to pinpoint factors governing variations in the flow profiles. Hence, the shape of the blood flow profile was discussed in terms of two common profiles seen in fluid mechanics: parabolic, and plug flow (i.e. pluggish in shape). This is attributed to turbulent flow, that is pluggish in shape. The control cases are less pluggish in shape than the anaemic, EPO, transgenic, and tumorous mice models, indicating that turbulence due to fluctuations is of secondary importance.

The role of hemodynamic forces was first proposed with observations of initial atherosclerotic lesions typically at arterial bends and branches with a disrupted flow. This flow pattern included recirculation eddies and change in direction with respect to space (reattachment and flow separation) and time (reciprocating flow).[39-42] Recent research also shows that this kind of flow and the associated reciprocating and low shear stress bring about a constant activation of several atherogenic genes in ECs. Through our studies, we found that changes in morphology of red blood cells is related to the size and shape of the cells. These are direct factors that change blood flow and the mode of interactions of cellular components of blood with the vascular wall.

We also observed that a high RDW is a predictive factor for interaction between cellular components of blood and the vascular wall. These interactions can lead to increased inflammation in the vessels and initiation of thrombosis.

Study 3:

Cytomegalovirus (CMV) is a virus that remains latent in the body after a primary infection. Studies have shown its potential involvement in several types of cancer. CMV belongs to the family Herpesviridae and the subclass Betaherpesviridae. Emerging evidence indicates that CMV, which is not considered as an oncogenic virus, is highly present in several types of cancer. For instance, over 90% of glioblastoma, neuroblastoma, medulloblastoma, colon, breast and prostate cancers are positive for CMV proteins and nucleic acids. The viral proteins are also detected in lymph node and brain metastases of colon and breast cancer patients. However, it is rarely found in healthy tissue surrounding the primary tumor[348, 349]. Although HCMV is found in lymph node and distant metastases, the potential virus related

mechanisms of metastasis promotion are not understood. [221, 222] Through this study, we investigated the role of murine CMV (MCMV) on the progression of colon cancer using a combination of *in vivo* and *in vitro* approaches. *In vitro*, we verified that MCMV could infect CT26 murine colon cancer cells and that the cells express immediate early (IE) proteins. We found that the percentage of cells expressing IE increased in a dose dependent manner with increasing MOI's. 12 – 20 % of the cells expressed IE proteins at 3 days' post infection, as determined by immunofluorescence staining and flow cytometry, respectively. The IE proteins are regulatory proteins that are expressed at a very early stage in the virus cycle. They regulate the expression of other viral and cellular genes and are important in mediating tumor transformation. For instance, studies show that IE1 induces high telomerase activity by interacting with SP1 binding sites in the hTERT promoter, a key step in tumor transformation.[350] The IE 72 gene also promotes proliferation of dormant cells by alleviating the repression of transcription factor p 107 which in turn activates E2F promoters. IE2-86 can also drive cells into S phase and prevent cellular DNA synthesis. This can possibly occur through interaction with minichromosome maintenance 3 (MCM3)-associated protein (MCM3AP).[351]

We next assessed the effect of MCMV infection on cell proliferation. Cells were infected with MCMV at M.O. I's 0.5, 1 and 5, and were assessed for cell proliferation on days 1, 3 and 6-post infection. We observed that MCMV infected cells proliferated at a lower rate compared to the uninfected cells. HCMV is known to arrest the cell cycle and block DNA

replication. Studies have shown that HCMV halts the cell cycle in G1 phase followed by a block in replication of cellular and viral DNA. IE72 blocks the cell cycle in G2/M phase while IE86 can block the cell cycle at the G/S boundary.[352, 353] Hence the decrease in cellular proliferation *in vitro* is consistent with the effects the virus has on the host cell. We then assessed the effect of MCMV infection on invasion and observed that the percentage of infected cells that invaded and migrated through a porous membrane was significantly higher compared to the non-infected cells. These results indicated a possible enhanced invasive capacity of CT 26 tumor cells.

Next, we used an animal model of colon cancer and examined the effect of MCMV infection on tumor growth, metastatic potential and tumor characteristics. Three groups were assessed the first group (MCMV infected mice) were injected with 200 μ l of MCMV intraperitoneally. Three days later CT26 colon cancer cells (3×10^6 / mouse) were subcutaneously implanted on the flank of balb/c mice. In the second group (MCMV infected cells), we infected CT26 cancer cells (3×10^6) with an MOI of 0.5 (virus titer 1.8×10^6) three days prior to implantation in mice. The cells contained a mixture of uninfected and infected cells as (ratio 0.25:0.75- Infected – Uninfected). The third group served as the control group; mice were injected with the same number of uninfected CT26 cells into the mice as both the other groups. We assessed tumor growth over a period of two weeks (measured on alternate days starting on day 5) in all the animals. We found that the control group and the group infected with MCMV had similar tumor growth rates, while the MCMV infected cells group had no visible tumors after 15 days. Thus, MCMV did not enhance tumor growth at the injection site

We then quantified the number of circulating tumor cells (CTCs) in the bone marrow and found that this number was significantly lower in the infected groups (MCMV infected mice and MCMV infected cells) compared to the control group. However, when we quantified the number of metastatic loci in the lungs, we found a significant increase of micro metastasis in the infected groups compared to control mice. In agreement with this observation, *in vitro* infection demonstrated increased ability of infected cells to invade and migrate. A probable explanation is that the migratory cells from infected mice are capable of rapidly colonizing metastatic niches and spend little time in the circulation. This could be due to CMV affecting the cadherin function, which *in vivo* may lead to induction of trans-endothelial migration, and possibly to metastasis. Also, CMV infection leads to enhanced invasiveness of the infected cells, for instance, CMV infected neuroblastoma cells exhibit increased invasive capacity mediated via VLA-5 $\alpha 1 \beta 5$ integrin along with neural adhesion

cell molecule (CD56). [349]The viral receptor M33, the murine counterpart of the HCMV US28 receptor stimulates cell migration through a ligand independent process. [252]

From our animal models, we observed that while the infected cells did not give rise to tumors, the uninfected and systemically infected mice grew tumors at similar rates. We could not detect viral particles in tumors using quantitative PCR, IHC on tumor tissues. However, they formed significantly increased number of micro metastases in the lung. It is possible that the expression levels were much lower than the sensitivity of the methods used. Another possible explanation is that CMV modulated cancer through a hit and run mechanism, as has been described by other investigators.

We characterized the tumor vasculature to assess differences in tumors from infected and uninfected models by immunohistochemical staining for smooth muscle alpha (SMA) and lectin. We found a significant increase in the expression of the SMA protein in the infected group compared to the uninfected group of mice whereas expression of lectin was not significantly different. We also assessed the presence of CD45+ leukocytes and CD68+ macrophages and found a significant increase in the presence of both these cell types in the infected group compared to the control which indicate an enhance immune response to the virus.

We further conducted an RNA array analysis on 5 samples from each group collected post the metastatic study. The micro-array data highlighted changes in expression of angiogenic and inflammatory cytokines along with several chemokines and apoptotic factors which were upregulated in the infected groups compared with the controls. Surprisingly, the family of proteins that was mostly upregulated in tumors from infected mice were cytokeratins. By immunostaining, we verified that cytokeratin expression was upregulated also at protein level in the tumors. Hence, we hypothesized that MCMV infection leads to upregulation of cytokeratins (CK) and results in metastasis.

To evaluate this, further we conducted an RNA array analysis on CT26 cells that were infected in vitro. While we found an upregulation of several angiogenic factors and inflammatory cytokines in the MCMV infected CT26 cells in vitro, we did not observe a direct upregulation of any of the cytokeratins that were observed to be affected in the MCMV infected mice. Instead, the virus appears to induce high cytokeratin expression by indirect effects. To assess the association of CMV related induced CK expression in tumor samples from patients, we studied tissue micro array (TMAs) tumor samples from colon cancer patients. Tissue sample sections were stained for HCMV IEA, pp65 and LA proteins and CK 1, 2 and 14.

All the samples were graded as negative, low and high expression. We found a strong association between the level of expression of CKs and CMV proteins. These observations indicate that the virus induces cytokeratin expression via indirect mechanisms that may involve the immune response against the virus *in vivo*. We found no direct evidence of virus induced expression of these *in vitro* Cytokeratins have been utilized as tumor markers for many decades.[354] Recent studies have shed light into their role during progression to metastasis. For example, Keratin 14 as a key regulator of metastasis; polyclonal breast cancer metastasis arises from collective dissemination of these K-14+ tumor clusters. The study revealed systematic changes in the relative proportions of K14⁺ and K14⁻ cells from micrometastatic to macrometastatic stages. Keratin was also shown to be involved in salivary adenoid cystic carcinoma, where elevated levels of keratin expression were associated with bad prognosis. The study suggested that K14 expression enabled collective cancer cell invasion. Another study reported the involvement of keratin in metastasis of lung carcinoma. The study showed that in lung squamous cell carcinoma(LSCC), CK 14 was expressed in the tumor cell nests showing stromal invasion with fibrosis and lymph node metastases. This indicated its involvement in proliferation and metastasis of LSCC[355]

In agreement with this observation, we also propose the involvement of cytokeratins in cancer metastasis. The metastases of infected animals (brain and lungs, data now shown) had highly enhanced expression levels of cytokeratins.

In conclusion, our data suggests that *in vitro* CMV promotes invasion and migration of CT 26 murine colon cancer cells, while CMV does not affect tumor growth but increases incidence of metastasis in the lungs. We also found through RNA array analysis that cytokeratins 1, 2 and 14 are upregulated between sixty to hundred times in the infected models compared to the uninfected. Collectively, through our study we suggest that CMV may promote colon cancer metastasis through a cytokeratin mediated pathway.

Study 4:

The CCAAT-enhancer binding protein β (C/EBP β) is a transcription factor that plays a key role in the development of the mammary gland and it is also involved in breast cancer progression. *In vitro* and *in vivo* studies have showed a potential substantial role of C/EBP β isoforms in mammary gland development and breast cancer.[305-307] Studies, using C/EBP β -/- mice, revealed that C/EBP β is vital for ductal morphogenesis, functional differentiation in the murine mammary gland and epithelial cell proliferation.[308-310]

A previous study by our group showed that loss of C/EBP β enhances the metastatic spread of mouse mammary tumor cells but did not elucidate the mechanism behind this observation. Therefore, through this study, we intended to further explain link between C/EBP β and metastasis formation along with defining the relationship between C/EBP β and survival of breast cancer patients.

To test the hypothesis that C/EBP β is involved in breast cancer progression, we first analysed a tissue microarray containing 137 breast cancer patient samples that were immunohistochemically stained for C/EBP β . This was followed by a qualitative scoring of C/EBP β staining in relationship to breast cancer progression. All samples were graded on 0-2 based on expression and analysed by a pathologist. We observed the presence of a strong nuclear positive staining in the normal breast tissue and in some ductal cancer in situ (DCIS) cases. However, the expression of C/EBP β was lower in DCIS compared to the expression in normal breast tissue. In many nodules, the expression was typically present in the basal layer surrounded by desmoplastic (growth of connective / fibrous tissue) stroma with lymphocyte infiltration. Thus, the intensity of C/EBP β staining was reduced in case of inflammation combined with desmoplastic reaction. Reduced C/EBP β staining was strongly evident mainly in areas of micro invasive cancer. Thus, our results indicate that C/EBP β may play a role in breast cancer progression and its association to inflammation.

We next assessed the relation of C/EBP β expression to overall survival of patients using Cox proportional hazards and Kaplan-Meier analysis in univariate and multivariate analysis. The samples were adjusted for established prognostic factors. Two factors, tumor size and C/EBP β expression were independent predictors of overall survival (OS). Lack of C/EBP β expression was associated with shorter OS of breast cancer patients compared to higher C/EBP β expression. These results show that C/EBP β expression is associated significantly with OS, and relapse-free survival (RFS).

We used a 4T1 mouse model to study the effect of C/EBP β on breast cancer. We implanted two groups of 4T1 cells– one referred to as sh Control that expressed wild type C/EBP β and the second one -sh C/EBP β , with silenced expression of C/EBP β . These cell lines were implanted into two groups of isogenic BALB/c mice. We measured, tumor growth, CTC dissemination and metastasis formation between the groups. We found that loss of C/EBP β expression influenced tumor growth and morphology. C/EBP β -silenced tumors were smaller compared to the non-silenced tumors, despite no difference in proliferation rate assessed by the *in vitro* assay. These results indicate that C/EBP β does not directly control tumor growth, but is rather affected by other *in vivo* factors. Furthermore, we observed a striking

morphological difference between the tumors. The group with the wildtype C/EBP β grew tumors that were characterized by a large central necrosis, whereas the C/EBP β -silenced group grew solid tumors with an expansive growth pattern and inflammation surrounding them. This observation can be explained by the former study where we showed the involvement of the C/EBP family in vessel formation, here C/EBP δ was shown to regulate VEGF C autocrine signaling in lymphangiogenesis in lung cancer.[357]

To further characterize the effect of C/EBP β on the tumor, tumor vasculature was primarily analyzed by immunohistochemical staining for endothelial cell marker CD31. We observed an increase in the number of vessels in C/EBP β -silenced tumors compared to the wildtype. We next stained for both CD31 and a pericyte marker NG2. The results were analysed using Visiopharm software. Upon evaluating features such as vessel length, area, number of branchpoints, pericyte coverage of the vessels no significant difference was found between the two groups.

To further study the effect of C/EBP β on metastasis formation, we focused on the presence of CTCs in the bone marrow and the blood along with the metastatic nodules in distant organs. We labelled cells with GFP for this purpose. No significant differences were observed in terms of CTCs in the blood and bone marrow between the groups. We next evaluated if C/EBP β knockdown affected metastatic spread by analysing the lungs of tumor-bearing mice. Our results indicated that the ratio of mice with lung metastasis/without metastasis was significantly higher in the sh C/EBP β group than the controls. This indicated that loss of C/EBP β promotes metastatic spread of mammary 4T1 tumors. Furthermore, morphological analysis through H & E staining indicated evident chronic inflammation in lungs of mice with C/EBP β knockdown tumors compared to lungs of C/EBP β expressing mice. This observation agrees with the fact that C/EBP β affects development of an inflammatory process. This was also confirmed in the TMA analysis, where inflammation was more prominent in tumors with lower C/EBP β expression

To further study the mechanism of how C/EBP β affects formation of metastasis, we conducted a microarray analysis using RNA extracts from 4T1 cells expressing C/EBP β shRNA and control shRNA. The analysis divulged 559 statistically different sets of genes between sh control and sh C/EBP β cells. Analysis done using Panther database service showed that the group of genes most significantly changed amongst them were genes related to inflammation. Representative genes included MHCII α , MHCII β and HLACII γ . Chemokines such as CCL5, CCL7 and CCL8 were also among the highly-affected genes.

To see if a similar pattern is observed in the tumor, we also performed a microarray analysis on the tumors dissected from mice, using RNA extracts from 4T1 tumors expressing C/EBP β shRNA and control shRNA. In this setup, 135 genes were upregulated by more than 1.3-fold. Upon analyzing the results further, we observed that inflammation group of genes were the most representative affected. Most of the genes are yet to be characterized and CD3⁺ lymphocytes were first on the list with difference equal 5 times fold. These results clearly demonstrate that C/EBP β is involved in alteration of an immunological response in the tumor by directly regulating MHCII expression and indirectly amassing CD3⁺ lymphocytes in the tumor.

To confirm this observation, we stained sh C/EBP β tumors and sh control tumors were stained for CD3, CD45, CD4 and MHCII. The results indicated that C/EBP β inhibition led to an increased expression of MHCII followed by an accumulation of CD45, CD3 and CD4-positive lymphocytes in the tumors. This was by far, the most significant difference observed between the tumors from morphological, microarray data and immunohistochemical analysis. Inflammation is known to play an important role in cancer progression. Tumors can induce T-cell tolerance against tumor antigens, resulting in an immunosuppressive environment that is highly advantageous for the tumor development. For instance, extreme changes in myelopoiesis brought about by tumor growth resulted in recruitment of myeloid-derived suppressor cells. The immunoregulatory activity of these cells depend on C/EBP β . [358]

A probable connection between C/EBP β , inflammation and metastasis can be provided through chemokines. Numerous studies demonstrated that tumor-associated chemokines and their receptors have an important role in shaping the metastatic process of cancer cells. Also, chemokines are involved in inflammation regulation within tumors and stop the host's immune system from rejecting cancer. In this study, interestingly, both chemokines such as CCL2 (MCP-1) or CCL5 have already been linked to breast cancer metastasis. CCL2, produced by stromal tumor cells, is involved in promotion of lung metastasis of 4T1 cells. [359] Alternatively, CCL5/CCR5 promotes invasion and metastasis in the aggressive, basal subtype of breast cancer. [360]

In summary, we found that C/EBP β is a predictor for overall survival in breast cancer patients, and that lower C/EBP β expression affects tumor growth, morphology and lung metastasis formation in murine 4T1 breast cancer model.

4 CONCLUSIONS

In this thesis, we have focused on understanding factors contributing towards intimal formation post vascular injury, anisocytosis as a factor affecting blood flow in the vascular endothelium and factors contributing towards cancer metastasis.

- Through study 1, we suggest that bone marrow derived cells provide reparative function in the initial stages of arterial injury and were subsequently eliminated probably through apoptosis or had a low survival rate in the endothelium. The BM derived cells localized in the intima and most of them expressed endothelial cell markers indicating their endothelial phenotype although their distribution was unequal. In the areas where the intima was thicker the cells expressed smooth muscle cell actin and myosin. Moreover, the bone marrow derived cells did not fuse with the intima. We observed that the local delivery of BM- derived endothelial cells to the sites of arterial injury resulted in a 1.4-fold reduction in the intimal lesions area. These results show that these cells are a key factor in the inhibition of intimal formation and likely play role in healing. The results also suggest that the BM derived ECs are of high importance in the initial stages of intimal formation and put forth the idea that other sources of vascular progenitor cells apart from bone marrow are likely the major composition of the developed intimal lesions. These results define the role of these cells in the development of intimal lesions post vascular injury and this information contributes to the existing understanding of the pathogenesis of intimal hyperplasia. Nonetheless, additional studies are needed in both cell culture systems and translational animal models to better define the phenotype of BM-derived endothelial cells and their progenitor cells, and characterize the specific function of these cells in the injured vasculature.

- Recent research show that disturbed blood flow and the associated reciprocating and low shear stress bring about a constant activation of several atherogenic genes in ECs and contribute towards development of atherosclerotic lesions. RDW might be another factor that contributes to disturbed blood flow and is considered a risk factor for death in patients with cancer and CVD. Through our study, by using blood flow in micro -channels and analysis with optical coherence tomography, we have, for the first time, shown that changes in morphology of red blood cells is related to the size and shape of the cells and that these are direct factors that change blood flow and the mode of interactions of cellular components of blood with the vascular wall. We also observed that RDW is a predictive factor for interaction between cellular components of blood and vascular wall. These interactions can lead to increased inflammation in the vessels and thrombosis initiation.

Therefore, anisocytosis measured by RDW may be a useful predictive factor for patients with vascular diseases.

-Cancer metastasis is a leading cause of death worldwide despite improvements in diagnosis, adjuvant therapies and surgical techniques. An aberrant vasculature (tumor vasculature) leads to metastasis. Through study 3 and 4 we investigated factors contributing towards metastasis.

Through study 3 we investigated the role of murine CMV in colon cancer metastasis. We found that *in vitro*, MCMV promoted the migration and invasive ability of CT26 colon cancer cells. *In vivo*, MCMV increased the expression smooth muscle alpha actin and lectin and significantly increased the metastatic potential of CT26 cells. RNA array analysis indicated that MCMV infection increased the expression of high molecular weight cytokeratin in tumors along with various chemokines and angiogenic factors. Analysis of human colon cancer specimens confirmed a significant association between CMV protein expression and cytokeratin 1, 2 and 14 expressions. Collectively our data suggest that CMV may promote colon cancer metastasis through a cytokeratin mediated pathway. The mechanism warrants further investigation. Understanding this mechanism could provide new therapeutic advances for colon cancer patients.

-Finally, through study 4, our data suggests that C/EBP β is a predictor for overall survival in breast cancer patients, and that lower C/EBP β expression affects tumor growth, morphology and lung metastasis formation in murine 4T1 breast cancer model. The mechanism is related to immunological response, and occurs through activation of MHCII and recruitment of CD4+ lymphocytes mediated by C/EBP β . Our results suggest that C/EBP β may be a new predictive factor for OS of breast cancer patients and provides new insights on C/EBP β 's role in breast cancer progression.

In summary, this work contributes to broadening the knowledge related to implications of a damaged vasculature in different pathologies in vascular disease and cancer and could provide new breakthroughs in therapies for patients.

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