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**RADIATION-INDUCED
VASCULAR INFLAMMATION
- TRANSLATIONAL STUDIES**

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Radiation-induced vascular inflammation
- translational studies
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*“Live as if you were to die tomorrow
Learn as if you were to live forever”*

Mahatma Gandhi

To my family

ABSTRACT

Radiotherapy has been shown to increase the risk for localized cardiovascular disease in a growing population of cancer survivors. However, irradiation, as a risk factor for vascular complications in free flap surgery, has been debated. Furthermore, the mechanisms behind radiation-induced vascular disease are not fully understood, and there is yet no available targeted treatment.

We investigated vascular complications in preoperatively irradiated microvascular reconstructions and the vascular inflammatory response in the human blood vessels and evaluated if interleukin-1 blockade could ameliorate radiation-induced vascular inflammation in Apoe^{-/-} mice.

Paper I is a retrospective cohort study supporting that radiotherapy increases the risk for flap failure in microvascular autologous reconstructive surgery. There was a lower surgical complication rate in reconstructions performed at less than 6 weeks compared to delayed reconstructions performed 6-15 weeks after the last radiotherapy session.

Paper II-IV are experimental studies analyzing gene and protein expression patterns in human-irradiated blood vessels. Irradiated and non-irradiated biopsies were collected from the same patient at the same time during microvascular reconstructive surgery and analyzed pairwise.

Paper II shows that radiotherapy induced vascular inflammation in both arteries and veins years after last radiotherapy treatment as measured by pentraxin 3 (PTX3). Irradiation induced PTX3 expression in endothelial cells, smooth muscle cells and macrophages in the arterial vessel wall.

Paper III demonstrates the involvement of the pro-inflammatory 5-LO/leukotriene axis and vasa vasorum expansion together with macrophage accumulation in the adventitia of irradiated human arteries.

Paper IV shows an up-regulation of the NLRP3 inflammasome/IL-1 β axis and macrophage accumulation in irradiated human arteries. Treatment with the recombinant IL-1Ra anakinra dampened the radiation-induced inflammatory response in locally irradiated Apoe^{-/-} mice.

In conclusion, we demonstrated that irradiation induces an inflammatory response in human blood vessels that may contribute to the observed vascular complications after free flap transfer in irradiated subjects. Chronic vascular inflammation was seen in all layers of irradiated human arteries, and anti-IL-1 may be a potential treatment based on our animal study. However, further studies are needed before an intervention could be tested in a cancer setting.

LIST OF SCIENTIFIC PAPERS

- I. Tall J, **Björklund T** **Christersdottir**, Skogh AC, Arnander C, Halle M. Vascular complications after radiotherapy in head and neck free flap reconstruction: clinical outcome related to vascular biology. *Annals of Plastic Surgery*. 2015;3(309-15).

- II. **Christersdottir** **Björklund T**, Reilly SJ, Gahm C, Bottazzi B, Mantovani A, Tornvall P, Halle M. Increased long-term expression of pentraxin 3 in irradiated human arteries and veins compared to internal controls from free tissue. *Journal of Translational Medicine*. 2013;11(223).

- III. Halle M, **Christersdottir T**, Bäck M. Chronic adventitial inflammation, vasa vasorum expansion, and 5-lipoxygenase up-regulation in irradiated arteries from cancer survivors. *The FASEB Journal*. 2016;11(3845-52).

- IV. **Christersdottir T**, Pirault J, Gisterå A, Bergman O, Baumgartner R, Lundberg AM, Eriksson P, Yan ZQ, Paulsson-Berne G, Hansson GK, Olofsson PS, Halle M. Prevention of radiotherapy-induced arterial inflammation by IL-1 blockade. Accepted 11 February 2019 for publication in *European Heart Journal*¹.

Note: The two first authors contributed equally to the work in Paper IV.

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LIST OF RELATED PUBLICATIONS

Eken SM, **Christersdottir T**, Winski G, Sangsuwan T, Jin H, Chernogubova E, Pirault J, Sun C, Simon N, Winter H, Haghdoost S, Hansson GK, Halle M, Maegdefessel L. miR-29b mediates the chronic inflammatory response in radiotherapy-induced vascular disease. *JACC: Basic to Translational Science*. 2019 Feb 25;4(1):72-82.

Note: The two last authors contributed equally to the above manuscript.

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LIST OF ABBREVIATIONS

Apoe-/-	Apolipoprotein E knock-out
APC	Antigen presenting cell
BioGRID	The biological general repository for interaction datasets
BiRKA	Biobank of radiated tissues at Karolinska
BLT ₁	Leukotriene B ₄ receptor 1
CANTOS	The Canakinumab Antiinflammatory Thrombosis Outcome Study
CASP	Caspase
CCL	Chemokine C-C motif ligand
CD	Cluster of Differentiation
CRP	C-reactive protein
Ctl	Non-irradiated control
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
Gy	Gray
i.p. inj.	Intraperitoneal injection
IHC	Immunohistochemistry
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
KEGG	The Kyoto Encyclopedia of Genes and Genomes
LO	Lipoxygenase
LT	Leukotriene
MCP	Monocyte chemoattractant protein
MHC class	Major histocompatibility complex class
MI	Myocardial infarction
miR-29b	microRNA-29b
MSigDB	Molecular Signature DataBase

NaCl	Sodium chloride
NF- κ B	Nuclear factor kappa B
NLRP3	Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3 or NOD-like receptor family, pyrin domain containing 3
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular pattern
PGK	Phosphoglycerate kinase
PRR/PRM	Pattern-recognition receptor/molecule
PTX	Pentraxin
qRT-PCR	Real-time polymerase chain reaction (Taqman®)
RANTES	Regulated on activation, normal T cell expressed and secreted
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
SMC	Smooth muscle cell
TF	Tissue factor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
VCAM1	Vascular cell adhesion molecule-1
VTE	Venous thromboembolism
vWF	von Willebrand factor
XRT	Radiotherapy/radiation/x-ray therapy

1 INTRODUCTION

1.1 The vasculature

1.1.1 The normal vascular wall

The vascular system plays an important role in human health and disease. In order to understand the pathogenesis behind cardiovascular disease (CVD), it is important to comprehend the structure of the normal vascular wall. The vascular system consists of different types of blood vessels such as arteries, veins and capillaries. The normal vascular wall of all types of blood vessels shares similarities but also have important differences in function and morphology (1).

This subsection gives a brief overview of the vessel wall layers but also the vessel embryology with a focus on human characteristics. The vascular system develops early during embryogenesis in order to support tissue growth by transporting essential products necessary for vessel growth. The blood vessels adapt and adjust during development to fit the anatomical and physiological needs of surrounding tissues and target organs (2). During the initial phase, the three germ layers (i.e., endoderm, ectoderm and mesoderm) of the embryo are established. The mesoderm is the origin of the heart and vascular system of the limbs and trunk. The vasculature develops through activation of both angiogenesis and vasculogenesis. The mesoderm evolves into a three-branched aorta. The first branch is the *dorsal* aortic branch, which distributes vessels to the brain, back muscles and limbs. The *dorsal* aortic branch together with the *ventral* aortic branch form the aortic arch and the carotid arteries. The second branch is the *lateral* aortic branch that covers the retroperitoneal organs. The third branch is the *ventral* aortic branch, which supplies the intestines with vasculature (3-6). The signaling system responsible for the development of the vascular system is complex, and it is not fully understood, but several different growth factors are known to be involved (7-10).

The normal vascular wall of arteries and veins consists of three layers being the tunica intima, tunica media and the adventitia (Figure 1). Capillaries differ from arteries and veins as their tunica intima is covered by pericytes only (1). This thesis will focus on conduit blood vessels and capillaries will not be discussed in detail. A monolayer of endothelial cells (EC) covers the most inner side of the normal vessel wall of all blood vessels (11). The inner luminal layer, also named the tunica intima, exposes the vascular wall to the circulation and its content and therefore plays an important function in cell recruitment and hemostasis (12). The second layer is named the tunica media. Smooth muscle cells (SMCs) are the most common cell type in the normal tunica media of arteries and veins, but the volume and type differ between vessels. In the large elastic arteries, such as the aorta, there are multiple layers of circular SMCs, whereas conduit arteries and arterioles contain less SMC layers. The veins and venules have a thin tunica media in comparison to size-matched arteries. The SMCs in the tunica media layer play an important part in maintaining the vascular tone (13). All blood vessels, excluding the capillaries, have an additional outer layer, the tunica adventitia. The

adventitia consists of fibroblasts, resident macrophages, a collagen-rich extracellular matrix and progenitor cells (1, 13). Larger blood vessels such as elastic arteries and the vena cavae have their own network of small blood vessels for additional blood supply called the vasa vasorum, which is the Latin term for “vessels of vessels” (14, 15). The vasa vasorum is distributed within the adventitial layer in large- and medium-sized blood vessels, and in some vessel types, the vasa vasorum is present in the media layer. The distribution of vasa vasorum depends on vascular type, size and vessel wall thickness (15, 16). The vasa vasorum is more common in veins than arteries (15). The vasa vasorum enters either from the luminal site (internal) or adventitial layer (external) in order to function as an entrance for the circulation to the outer layers of the vascular wall (14). In the large elastic artery aorta, the vasa vasorum distribution changes with size, and the abdominal aorta has a thinner wall with no vasa vasorum below the arterial renalis (17). The vasa vasorum is able to dilate, constrict and increase in number as other small vessels of the vascular system (14).

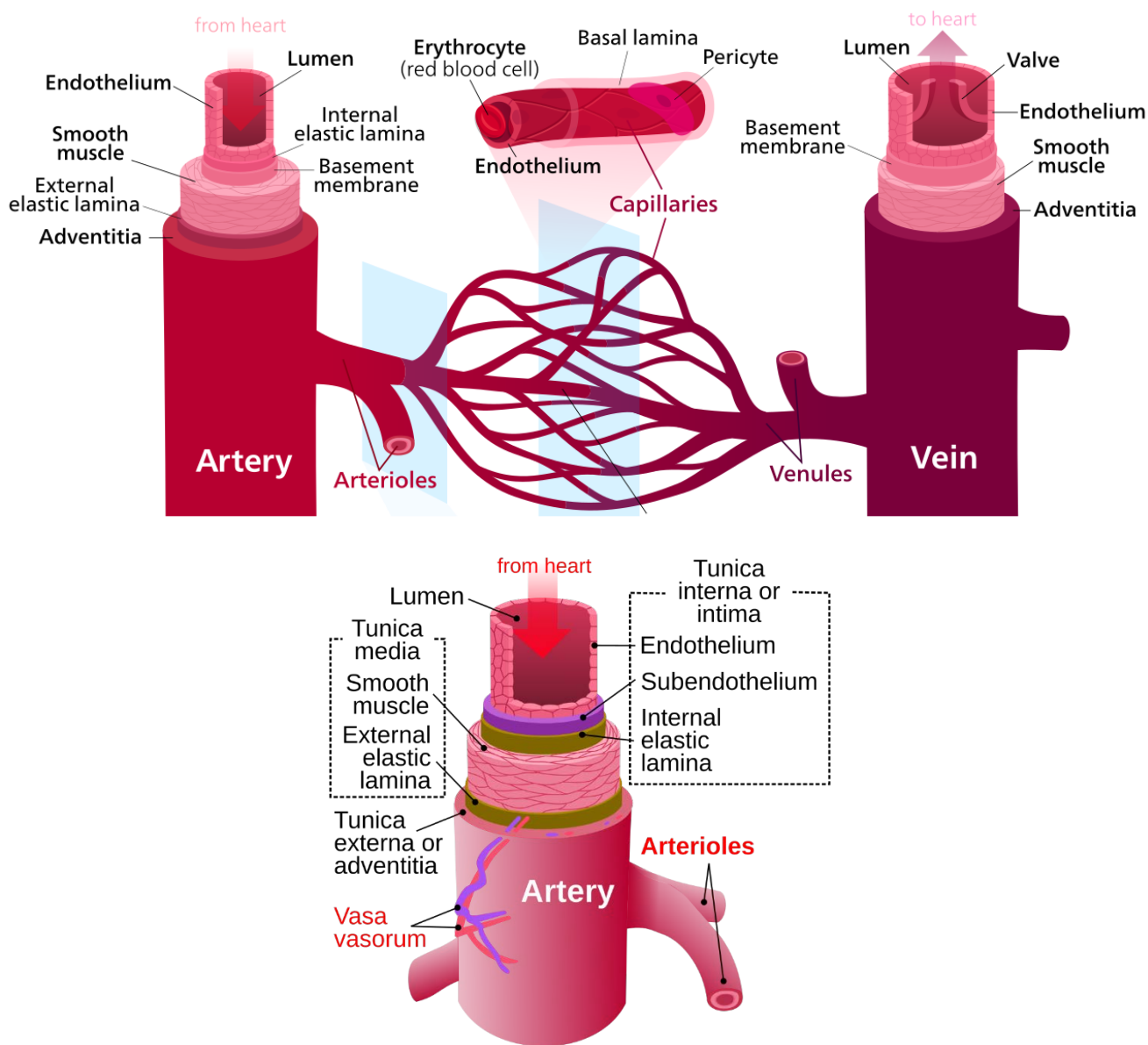


Figure 1. An overview of the different vessel types and layers. The figure blood vessels is available via license: CC by 3.0

1.1.2 Function and morphology in arteries and veins

The endothelial layer covers the luminal site of the vascular system including arteries and veins. However, there are morphological and functional differences in the endothelial cells depending on sites in the vasculature. The main function of the ECs in post-capillary venules are leukocyte trafficking, while in arteries, it is vascular tone (18). Veins have several properties that facilitate recruitment of cells such as lower flow velocity, thinner walls and looser tight junctions in comparison to arteries. In the event of inflammation, veins are the primary site of leukocyte recruitment (19, 20). The potential to induce vascular tone in veins is limited due to their reduced amount of SMCs in the tunica media. Furthermore, is the vascular tone capabilities less in veins compared to arteries due to the lack of stabilizing an internal and external elastic lamina in veins together with a less organized tunica media. Veins have valves to reduce back flow and are able to store larger volumes of circulating blood than arteries. The adventitia represents approximately 50% of the vascular wall thickness in arteries, while it represents the largest part of the vascular wall in veins (18).

1.1.3 Microvascular reconstructive surgery after cancer resection

Transplantation of healthy tissues from a donor site to a defected recipient site for reconstruction purposes is named *microvascular free tissue transfer*, also called *free flap surgery* or *autologous tissue transfer* (Figure 2). In order for the transferred tissue to survive, the circulation needs to be restored by vessel anastomoses. The donor blood vessels are sutured to the recipient blood vessels under the microscope or through loop magnification (21). The first microvascular free tissue transfer was performed in 1971 by McLean and Buncke (22). However, microvascular surgery was not considered a standard procedure reconstructive plastic surgery until the 1980s. Microvascular autologous free flap surgery for cancer reconstruction is mainly used for breast cancer together with head and neck cancer patients (22). The main indication for microvascular free tissue transfer surgery is a large defect that is not possible to cover with a local flap.

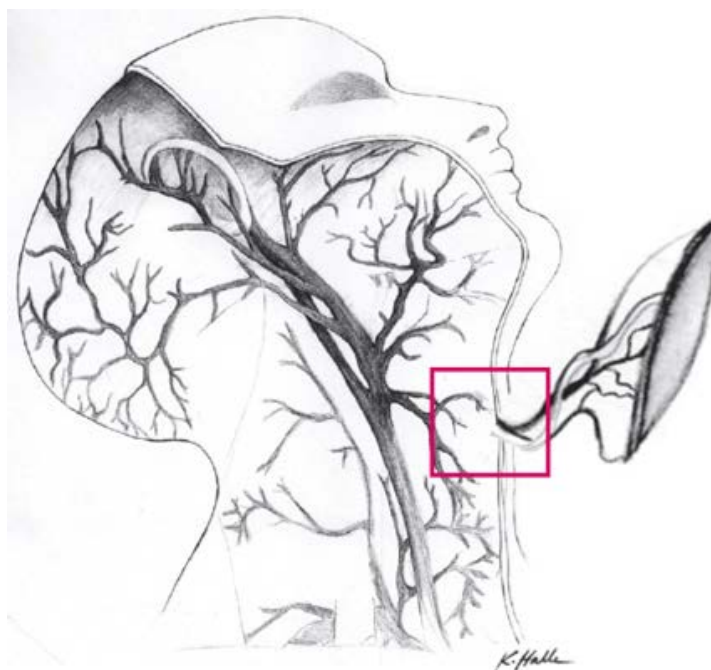


Figure 2. Illustrative figure of autologous free tissue transfer. Illustration by Kim Halle.

Head and neck cancers are most commonly squamous cell carcinomas originating from the tongue, lip, oral cavity, oropharynx, hypopharynx, nasopharynx and larynx. Therefore, resections of head and neck tumors often leave large defects in locations with high functional requirements and often require reconstructive surgery in order to restore function and esthetics (21). Microvascular free tissue transfer surgery is today considered the golden standard for reconstructions after head and neck tumor resections (23). The most common free flaps for head and neck reconstructions are the radial forearm flap, the fibular flap and the anterolateral thigh flap (23). A minimum of one vein and one artery is prepared at respective sites and thereafter connected through microsurgical anastomoses (Figure 3) (23). The vessel diameters within microvascular free tissue surgery usually range from 1-3 mm (21). The free flap vessel diameter is comparable to the size of the left anterior descending coronary artery (3-4 mm) and the middle cerebral artery of the brain (2.5-4 mm), which are both well-known locations for arterial occlusion with subsequent myocardial infarction (MI) and ischemic stroke, respectively (24). Before a connecting vessel ends at an anastomosis, it needs to be cleanly cut, and the otherwise discarded piece has thus been collected, saved in the Biobank of radiated tissues at Karolinska (BiRKA) (25) and used in the current thesis. One often used instrument during surgery is the double clamp. The double clamp plays an important role in the maintaining of hemostasis, low vessel tension and the enabling of vessel alignment in the anastomosis, which all simplifies suturing. A single vessel clamp can be used to isolate the flap from the systemic circulation in order to enable the administration of intravascular drugs without systemic effects. For example, the clamp can be used to treat a thrombosed free flap with thrombolytic agents during salvage surgery. In theory, treatment with a thrombolytic agent during salvage surgery could improve flap salvage rates. However, the clinical evidence for this is inconclusive (26, 27).

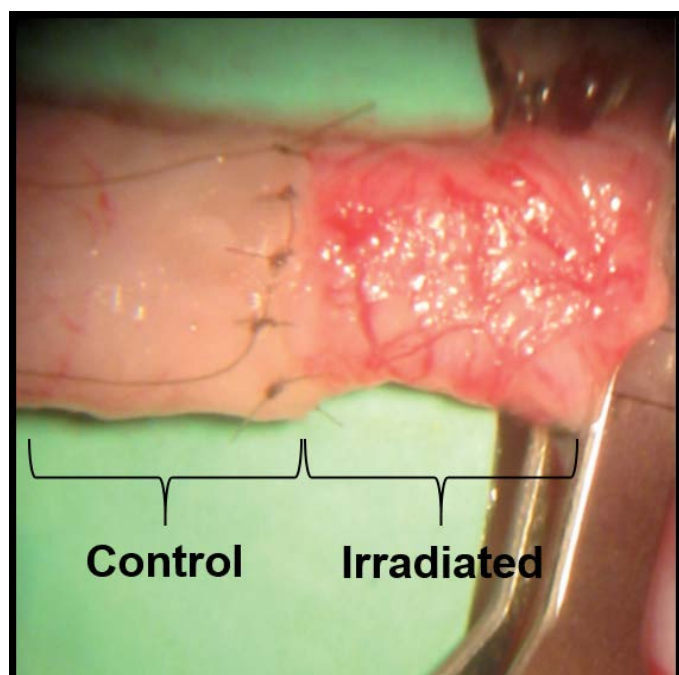


Figure 3. A picture of a microvascular anastomosis observed in an operation microscope. To the left, the non-irradiated donor vessel and to the right the irradiated recipient vessel. Adapted and reprinted with permission from FASEB J (**Paper III**).

1.2 Vascular inflammation

The vasculature is of great importance in the inflammatory response by functioning as a transport system of immune cells and other components of the immune system to the site of infection or damage. Furthermore, vascular cells can activate and induce immune responses with subsequent recruitment of immune cells (28). The traditional cardinal signs of inflammation, first described by Celsius, are calor, dolor, rubor and tumour (29), and all are highly related to vascular changes. The immune system has also been linked to inflammatory diseases, when the system fails to distinguish between self and non-self or when the balance between an adequate inflammatory response and resolution is disturbed. Several diseases have been associated with vascular inflammation, i.e., atherosclerosis, vasculitis and venous thromboembolism (VTE) (30-33). Inflammation can be divided into acute and chronic inflammation. Acute inflammation is transient and mediated through granulocytes and resolves in the case of stimulus removal. Chronic inflammation is primarily mediated by monocytes/macrophages and lymphocytes. Chronic inflammation induces tissue damage, neovascularisation, fibrosis and impaired inflammatory resolution regardless of elimination of initial stimuli (34, 35).

1.2.1 The innate and adaptive immune system

The immune system plays a major role in tissue repair, tumor surveillance and host defense (36-38). In the event of infection or host cell injury, the immune system triggers an inflammatory response in order to fight and clear threats. After a successful clearance, when the immune response is no longer needed, the inflammation is resolved (29, 38). The immune system is often divided into two parts being the innate and the adaptive immune systems. The innate immune defense system is constantly ready and identifies common molecular patterns of pathogens, foreign materials and damage cells allowing for an unspecific and fast immune response.

The innate immune system is divided into a cell-mediated arm that contains mainly granulocytes, monocytes and macrophages and a humoral arm including circulating components of the complement system and soluble pattern-recognition receptors/molecules (PRRs/PRMs). PRRs are crucial for the innate immune systems recognition of pathogen-associated molecular patterns (PAMPs) and debris from damaged or dying host cells termed damage-associated molecular patterns (DAMPs) and subsequent activation (39-41). PRRs are also expressed on innate immune cells as part of the innate cell-mediated response. PRRs have been shown to play a part in atherosclerosis development by ligation to self-antigens and oxidized-low density lipoprotein particles (42).

The adaptive immune defense is partly activated by the innate immune system and is therefore slower, but the communication between the two systems enables a more specific and adaptive response. T- and B-lymphocytes together with antibodies are the main components of the adaptive immune system (43, 44). Antibodies are part of the humoral arm

of the adaptive immune system. Macrophages, dendritic cells and B cells are the primary antigen presenting cells (APC) of the innate immune system and responsible for cell-to-cell communication with the adaptive immune system. APCs are able to present internalized antigens through the peptide-major histocompatibility complex (MHC) II complexes on the cell surface allowing for CD4+ T-lymphocyte recognition and activation of the adaptive immune system. In humans, there are three different MHCII molecules (i.e., HLA-DP, HLA-DQ and HLA-DR), and their mouse equivalents are H2-M, H2-IA, H2-IE (45).

1.2.2 Vascular inflammation in cardiovascular disease

The main cause of CVD is atherosclerosis. Atherosclerosis has been described as a chronic inflammatory disease, because increased plasma levels of C-reactive protein (CRP) are an independent risk factor for future CVD (46-48). The role of inflammation in CVD progression has recently been supported by Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) (49-52). In addition, several inflammatory diseases have been associated with an increased risk for CVD (53, 54).

Atherosclerosis is a slowly progressive systemic disease engaging the whole vessel wall of large- and medium-sized arteries and characterized by chronic inflammation and lipid accumulation (55). Acute manifestations of atherosclerosis by plaque rupture or erosion with subsequent thrombus formation can obstruct the vessel lumen leading to MI or ischemic stroke (55, 56). There are different plaque phenotypes where the vulnerable plaques prone to rupture are characterized by a large lipid core, a thin fibrous cap and infiltration of inflammatory cells that are mainly macrophages (57, 58). Macrophages weaken the fibrous cap by secretion of proteases, stimulate immune cell recruitment by cytokine production and promote thrombosis by expression of tissue factor (TF) (55, 59, 60). Monocytes and macrophages are the major cell population in atherosclerotic plaques (61), and they are important players in atherosclerosis progression due to their ability to express PRRs (62). Tissue-resident macrophages are able to produce interleukin (IL)-1, IL-6, tumour necrosis factor alpha (TNF α) and chemokines in response to tissue injury. Cytokines, such as TNF α , IL-1 and chemokines, are all downstream signals of Nuclear factor kappa-B (NF- κ B) activation and are involved in ECs activation (63), monocyte and neutrophil recruitment and extravasation to the lesion site (64). Activated ECs express adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and P- and E-selectins that promote monocyte and neutrophil rolling and adherence to the vascular wall (65-69). Furthermore, cell transmigration into the vessel wall occurs in response to locally produced chemoattractants such as the C-C motif Chemokine Ligand (CCL) 5 and CCL2 also known as monocyte chemoattractant protein 1 (MCP-1) (70). Monocytes are then differentiated into active phagocytic macrophages by the macrophage colony-stimulating factor and the granulocyte-macrophage colony-stimulating factor (71). Activated macrophages are able to further promote production of pro-inflammatory mediators

that could induce further atherosclerosis formation with later stenosis or plaque rupture with subsequent clinical event such as MI (33).

1.2.2.1 Pentraxins

Pentraxins are multimeric soluble PRRs that, together with components of the complement system, are part of the humoral arm of the innate immune system (72-74). Pentraxins primarily bind to DAMP/PAMP and subsequently to soluble pattern recognition components of the complement system, antigen-antibody complexes and other PRRs-presenting cells in order to facilitate phagocytosis and thus promote clearance of foreign and damaged host material (75-83). In addition, pentraxin 3 (PTX3) plays a part in tissue repair by promoting an appropriate balanced remodeling process by interaction with both a fibrin matrix and plasminogen (84).

Pentraxins are divided into short and long pentraxins according to structural differences in their subunits (85). The most known pentraxin is the short CRP (73, 86), which is used daily in the clinic as a plasma biomarker for inflammatory and infection states such as CVD and sepsis (87). CRP is produced by the liver in response to IL-6 (73, 86). The CRP promoter does not have a clear binding site for NF- κ B. Despite the lack of a binding site on the CRP promoter, NF- κ B could induce CRP expression through indirect channels. CRP is an acute phase protein and can therefore rapidly accumulate in plasma from baseline levels of 1-2 mg/L up to 1000-fold in 48-50 hours following the event of severe inflammation (73). The other short pentraxin called serum amyloid P component (SAP) shares structural similarities with CRP, however, it is not an acute phase protein in humans. CRP is not well-preserved from mice to humans and does not act as an acute phase protein in mice. Instead, SAP has been widely used to study acute phase proteins in mice, where it shares similarities with human CRP.

In recent years, there has been an increasing interest in the long PTX3, an acute phase protein locally produced by several cell types in the vessel wall, i.e., ECs, in response to IL-1 and TNF α (88), SMCs, macrophages, monocytes and fibroblasts in response to TNF α (88-91). The PTX3 promoter contains a binding site for NF- κ B, and NF- κ B is therefore essential for PTX3 transcription in response to pro-inflammatory cytokines such TNF α and IL-1 β (92). In contrast to CRP, PTX3 is well preserved from mice to humans, which makes it an excellent candidate to study in both species (73, 88, 93). Both PTX3 and CRP plasma levels have been shown to be increased in cases of a major CVD event (73, 94). PTX3 has an even more rapid acute response than CRP and reaches peak values after 4-8 hours during inflammation (94). Both PTX3 and CRP expression are up-regulated in coronary lesions of instable plaques (95), but their distribution within the lesion differs. CRP is more prominent in lipid-rich plaques (95), while PTX3 is primarily increased in fibroatheroma-like complicated plaques with intra-plaque hemorrhage and in areas of anti-inflammatory macrophages (95-97). PTX3 has also been shown to be up-regulated in other vascular inflammatory diseases such as vasculitis (98). IL-1 β and TNF α are both known inducers of PTX3 production in ECs, macrophages and SMCs in CVD (99, 100). A growing body of evidence suggests a protective role of PTX3

rather than an atherogenic role (94, 101). PTX3 knock-out (KO) mice have larger lesions, increased tissue damage, macrophage accumulation and increased apoptotic cardiomyocytes than their littermate controls, and the PTX3 KO mice phenotype is reversed by PTX3 treatment (101). P-selectin has been suggested as a target for PTX3 by inhibiting leukocyte recruitment (67). The athero-protective concept has been challenged by PTX3 studies demonstrating a lack of a protective role of PTX3 in mesenteric arterial occlusion in mice (102, 103). The role of PTX3 in radiation-induced vascular damage is still unknown.

1.2.2.2 Leukotrienes

Lipoxygenases (LOs) are mainly present in the cytosol as enzymatic oxygenases that metabolize fatty acids especially polyunsaturated fatty acids like arachidonic acid (AA) and are expressed in many tissues (104). LOs play a role in both cancer and inflammatory diseases (105, 106). In particular, 5-LO, an enzyme that catalyzes the formation of AA to bioactive leukotrienes (LT), plays a key role in human CVD (107). AA can be oxidized to anti-inflammatory and pro-resolving lipid mediators, namely the lipoxins as well as pro-inflammatory lipid mediators such as prostaglandins and LT (108, 109). LT can be synthesized by both resident and recruited leukocytes. First, an unstable intermediate epoxide called LTA₄ is formed that is then further hydrolyzed to LTB₄, which regulates EC permeability, vascular tone (110, 111) and leucocyte recruitment and activation (112). LTA₄ is also an intermediate for other LTs such as the cysteinyl-LTs (LTC-E₄) (107).

5-LO activation in the adventitia has been recently suggested to be involved in various CVD disorders (107, 113-117). Interestingly, immune cells are both the producer and target cells of LTs (107). 5-LO is expressed in monocytes and macrophages (118), but during pathological situations, for example, during atherosclerosis, also structural vascular cells such as ECs and SMCs can express 5-LO (119-121). In monocytes, 5-LO expression is stimulated by cytokines such as IL-1 β (122).

The leukotriene B₄ receptor 1 (BLT1) is a G-protein-coupled high affinity receptor expressed on mainly leukocytes that bind to LTB₄ released from macrophages, ECs and SMC (123). In atherosclerotic lesions, vascular cells are able to express the BLT1-receptor (107). LTB₄ is secreted by macrophages and has been shown to be an effective chemoattractant within atherosclerotic lesions (123). LTB₄ can also bind to the low-affinity receptor BLT2 (123) and through others (124). Both the LTB₄ receptors, BLT1-2, have been detected in areas with a high density of macrophages within atherosclerotic lesions (125). The LTB₄-BLT1 signaling pathway induces adhesion through integrins and migration by CCL2 production in macrophages (126-128). BLT-1 receptor expression has been linked to NF- κ B activation and is increased in the event of vascular injury (125, 129).

1.2.2.3 NLRP3 inflammasome and interleukin-1

The multi-protein complex named NOD (nucleotide oligomerization domain)-, LRR (leucine-rich repeat)- and PYD (pyrin domain)-containing protein 3 inflammasome is shortened as NLRP3. The NLRP3 inflammasome plays an important role in innate immunity

and inflammation by promoting IL-1 β production with subsequent leucocyte recruitment and activation, which are all important features in atherosclerosis development (130).

Furthermore, the NLRP3 inflammasome induces pyroptosis, an inflammatory form of programmed cell death.

The NLRP3 inflammasome induces pyroptosis and cleavage of the pro-IL β to the bioactive and mature IL-1 β in response to IL-1, DAMPs/PAMPs and/or ROS (131). Two independent signaling pathways are needed to activate the NLRP3 inflammasome in macrophages. The first priming signal is through PRRs (i.e., Toll-like receptor) or cytokine receptors (i.e., IL-1R) by DAMP/PAMPs or IL-1 α /b, which activates NF- κ B and subsequent transcription of IL-1 β and NLRP3. The second activation signal is thought to be through DAMPs/PAMPs or reactive oxygen species (ROS) stimulation and later, the NLRP3 inflammasome complex formation by NLRP3, the protease caspase-1 and the adaptor molecule apoptosis-associated speck-like protein containing CARD often called ASC (131). The formation of pro-caspase-1 to caspase-1 requires NLRP3 inflammasome activation (130).

A considerable amount of literature has been published on IL-1 β in CVD (132-134).

Furthermore, the NLRP3 inflammasome has been shown to be the main producer of IL-1 in atherosclerosis and CVD (130). IL-1 β seems to promote atherosclerosis, for example, by inducing:

- ECs activation with subsequent monocyte recruitment and migration (135, 136)
- Pro-thrombotic properties by increased plasminogen activator inhibitor-1 (PAI-1), reduced thrombomodulin and tissue plasminogen activator (tPA) (135-137)
- SMCs proliferation (138)

Several mice studies support the pro-atherogenic role of IL-1 β signaling and the therapeutic effects of inhibiting the IL-1 signaling pathway as summarized in the review by Grebe et al. (130). Previous animal studies show reduced apoptosis and cardiac remodeling when treated with the IL-1Ra drug called anakinra (139-141). The CANTOS trial has furthermore confirmed the therapeutic effects of IL-1 inhibition within CVD in the clinical setting (49-52). However, little is known about IL-1 signaling in radiotherapy-induced vascular disease.

IL-1 α compared to IL-1 β is already in its bioactive and mature form after transcription and does not need the NLRP3 inflammasome for activation (32). IL-1 α is constantly expressed and usually membrane bound to the cell surface of primarily monocytes and macrophages and also ECs. The cytokine IL-1 α can be released in the event of tissue damage or cell death and functions in local inflammation (142-144). IL-1 α and IL-1 β possess equal affinity to the same receptor named IL-1R thereby having a similar effect on inflammation. IL-1 α and IL-1 β possess equal affinity to the same receptor named IL-1R thereby having a similar effect on inflammation.

1.2.3 Thrombus formation

In the normal condition, circulation avoids clotting in order to sustain blood flow, however, in the case of endothelial injury, hemostasis is activated. Inflammation and tissue damage are key factors in the triggering of thrombus formation. In the event of superficial vascular injury, an initial primary hemostasis induction of platelet adherence to von Willebrand factor (vWF) or to collagen receptors in the subendothelial layer occurs, especially in the capillaries and small stenosed arteries that are present. This promotes platelet aggregation but also enhances fibrinogen binding (145, 146). In the case of deep vascular injury, tissue factor is exposed to the circulation (147-149). The binding of TF to circulating coagulation factor VIIa activates the coagulation cascade (150, 151). Thrombin is the end product of the coagulations cascade (152) and promotes coagulation by conversion of fibrinogen to fibrin and promotes continuous activation of the coagulation cascade by activation of the coagulation factors V and VII (152). Fibrinogen is a plasma protein present in the circulation that can rapidly increase in the case of inflammation (153-155). Hemostasis is a double-edged sword, where there is a fine balance between coagulation and anticoagulation. The transmembrane glycoprotein thrombomodulin is a high-affinity receptor for thrombin that is expressed on the normal endothelium surface of arteries, veins and capillaries (156-158). An anticoagulant complex is formed by thrombin and thrombomodulin in the normal intact vasculature counter-act thrombosis (159-161). Increased plasma levels of TNF α and IL-1 β may decrease thrombomodulin expression on the endothelial surface thus promoting thrombus formation (162). Fibrinolysis is an anticoagulation system initiated in parallel with the production of fibrin (163). The plasma protein plasminogen is a key player in fibrinolysis and clot dissolving. Plasminogen is converted to plasmin by the serinprotease tPA. Plasmin binds to fibrin and initiates fibrinolysis (164, 165). tPA can be inhibited by PAI-1 (166, 167). The PAI-1 promotor contains sequences for IL-6 and NF-Kb that promote transcription of PAI-1 and could thus inhibit fibrinolysis (168). Today, there are several available drugs that work in order to induce fibrinolysis of clots, such as alteplase, a human recombinant tPA and urokinase (169).

1.2.3.1 *Differences between arterial and venous thrombus formation*

Platelets, circulating proteins and ECs are all crucial in thrombus formation in both arteries and veins. However, the individual thrombus components and clinical events differ between arteries and veins. Arterial thrombosis is usually initiated through plaque rupture and is common during a subsequent MI or ischemic stroke depending on the thrombus site. Vein thrombus formation, on the other hand, can occur even with an intact endothelial layer and could lead to clinical events such as VTE (12). An arterial thrombosis is platelet-rich and often located in close range to the ruptured atherosclerotic plaque. Venous thrombus, on the other hand, is a fibrin-rich thrombus or embolus detected in areas of even intact endothelium (12). The main prevention for arterial thrombosis today is to use different types of platelet inhibitors, while VTE is treated with drugs based on inhibition of the coagulation cascade.

1.2.4 Arterial versus venous vascular disease

Arteries and veins have morphological and functional differences and similarities, and therefore there may be differences in response to injury. One of the major differences between arteries and veins is the temporal aspects in response to cell damage and inflammation. Another is the difference in clinical presentation. Arterial occlusion is often an acute life- or limb-threatening clinical condition resulting in ischemic stroke, MI and central retinal artery occlusion, all of which need instant treatment. In the event of an acute venous thrombosis, the symptoms could develop slower depending on thrombus size and location. Deep vein thrombosis and pulmonary embolism are the most common locations for VTE. VTE is usually not caused by chronic inflammation but rather triggered by a temporary underlying cause such as sepsis, immobilization and pregnancy and can also be triggered by malignancies. However, heredity and genetic variation such as a pro-thrombin mutation, anti-thrombin or protein C deficiency increase the risk of VTE. Patients with VTE also have a higher risk to develop CVD (170, 171).

1.3 Radiotherapy-induced vascular disease

1.3.1 Clinical background

1.3.1.1 Increased cancer survival

In 2018, the worldwide cancer incidence was 18 million according to the WHO IARC-GLOBOCAN database. Breast cancer, Hodgkin's lymphoma, brain malignancies and head neck cancer represented 18% of these new cases in 2018. The 5-year cancer survival rate worldwide has improved during the last decades due to early detection and improved cancer treatment (172, 173). In the US alone, there were 15.5 million cancer survivors in year 2016, and two-thirds of these had reached the 5-year relative survival rate (174). The 5-year relative survival rate for breast cancer was 89% and 84% for children/adolescence tumors in 2016 (174). Twenty-nine percent of the long-term cancer survivors had received radiotherapy treatment at least once (Figure 4) (175). The increased number of long-term cancer survivors has also led to new long-term side effects caused by the cancer treatment itself (176, 177).

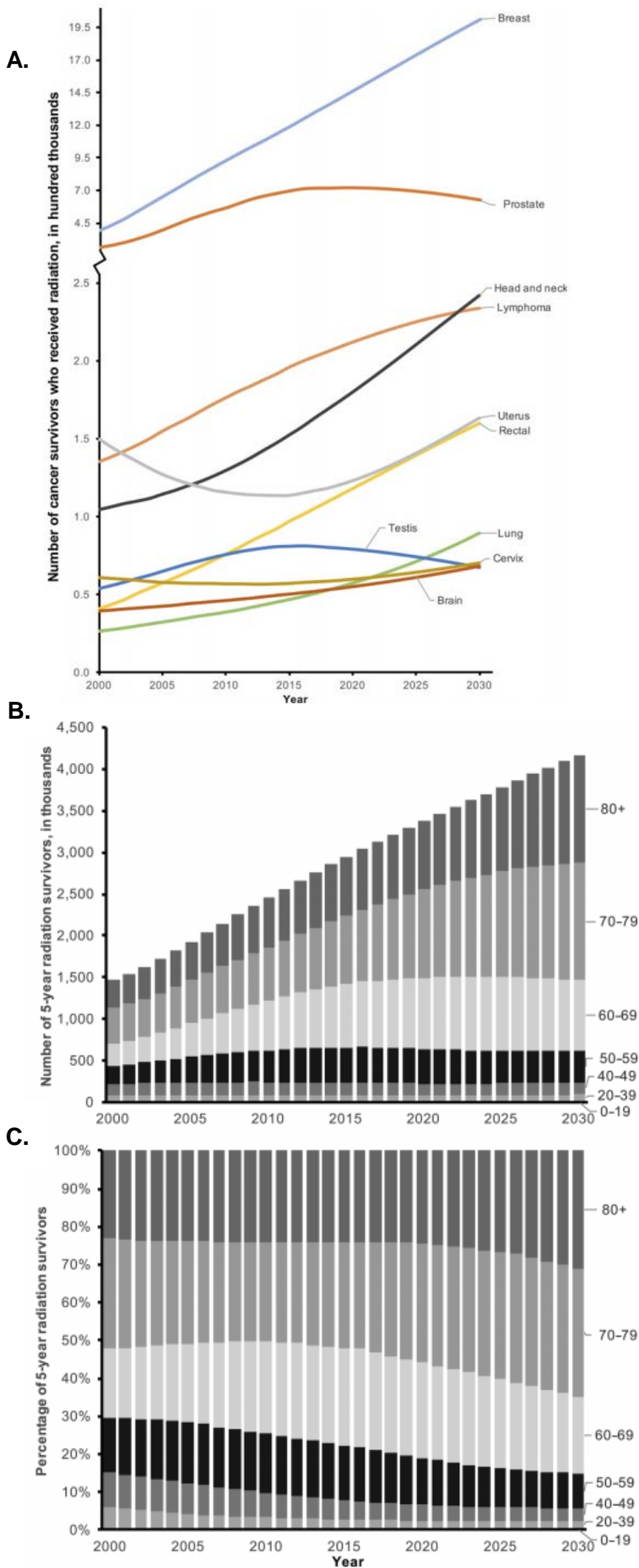


Figure 4. The number of cancer survivors treated at least once with radiotherapy over time and are divided according to cancer diagnosis (A) and divided according to age (B-C). Reprinted from Publication *Cancer Epidemiology Biomarkers & Prevention*, 2017, 26/6, 963-70, Alex K Byrant et al, Trends in Radiation Therapy among Cancer Survivor in the United States, 2000-2030, with permission from American Association for Cancer Research.

1.3.1.2 Radiotherapy

Radiotherapy was initiated as a cancer treatment at the end of 19th and early 20th century (178, 179), and studies showed beneficial effects on cancer patient survival (180, 181). A large number of cancer patients are estimated to benefit from radiotherapy treatment as curative or palliative relief (182-184). Radiotherapy is used as monotherapy but also used together with surgery and/or chemotherapy (174) and can be delivered in fractions pre- or postoperatively (183, 185, 186). Radiotherapy can be delivered externally by an external beam or by from a radiation-emitting source placed within the tumor (called brachytherapy) (178).

Radiotherapy treatment is a fine balance between successful cancer treatment and simultaneously avoiding side effects on surrounding healthy tissues.

1.3.1.3 Radiotherapy-induced vascular disease

Radiotherapy is a key player in several cancer treatments (183, 184, 187), and the major comorbidities of irradiation are the long-term effects (175, 188). The improved and refined cancer therapy has become more targeted after every decade, however healthy tissues are inevitably exposed to radiotherapy (189). One of the often overlooked clinical side effects of radiotherapy treatment is an increased risk for CVD at the site of radiation (Figure 5). Epidemiological studies have shown an increased risk for MI after left-side thorax irradiation for breast cancer or Hodgkin's lymphoma (190-194) and stroke after radiation for brain tumours, Hodgkin's lymphoma and head and neck cancer (195-200). Furthermore, preoperative radiation has been associated with an increase in postoperative morbidity, and surgeons sometimes refrain from performing surgery in previously irradiated tissues (201, 202). Endovascular surgery seems to limit the surgical risks compared to open surgery in previously irradiated carotid arteries (203), probably due to less tissue damage in need of repair (204). Radiotherapy has been debated as a risk factor for free flap necrosis due to vascular complications (205, 206). However, some clinical studies have indicated an increased number of complications (206-211) that are supported by experimental studies showing impaired patency in the radiated vessel area of the anastomosis (212-215). It has been shown that radiated tissues have impaired wound healing capability in flap surgery that is associated with impaired microcirculation and fibrosis development (189, 204, 216, 217). Radiotherapy treatment doses differ between breast cancer and head and neck cancer patients. Traditionally, breast cancer is treated with a lower total radiation dose of around 50 Gy (218), while head and neck cancer patients often acquire more than 60 Gy, (219). Furthermore, the



Figure 5. Radiotherapy-induced cardiovascular disease. Illustration by Tinna Christersdottir, reprinted with permission from European Heart Journal (**Paper IV**).

radiation field during head and neck treatment more often includes vital vessels compared to breast cancer fields (207), which may affect the surgical outcome.

1.3.2 The pathogenesis of radiation-induced vascular disease

1.3.2.1 General tissue damage

The development from normal to cancer cells has been explained by genetic and epigenetic changes that lead to disruption of normal cell function with cancer cell proliferation, growth and finally manifestation of cancer disease (220). Radiotherapy promotes cancer cell death by high-energy ionizing radiation through most commonly x-ray or gamma radiation (180). The mechanism of action is through radiation-induced cell death by direct DNA damage, e.g., double- or single-strand breaks or indirectly through production of ROS by ionizing or excitation of water particles (180). High proliferative cancer cells are more sensitive to radiation-induced DNA damage than other cells (181). Furthermore, normal tissue with highly proliferative cells, located around the cancer, are also affected. Cells with high proliferation and potential to replace damaged or dying cells are vascular cells like ECs and SMCs, epithelial cells (from e.g., lung, breast, liver and fibroblasts) present in several tissues types (221). Other epithelial cells present in skin and gut have a short life span and are normally replaced by a differentiation of stem cells (221). In the event of radiation-induced damage to tissues with epithelial cells with high normal turnover such as the skin and intestine, patients often develop *acute symptoms* during radiotherapy. However, stem cells are more radiation-resistant than epithelial cells and, in these tissues, able to proliferate and differentiate to new functional cells. Cells in tissues such as lung, liver and blood vessels normally have a slow turnover rate but also contain cells with high proliferative capabilities that are more prone to the *late side effects* of radiotherapy (189). Because the human vasculature contains ECs and SMCs, this tissue is at risk for late and chronic radiation-induced damage.

1.3.2.2 Radiation-induced vascular injury

Clinical studies support the notion that radiotherapy is a risk factor for CVD, however uncertainty still exists about the relationship between radiation and vasculopathy. Experimental cell studies are often limited to acute experiments. Animal models on the other hand often offer longer experiments, where few long-term studies are published that use mouse models (222-224). Studies in atherosclerosis-prone mouse models have shown that radiation induces an inflamed macrophage-rich plaque with intra-plaque haemorrhage (223, 225), which are all features of a vulnerable plaque that is more prone to rupture and are therefore at risk for clinical events such as MI and stroke (55).

Human materials are scarce due to ethical and surgical constraints. Human arterial and vein biopsies harvested during autologous free tissue transfer surgery provides a unique possibility to investigate gene and protein changes in humans months to years after final radiation

exposure (25, 226). CVD has been linked to a progressive chronic vascular inflammation that involves both the adaptive and innate immune systems (55). Recent studies of mouse carotid arteries and human conduit arteries support a similar inflammatory response in radiation-induced vascular disease (25, 223). Irradiation of human arteries induces a sustained chronic inflammation driven by chronic activation of NF- κ B as previously described (25) and acute up-regulation of NF- κ B and NF- κ B-associated cytokines and adhesion molecules in veins (226). NF- κ B is a major mediator of the innate immune response and notably regulates the expression of PTX3 (92), cytokine IL-1 β (227), IL-6 (228), VCAM1 (229), ICAM-1 (230), E-selectin (231), CCL2 (232, 233) and PAI-1 among others (234), which are all targets associated with atherosclerotic vascular disease (25, 226, 235). Radiation has been shown to up-regulate expression of NF- κ B and the downstream target IL-1 β in irradiated human arteries and in other tissues such as lung and brain (25, 236-238). IL-1 is an important mediator of innate immunity and inflammation, and several studies have provided evidence for a role of IL-1 in radiotherapy-induced vascular tissue damage (25, 239). IL-1 β increases the inflammatory response, as reflected by an increased expression of adhesion molecules such as VCAM1, a key player in monocyte and T-lymphocyte recruitment (25, 222, 240) and the monocyte chemokine CCL2. Hoving et al. found increased transcription of CCL2 in carotid arteries from mice 4 weeks after radiation exposure (222). CCL2 has furthermore been connected to radiation-induced vascular disease in a computational model (241).

Experimental studies in human and mice have provided evidence for a pro-thrombotic state of irradiated endothelium (226, 242-245), which could promote thrombotic clinical events in both arteries and veins. In addition, radiation induces inflammation, EC dysfunction and activation of the coagulation cascade, which all promote vascular damage and CVD (25, 176, 189, 242, 245-249). Traditional treatments such as the platelet inhibitors including clopidogrel and acetylcysteine and the lipid-lowering drug, atorvastatin for CVD, fail to prevent radiotherapy-induced atherosclerosis in apolipoprotein E knock-out (*ApoE*^{-/-}) mice (222, 224). Anti-inflammatory treatment with Thalidomide initiated weeks after radiation exposure did not manage to limit the radiation-induced increased expression of the pro-thrombotic vWF in cardiac vessels or other cardiac changes in *ApoE*^{-/-} mouse model (250). Further studies on radiation-induced vascular disease are needed in order to understand the underlying biology and to innovate future targeted treatments.

1.3.3 Management and therapy

Today there are no specific treatment protocols for CVD in patients previously exposed to radiotherapy. Furthermore, there are no prophylactic treatment protocols. Irradiated patients with CVD are treated with the same therapeutic drugs as patients with traditional CVD. In cancer patients that will receive both radiotherapy and tumor resection surgery and need subsequent microvascular autologous free flap surgery, the treatment timeline has changed recently in order to reduce the risk for radiotherapy-induced vascular complications. The aim at Karolinska Hospital today is to perform surgery before or less than 6 weeks after

radiotherapy treatment in head and neck cancer patients, if it does not interfere with the tumor treatment (251, 252). Anti-thrombotic treatment in irradiated patients is the same as others with arterial or venous disease. tPA has been suggested as a potential candidate for thrombolysis also within salvage surgery for free flap with vascular complications as in stroke and CVD, but data are conflicting regarding the effect of the treatment during salvage surgery for free flaps (26, 27).

2 AIMS

The overall aim in the present thesis was to investigate the effect of high-dose ionizing radiation on blood vessels and to evaluate the clinical outcome, gene and protein expression patterns and the effect of anti-inflammatory treatment in an *in vivo* model.

The specific aims of each Paper were:

Paper I: To investigate how radiotherapy, and furthermore, the time elapsed from radiotherapy to surgery, would affect the risk for free flap vascular complications.

Paper II: To examine a radiation-induced inflammatory response in the vessel wall of human arteries and veins by means of expression of the innate inflammatory biomarker PTX3.

Paper III: To evaluate the involvement of leukotrienes in radiation-induced inflammatory response confined to the adventitia in human arteries.

Paper IV: To translate the previous findings in human arteries to an animal model of localized radiation-induced vascular inflammation in *Apoe*^{-/-} mice, in order to investigate the effect of IL-1 inhibition.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Study subjects

In total, 344 head and neck free flap reconstructions from patients enrolled in a preoperative radiotherapy treatment protocol were included in **Paper I**. All reconstructions were performed at Karolinska University hospital between 1984-2010. The arbitrary set time-points for the different groups (Figure 6) were based on previous clinical and experimental publications (189, 212, 216, 226, 251, 253) in combination with formation of acceptably sized-matched groups. Temporal data were incomplete in three reconstructive surgical cases and therefore excluded from the temporal analysis. Demographic characteristics were collected in order to identify potential confounders within the patient cohort. The median radiation dose was 64 Gy (40-94 Gy) and in one patient, the radiation dose was unknown. The registered complications are presented in Figure 7. The study was approved by the Ethical Committee of Stockholm and was performed in agreement with institutional guidelines and principles of the Declaration of Helsinki.

0 XRT	Early	Delayed	Late
<ul style="list-style-type: none"> •no preoperative radiotherapy •n = 61 	<ul style="list-style-type: none"> •< 6 weeks •n = 108 	<ul style="list-style-type: none"> •6-15 weeks •n = 77 	<ul style="list-style-type: none"> •> 15 weeks •n = 95

Figure 6. The reconstructions were divided into four different temporal groups. The weeks represent the time from the last radiotherapy treatment to surgery. n = the number of reconstructions.

Vascular complications	Hematoma	Late revision
<ul style="list-style-type: none"> • venous thrombosis • arterial occlusion • combination 		<ul style="list-style-type: none"> • salvage surgery not possible • vascular complication unknown.

Figure 7. The reconstruction surgical complications groups and subgroups.

3.2 The human Biobank of Radiated tissues at Karolinska (BiRKA)

The human arterial and venous biopsies were obtained during autologous microvascular free tissue transfer surgery for head and neck reconstructions after tumour resection and radiotherapy treatment. The irradiated vessels harvested at the recipient site were mainly collected from side branches of the external carotid artery and the internal jugular vein. The non-irradiated control vessels harvested at the donor site were either radial, fibular or lateral circumflex femoral arteries or/and veins. Demographic data, specific donor site and time elapsed from last radiotherapy session were collected. During harvest, one non-irradiated (donor site) and one irradiated (recipient site) vessel biopsy were collected from the same patient at the same time, allowing us to exclude inter-individual differences between groups, for example, in smoking (Figure 3, 8). Post-oncological reconstructions could be performed years after the last radiotherapy treatment enabling us to study long-term effects on gene and protein expression levels. The biopsies were either snap frozen or placed in allprotect for future protein extraction and subsequent western blot (WB) analysis (**Paper IV**), put into paraformaldehyde (PFA) and later paraffin embedded for future immunohistological staining

(**Papers II-IV**) or conserved in allprotect/RNA later (**Papers II-IV**) for future RNA purification, cDNA synthesis and qPCR or array analysis (Figure 8). The studies were approved by the Ethical Committee of Stockholm and were performed in agreement with institutional guidelines and principles of the Declaration of Helsinki. All enrolled subjects gave informed consent.

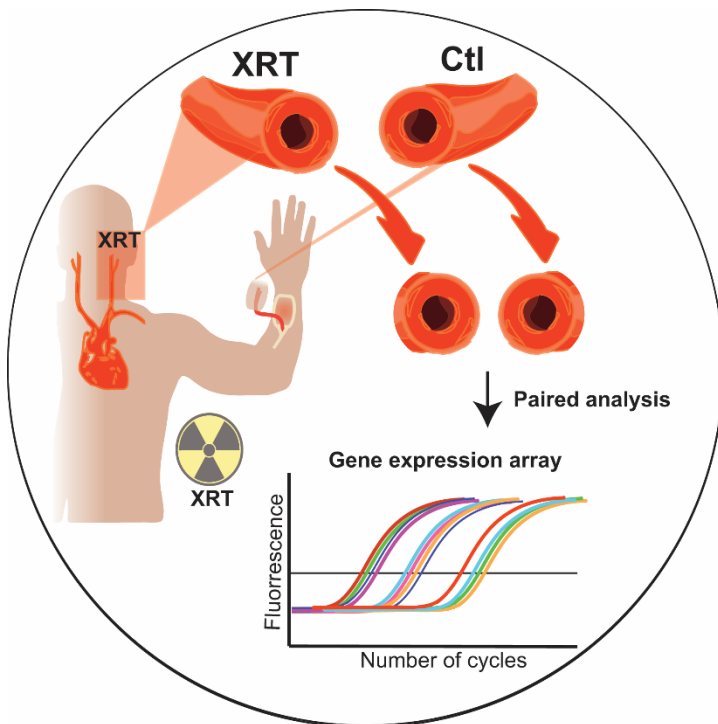


Figure 8. Paired human arteries and veins collected during free tissue transfer for cancer reconstruction. One irradiated and one non-irradiated vessel biopsy from the same patient are analyzed by real time qPCR. XRT=irradiated (recipient site). Ctl = non-irradiated (donor site). Illustration by Tinna Christersdottir, reprinted with permission from European Heart Journal (**Paper IV**).

3.3 Mouse model of radiation-induced vascular disease

Mice are well established as experimental laboratory animals within atherosclerotic research. The small size of the mouse facilitates storage and maintenance. The gestation period is short allowing for efficient breeding. Inbreeding allow for experimental mice with low genetic variation, well-defined genomes and in some cases, genetic modification (254). Most wild type strains are resistant to atherosclerosis development (254). Therefore, the need for genetically modified mice that share similar lesion development with humans is crucial. The wild type C57B1/6 strain does, however, develop small fatty streaks in response to a high fat diet, but they do not progress to complex lesions as observed in humans with atherosclerotic disease (255). Therefore, the C57B1/6 strain is a common background strain to many transgenic murine models within atherosclerotic research.

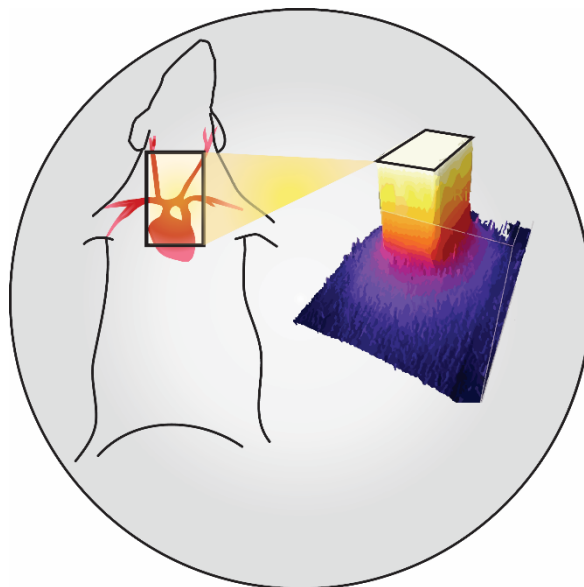


Figure 9. Radiation field- and scatter analysis in experimental mice. Illustration by Tinna Christersdottir, reprinted with permission from European Heart Journal (Paper IV).

Today, several of inbred genetically modified mice strains are available for atherosclerosis research (254). The *Apoe*^{-/-} and low density lipoprotein receptor^{-/-} are the two most widely used genetically modified mouse models within atherosclerosis research (254, 255). Both models suffer from hyperlipidaemia, and they do develop lesions throughout the aortic tree in similar locations as humans (256-258). The main disadvantage of a high fat diet-induced atherosclerosis is that the diet is thought to be pro-inflammatory and could therefore affect the final inflammatory response to irradiation (259). In contrast to many other transgenic murine models of atherosclerosis, the *Apoe*^{-/-} model develops atherosclerotic plaques at the age of 10-20 weeks on a regular chow diet, because *Apoe*^{-/-} mice have increased levels of pro-atherogenic lipoproteins in plasma due to impaired lipoprotein clearance (255), making it a more suitable candidate for radiation-induced vascular disease studies (254, 255). Transgenic mice open up the possibility to study biological mechanisms of CVD, however, there are some limitations to consider. The physiological condition of *APOE* deficiency is extremely rare in humans, the lipoprotein levels and profile do not reflect lipoprotein disturbances in the human setting and finally the mice rarely experience plaque rupture with subsequent thrombus and vascular occlusion as seen in clinical manifestations in humans (260). On the other hand, *Apoe*^{-/-} mice are well suited for studies on innate immunity and especially monocyte migration (254), and the innate immune system has been shown to be an important player in radiation-induced vascular disease (25). Previous studies on radiation-induced vascular disease have been performed successfully in *Apoe*^{-/-} mice, which further support their

suitability, but also enable the possibility for comparison (222-224, 261). Gender differences within CVD are well known. In **Paper IV**, only female mice were analysed and therefore excluded the potential to compare biological response differences between genders. The female mice model is the most athero-developmental model and therefore more suitable for proof-of-concept studies (262).

Radiation can be given as a one-time, single dose or in fractions until the total dose is reached. In **Paper IV**, a one-time, single high-dose was applied instead of several fractionated low-doses as done in the clinical setting. There are two main advantages with the single high-dose treatment. First, it limits the interference of anaesthesia and other stress factors such as injections and handling of the animal. In addition, we could reduce the suffering of the mice. Thirdly, Hoving et al. (261) has demonstrated similar results of single and fractionate irradiation protocols in *Apoe^{-/-}* mice. Lethal doses of irradiation in mice depend on the strain but are normally between 9-11 Gy depending on the radiation source (263). The selection of the dose 14 Gy was based on previous research that shows acceptable survival rates to local radiation of

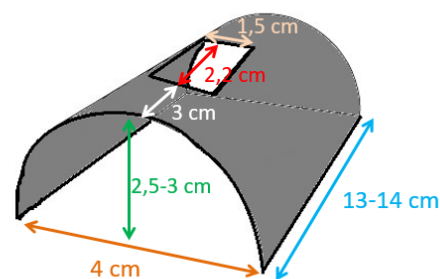


Figure 10. The custom-made lead shield collimator for radiation. Lead thickness was 7 mm.

14 Gy to study long-term effects (224, 261). Scatter is a well-known problem within radiotherapy treatment, because the spread of radiation might affect non-targeted areas. To reduce this, a customized lead shield collimator (Figure 10) was developed, and a fixed-beam collimator was used in our model. Through scatter analysis, we were able to confirm a low level of scatter in our mouse model (Figure 9). A radiation filter was used to further reduce off-target tissue effects such as skin injury and thereby mice suffering (264).

The anesthetic ketamine is a well-known sedative with a low risk of hypotension during anesthesia. Ketamine is therefore a favorable sedative to use in a radiation source with limited monitoring abilities. The anesthetic adjuvant domitor was found appropriate due to its reversal possibility by antisedan and analgesic effects with reduced suffering and therefore ethically beneficial.

Anakinra is a recombinant IL-1 receptor antagonist (IL-1Ra) that is able to competitively inhibit both IL-1 α and IL-1 β signalling by binding to the IL-1R. In comparison to canakinumab (CANTOS), which is a monoclonal antibody that targets IL-1 β only. In order to show proof-of-concept, the anakinra administration (*i.p.*) treatment period and dosage was based on the previous most efficient dose and treatment period within arteriosclerosis research in *Apoe^{-/-}* mice (139, 141, 265). All animal experiments were approved by Stockholm Regional Board for Animal Ethics.

3.4 Experimental methods

3.4.1 Gene expression analysis

3.4.1.1 RNA extraction, cDNA synthesis and real-time quantitative PCR

Gene expression is the process by which a gene within our DNA is able to transfer sequence information that is used for protein synthesis. The most popular described version of this process is the central dogma as describe by James Watson in 1965 (266). The central dogma is described as the process by which the information stored within our DNA is copied (i.e., transcribed) into mRNA, which is issued as a template for protein synthesis (i.e., translation). This process can be affected by intrinsic and external factors, before, during and after the different steps and thereby affect the functional outcome and phenotype (266). By differentiating the gene expression between irradiated and non-irradiated vessels, we can identify key components and reveal underlying mechanisms behind the functional differences. Nevertheless, mRNA does not *per se* measure cellular functionality, because proteins are the actors of cellular function. However, protein levels do not necessary predict effects and influence of inhibitors. Despite mRNA's shortcoming in measuring cellular functionality, measurements of mRNA levels provide unique insights into changes in cellular function and physiology.

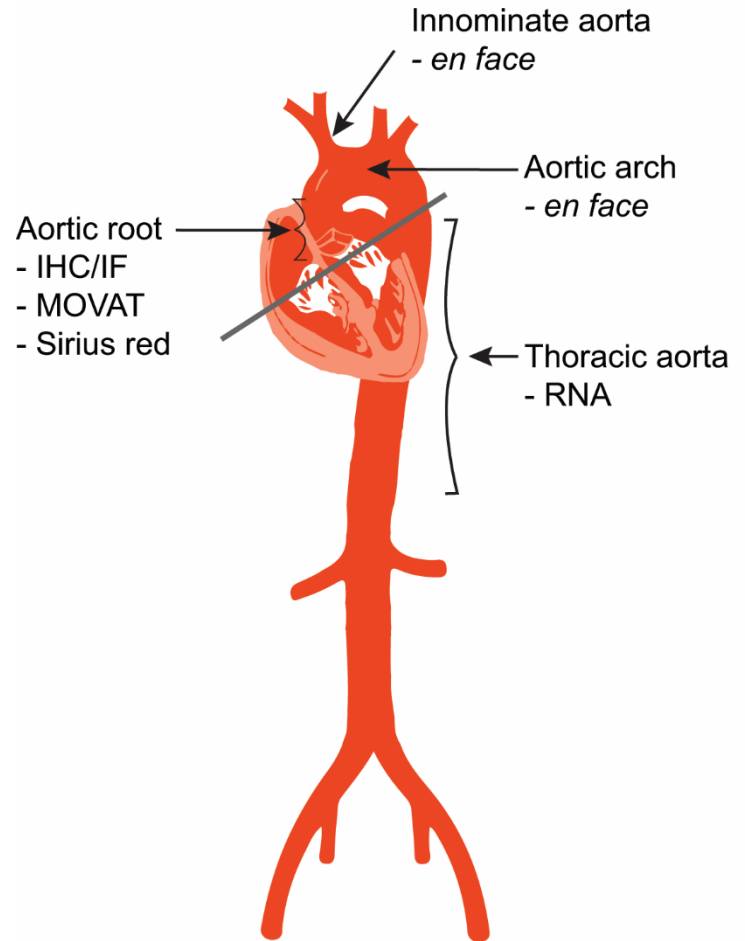


Figure 11. Anatomy of the aortic tree of experimental mice. IHC = immunohistochemistry. IF = immunofluorescence.

In **Paper II-IV**, human blood vessels from autologous free tissue transfers for head and neck reconstruction, and in **Paper IV**, thoracic aorta of experimental mice were harvested for RNA purification, cDNA synthesis and semiquantitative real time PCR (RT-PCR, Taqman) (Figure 11). In order to determine the quantity and quality of RNA, the NanoDrop 1000 Spectrophotometer and Agilent 2100 Bioanalyzer were used for analysis. Only mRNA samples with an acceptable RIN quality were included. Gene expression calculations were performed according to the well-described methodology by Livak et al. (267).

In order to compensate for intra- and inter-RT-PCR variations, housekeeping genes were used. Radiation is known for its genotoxic effect, which could interfere with several known

housekeeping genes. The housekeeping gene PGK1 has been previously tested and been shown suitable for irradiated human blood vessel (25) and was therefore used in **Papers II-IV**. In **Paper IV**, several housekeeping genes were used due to a large genetic variation in irradiated mice tissues and instead a geometric mean of several housekeeping genes was calculated. In order to have a more comparable result between human and mouse data, one additional housekeeping gene named ribosomal protein large P0, which is comparable to one of the mouse housekeeping genes was added in **Paper IV**. Standard curves have been used to test the reliability of the tests.

3.4.1.2 *Gene expression profiling*

Traditional gene expression analysis, as described above, requires tests of pre-determined single genes. In order to identify signalling pathways of interest, microarrays are well established and have reproducible and efficient methodologies, where the gene expression of thousands of genes can be analysed at the same time. The analysis limits experimental bias and allows for a broad analysis on a limited amount of tissues. Microarrays were used to detect gene expression differentiations between irradiated and non-irradiated vessels but also to determine gene expression levels and to calculate the fold change between irradiated and non-irradiated biopsies.

All genes with gene expression differences between radiated and non-irradiated vessels were selected for enrichment analysis. In order to investigate genes that may reflect the radiation-induced vascular disease phenotype in the the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Hallmark gene sets from the well-established Molecular Signature DataBase (MSigDB) database were used (268, 269). Nevertheless, all genes provided within the different gene sets were manually selected therefore allowing for human-related errors. All enrichment analysis with their genes sets have been controlled and trimmed by the MSigDB database. To reduce the risk of including genes that are not applicable within a certain gene set, the enrichment analysis was performed under the MSigDB database instead of their original separated databases. Of note, the genes included are only genes that to our knowledge today are involved in certain processes.

Genes with a known association with inflammasome biology were included as target genes (269-274). The researchers determined the gene set in this analysis, which makes this method more affected by selection bias than enrichment analysis. To limit this effect, all genes were predetermined and selected according the present knowledge of inflammasome signalling before any analysis. To allow us to identify interactions between the target genes and create a subnetwork, we used the well-established protein-protein interaction mapping system called the Biological General Repository for interaction database (BioGRID) together with the extraction method called the prize-collecting Steiner Forest graph optimization approach to include “bridge” genes (275, 276) instead of generating a subnetwork of genes, which was another option available. Proteins are the measure of cellular function, and therefore it is more interesting to evaluate potential interactions between the gene-equivalent protein.

3.4.2 Immunostainings

To establish if changes in gene expression patterns end up as biologically active proteins, further analysis was needed. Therefore, to verify if the proteins of interest showed differential expression in the irradiated compared to non-irradiated samples, we conducted immunostaining. The immunostaining models used within **Papers II-IV** were immunohistochemistry (IHC) and immunofluorescence (IF), in **Paper IV** western blot (WB) was used and in **Paper III**, an enzyme-linked immunosorbent assay (ELISA) was used. The general concept for all immunostainings used within **Paper II-IV** is that they are antibody-antigen-based methods. A specific epitope on the target protein, also called an antigen, is detected by a primary antibody. To enable visual detection of the antigen-primary antibody complex, a secondary antibody with either a detectable fluorescence dye, an enzymatic component that induces a coloured or chemiluminescence reaction or another colorimetric product is added. In some cases, the primary antibody could be directly labelled with one of the above-mentioned detection options, i.e., the I-Ab (MHC class II) staining in experimental mice. Direct labelling allows for a more efficient protocol option making multiple antibody staining possible, and also there is less non-specific binding and background in comparison to indirect labelling. However, there are few direct antibodies available, and the method is less sensitive in comparison to indirect labelling methods. The different methods have their specific application, but they all requires antibodies with high specificity and proper controls. Furthermore, IHC/IF staining needs proper tissue preparation and fixation in order to detain a decent tissue morphology and for optional antibody binding capacity. The fixation procedure was based on previous reports on antigen sensitivity to aldehyde. A blocking agent was used to ensure non-specific binding. IF staining allows for double labelling that enabled us to co-localize two different targets proteins in order to further understand the role of the target protein. All the different methods have their advantages and disadvantages. A limited number of samples have been included in the different analyses due to surgical and ethical restraints.

In **Papers II-IV**, immunohistochemistry and immunofluorescence staining was performed in arterial and/or vein sections in order to detect, quantify and localize the target proteins; PTX3 (**Paper II**); CD68-expressing macrophages (**Papers II-IV**); vWF, in order to visual ECs (**Papers II-III**); alpha-actin to visualise SMCs (**Paper II**); VCAM1; I-Ab and 5-LO; (**Papers II-IV**). Sudan black staining was used to reduce auto-fluorescence and DAPI for nuclei staining in **Papers III-IV**. Fluorescence, confocal and light microscopy were used for quantification and localization. In **Paper IV**, IF/IHC was performed according to previous publications by Gisterå et al. (277).

Samples used for WB analysis were tissue extracts. Therefore, WB allowed for an overall semi-quantification of target proteins of varying size in small amounts within the whole vessel wall. The WB values presented in **Paper IV** were normalized with total protein amounts, as it has been shown that normalisation to total protein is most robust for complex tissues (278), because radiation-associated effects on house-keeping proteins cannot be excluded.

3.4.3 Atherosclerotic lesion size and composition

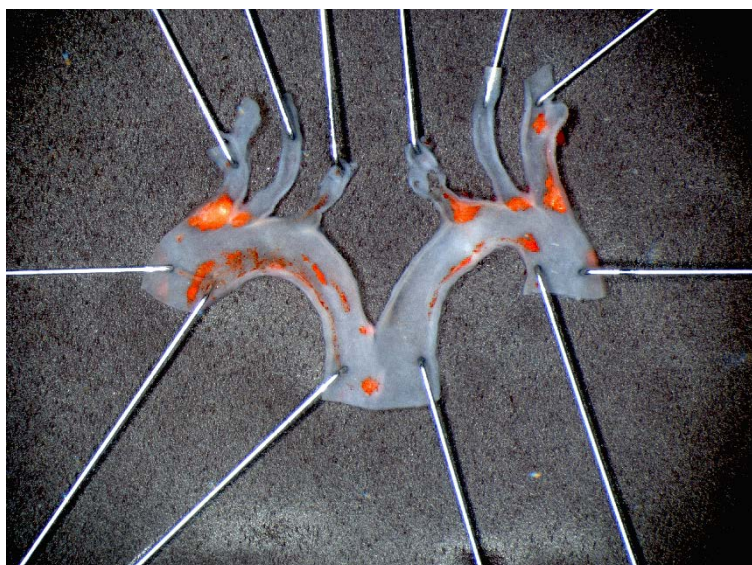


Figure 12. Quantification of lipid-laden lesions with *en face* analysis in the aortic arch of experimental mice.

In order to investigate atherosclerotic lesion size in experimental mice, two well-established analyses were performed at three different locations in the aortic tree. In the aortic arch and innominate artery, quantification of lipid-laden lesions was performed with *en face* analysis, while in the sectioned aortic root, the atherosclerotic lesion size was assessed by the special connective tissue stain called MOVAT (277, 279, 280). As others have highlighted, atherosclerotic plaque size in humans does not *per se* predict clinical events. Increased inflammation, on the other hand, is associated with adverse clinical outcomes, and IL-1 treatment had positive effects in the CANTOS (49). The combined evaluation of lesion size and composition better reflect the severity of the disease and risk for clinical events. Therefore, further analyses on atherosclerotic lesion composition were performed in the aortic root. The first 900 μm of the proximal aorta was cryosectioned and used for the above methods. Traditionally, lesion composition has been assessed by measuring, i.e., collagen content, cellularity, the lipid core, the presence of inflammatory components and also cells, i.e., macrophages and I-Ab+ (MHC class II)-expressing cells among others. The marker analysed in this study was a special collagen stain called Picrosirius red (281), but also other markers, which are further described in Section 3.3.3 of this thesis were used. To further assess the information regarding the functional effects of the lesions, in-depth measurements of residual lumen volume and the circumference of the artery at the aortic root were done as presented by Alexander et al. (282).

3.4.4 Plasma

In **Paper IV**, whole blood was collected in order to test for potential systemic effects of localized radiation in experimental mice. Localized radiation treatment inevitably expose non-target areas through incomplete delimited properties of collimators and internal body scatter (283). In addition, the local stress response to radiation has been shown to give systemic effects by, i.e., recruitment of inflammatory cells (283). Blood sampling was done

by pre-radiation tail puncture and by *post mortem* heart puncture. Hypercholesterolemia is known to promote atherosclerosis development. Inflammation is tightly linked to metabolic disorders, and the inflammatory response increases energy expenditure. Several plasma cytokines, such as IL-1 β and IL-6, are able to mobilize energy sources and raise plasma levels of these energy sources. To evaluate promoters of atherosclerosis other than radiation in our mouse model of localised irradiation, plasma analyses were performed. Samples were analysed for total cholesterol and triglycerides by enzymatic colorimetric kits by Randox Lab. To determine whether local irradiation affected bone marrow-derived cells, whole blood cell count was measured with an ABCTM Vet animal blood counter (Scil animal care company, Germany). Cytokine levels were measured in plasma by the electrochemiluminescence technique named mesoscale according to the manufacturer's protocol to determine potential inflammatory systemic effects of radiation and/or anakinra treatment. The general measured low levels of cytokines should mirror the generally low systemic effects by radiation in our mouse model. Furthermore, there were no significant differences in blood count levels, which further support the reliability of our mouse model to demonstrate localized irradiation.

3.5 Statistical analysis

In order to test our research hypothesis we had to choose appropriate statistical methods based on the outcome variables and variable distribution. The significant level, was set at the standard of 5%. Therefore, all p-values below 0.05 allowed us to reject the null hypothesis in **Papers I-IV**. In **Paper I**, a binary exact logistic regression was used to investigate the effect of time between radiotherapy and reconstruction on the probability of flap necrosis and vascular complications. In order to identify differences in gene expression levels, between irradiated and non-irradiated paired human samples, we performed statistical analysis with the Wilcoxon signed-rank test in **Papers II-IV**. In **Papers II-IV**, the human sample sizes were ranged from 7-20 paired samples. Both the parametric student's t test and the non-parametric Wilcoxon signed-rank test are possible comparative tests for paired samples. Comparisons between the paired samples were made using the Wilcoxon signed-rank test (284). The student's t test was chosen in one analysis in **Paper III** of paired samples, where absolute protein levels were measured. To allow us to compare multiple groups of similar sample sizes, we used the one-way ANOVA analysis for comparison of variables for experimental mice in **Paper IV**. All samples were adjusted for multiple comparisons with Tukey's multiple comparisons test to reduce the risk of a type 1 error.

4 RESULTS AND DISCUSSION

4.1 Radiotherapy, a risk factor in microvascular free flap surgery (Paper I)

This study showed that (i) total flap loss was more common in previously irradiated head and neck cancer patients; (ii) an early reconstruction had higher surgical success rates, lower number of vascular complications and better free flap salvage rates compared to delayed reconstructions in irradiated patients; (iii) tPA, an inducer of fibrinolysis, improved total free flap salvage rate and (iv) that venous and arterial occlusions as vascular complications are differently distributed over time.

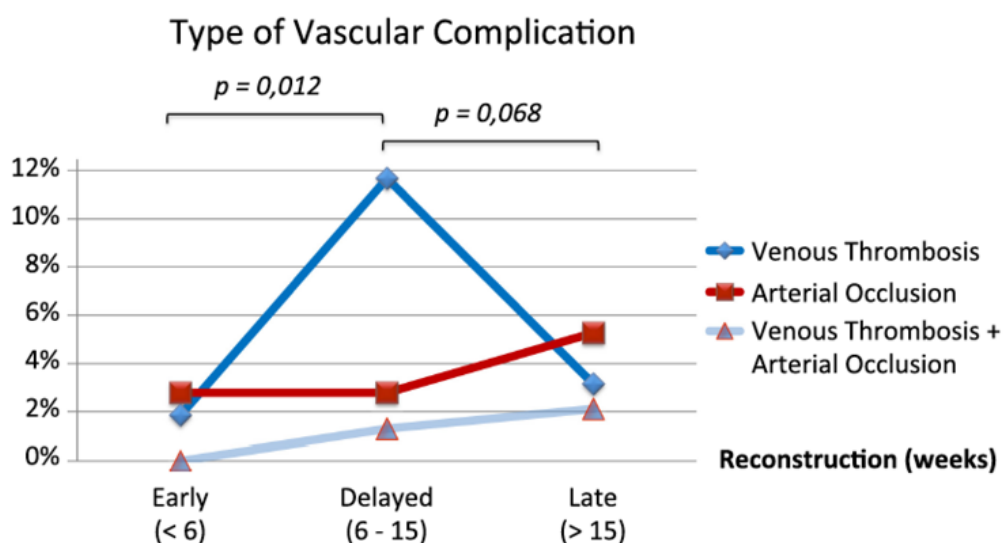


Figure 13. Vascular complication in preoperative irradiated head and neck cancer free flap reconstructions. Early = <6 weeks, delayed = 6-15 weeks, late = >15 weeks from last radiotherapy treatment to reconstructive surgery. Reprinted with permission from Ann Plast Surg (**Paper I**)

Out of 344 head and neck reconstructions, 283 were preoperatively irradiated with a median dose of 64 Gy. Compared to other studies, we were able to find a significant increase in free flap necrosis in the irradiated group (26, 205, 206). This may be partly related to the local treatment protocol with a high number of preoperatively irradiated cases compared to case series from other centers advocating postoperative radiotherapy. The reasons why clinical evidence regarding radiotherapy as a risk factor for free flap failure is lacking is probably the combination of high surgical success rates and limited samples sizes of irradiated cases together with mixed cohorts including breast cancer reconstructions (26, 207). Head and neck cancer patients receive higher radiation doses with more exposure of recipient vessels compared to breast cancer patients and are therefore more prone to develop irradiation damage. Our results have recently been supported by a meta-analysis of head and neck free flap outcome (285).

The risk for free flap necrosis in reconstructions after radiotherapy increased over time. Among vascular complications, venous thrombosis was more common in the delayed compared to the early group (Figure 13). Interestingly, the temporal curve (Figure 13, 14) presented in **Paper I** may reflect an early latent phase followed by a delayed acute pro-thrombotic and inflammatory state with a higher risk for venous thrombosis (189, 253).

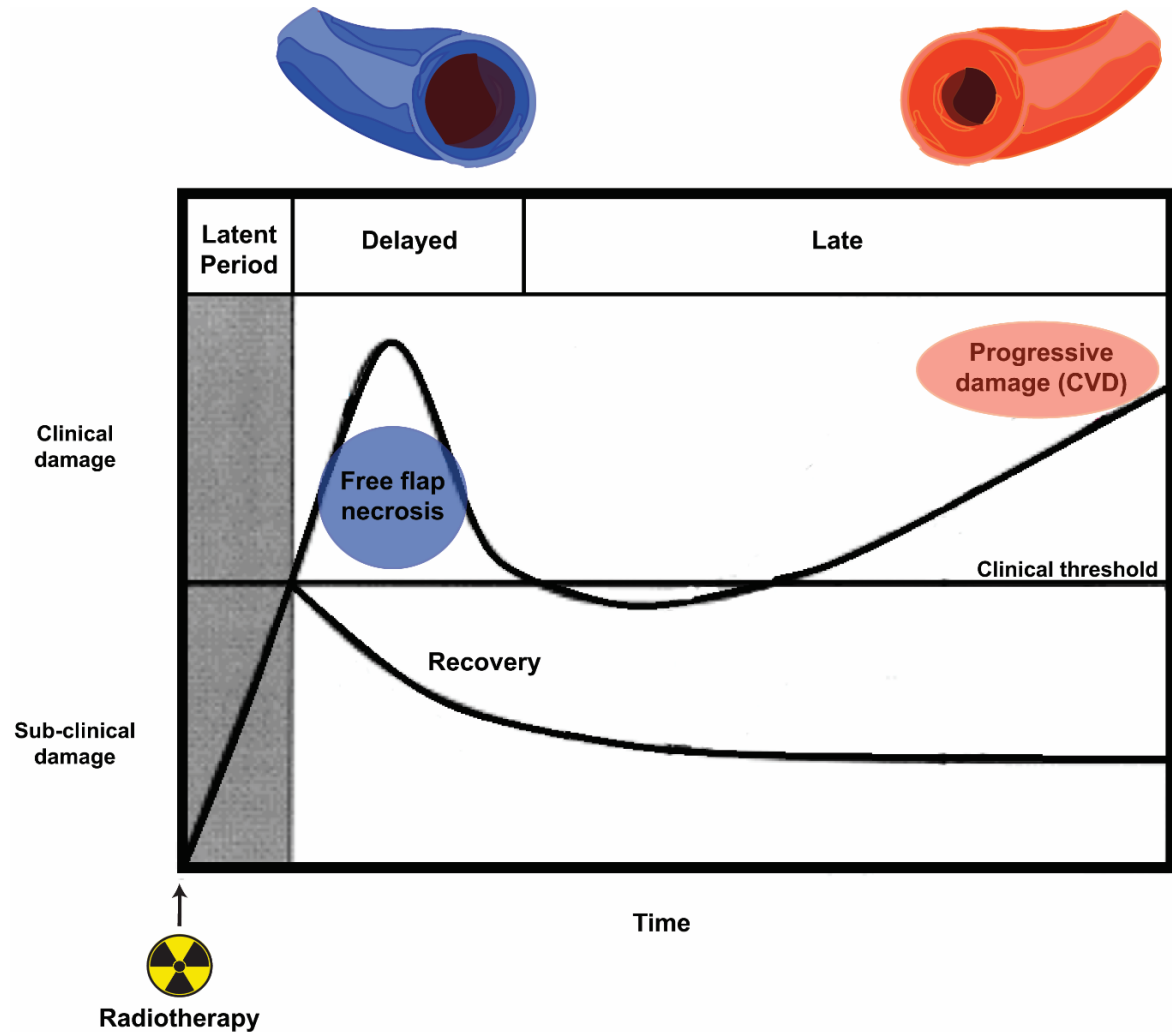


Figure 14. Vascular complications in the current study combined with an overview of general radiation-induced tissue damage. CVD = cardiovascular disease. Modified picture from Rubin, *Int J Radiat Oncol Biol Phys*, 1995.

Irradiated veins seem to be more prone to thrombosis during acute inflammation, unlike the chronic progressive inflammation that leads to arterial stenosis and occlusions in MI in previously irradiated left-sided breast cancer patients (190). The cohort included both primary and secondary reconstructions, which enabled investigation over a wide time-span after radiotherapy exposure. Nevertheless, no significant differences were observed between delayed and late reconstructions regarding arterial occlusion.

Experimental studies have showed evidence of up-regulation of pro-inflammatory cytokines and adhesion molecules together with activation of pro-thrombotic mediators in irradiated

human veins at similar time points as the occurrence of venous thrombosis in irradiated free flap anastomoses in our study (226, 235). Our data thus provided a link between clinical outcomes and biological findings. Other inflammatory conditions have also been linked to venous thrombosis in acute inflammatory states such as sepsis and trauma (31, 286), whereas arterial stenosis or occlusion often is a result of chronic inflammation. Thrombus formation and vascular irradiation damage have both been linked to endothelial dysfunction with subsequent inflammatory and pro-thrombotic properties (25, 31, 189, 226, 235, 242, 245-249, 287), which may be the promoter of venous thrombosis formation in patients with preoperative radiotherapy undergoing free flap reconstructions.

Paper I was limited by the retrospective design being that data were retrieved from a cohort collected over a wide timespan, which could be both an advantage and a disadvantage. A disadvantage is the fact that several surgeons with different surgical skills may affect the complication rate over time, however in 85% of the time, the same senior surgeon was present. The advantage is the possibility to increase the sample size, since a general problem is the rarity of vascular complications and reexplorations.

Taken together, we could, in this unique cohort of patients where more than 80% had undergone preoperative radiotherapy, show that radiotherapy is a risk factor for free flap necrosis in autologous free flap surgery in head and neck cancer patients. In the case of preoperative radiotherapy, we recommend microvascular reconstructions to be performed within 6 weeks after radiotherapy treatment in order to reduce the risk for venous thrombosis.

4.2 Inflammatory biomarkers in the vessel wall after radiotherapy (Paper II)

There were three major findings in **Paper II** as shown in Figure 15. First, we found a sustained upregulation of the acute phase protein and biomarker PTX3 in irradiated human arteries and veins compared to non-irradiated internal controls at a median of one year after radiotherapy exposure. Secondly, we demonstrated that PTX3 expression was predominantly seen into ECs in irradiated arteries and only occasionally in veins. Lastly, we noted a sustained up-regulation of IL-1 β with a linear correlation to PTX3 gene expression in irradiated arteries ($r=0.53$).

Pentraxin 3

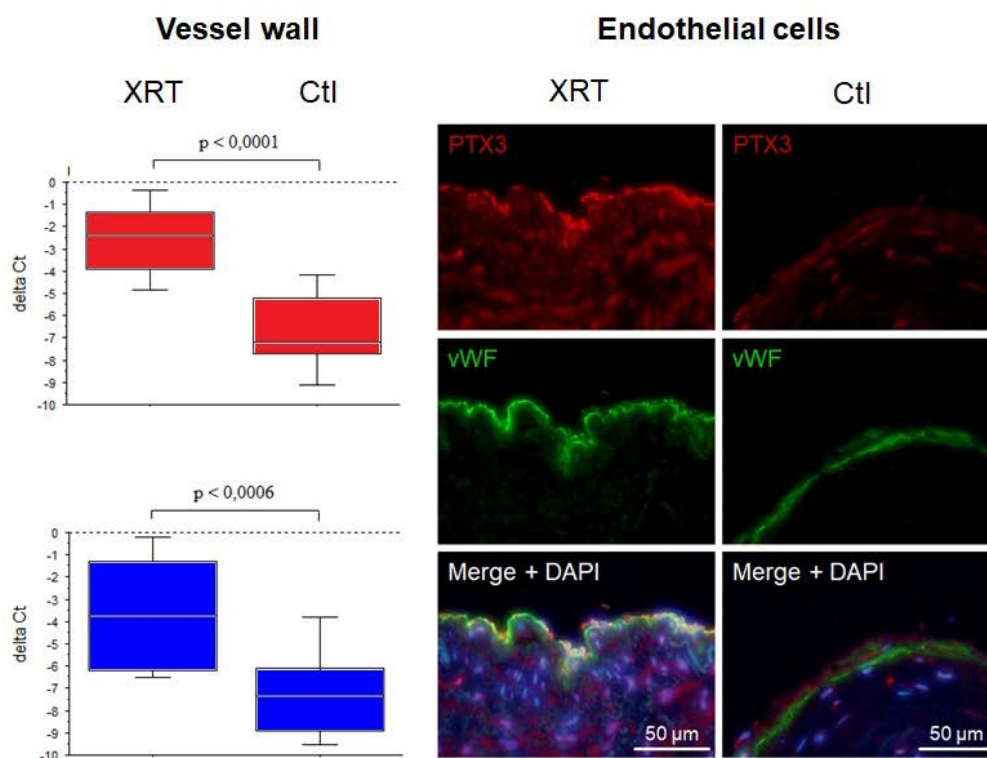


Figure 15. Paired human blood vessel biopsies of irradiated (XRT) and non-irradiated (Ctl) internal controls harvested at the same time during free flap surgery for cancer reconstruction. To the left, gene expression levels of PTX3, as measured by real-time PCR, for paired arteries (red) and veins (blue). To the right, immunofluorescence staining of PTX3 in paired arterial biopsies. DAPI was used for cell nuclei staining and vWF for endothelial staining. DAPI = 4',6-diamidino-2-phenylindole, PTX3 = pentraxin 3, vWF = von Willebrand factor. Adapted and reprinted with permission from J Transl Med (**Paper II**)

Radiotherapy-induced vascular inflammation was measured by PTX3 mRNA levels in both human arteries and veins at a median of one year after the last radiotherapy treatment. The results indicated that irradiation contributed to sustained innate inflammation of the vessel wall, which was mainly confined to the endothelium according to immunostainings. These are important features of both CVD and VTE development (32, 49, 288). Radiation caused lower PTX3 expression mRNA levels in veins compared to arteries, which may relate to either a thinner layer of PTX3-expressing SMCs or a generally lower sustained inflammatory response in veins (13). However, no further morphological double staining, with cell type markers, was performed in veins. The expression of PTX3 by arterial ECs may reflect ongoing ECs dysfunction (289, 290), which could promote inflammation (291, 292) and thrombus formation (99, 100, 293). In addition, the expression of PTX3 in the medial layer of irradiated arteries also supports the involvement of SMCs within radiation-induced arterial vascular disease (294, 295). The presence of PTX3 co-localisation with CD68+ macrophages further supports the observations of ongoing vascular inflammation in irradiated arteries, but could also reflect ongoing apoptosis of macrophages (296). Expression of IL-1 β mRNA was increased in human arteries after radiotherapy. Macrophages in the irradiated arterial wall may be the source of the pro-inflammatory cytokine IL-1 β (130), which can promote further PTX3 and IL-1 β expression not only by macrophages (96), but also PTX3 expression by SMCs and ECs (88-91). We could demonstrate a linear correlation between PTX3 and IL-1 β expression, which further supports IL-1 β -associated PTX3 expression. The cause of the increased expression of PTX3 and IL-1 β cannot be elucidated, but it is known that irradiation induces cell death and damage with the release of DAMPs, which may be the initiator of both PTX3 and IL-1 β expression by macrophages (297).

The posttranscriptional mediator microRNA-29b (miR 29b) is able to target PTX3 and thereby the innate immune response through repression of PTX3 protein translation (295, 298). We have recently shown that treatment with miR-29b enables a reduction of arterial inflammation as measured by a lower content of macrophages and reduced protein levels of PTX3 in irradiated *Apoe*^{-/-} mice (295). These results may suggest pro-atherosclerotic properties of PTX3 in radiation-induced vascular disease, however the role of PTX3 in CVD remains unclear (67, 299). Although both a detrimental (99, 100) and protective (67, 299, 300) role have been described, PTX3 has become an important biomarker of vascular inflammation diseases, such as CVD and vasculitis in both tissue and plasma (30, 96, 301-306).

One important limitation of the present study is that we performed gene expression analysis on the entire vessel wall, which makes it impossible to distinguish expression between different cells. However, this is partially compensated by IF, but further quantification analysis could reveal differences in expression by cells. Furthermore, comparison of gene expression levels between whole vessel wall arteries and veins is limited by the difference in cell composition, because arteries contain more SMCs. However, models investigating differences between human arteries and veins are lacking, and this model provides a unique possibility to compare chronic inflammation in human arteries with inflammation found in

veins. We believe that this study could contribute to a further understanding of not only radiation-induced vascular disease but also give insights into arterial versus venous chronic inflammation.

In summary, irradiation promoted a chronic vascular inflammatory response that involved arteries and veins found at different layers of the vascular wall. Especially, the morphological finding of EC expression of PTX3 in arteries was striking, and it reflects sustained EC inflammation, which may explain the clinical outcomes in **Paper I**. However, the role of PTX3 in radiation-induced vascular disease needs to be further investigated. The model used for this study may be a promising way to compare inflammatory responses between human arteries and veins.

4.3 The adventitia and leukotriene signaling in radiation-induced vascular inflammation (Paper III)

Paper III investigated the 5-LO/leukotriene signaling pathway in radiation-induced vascular inflammation. This was assessed by transcriptional and protein analysis of paired arterial biopsies of irradiated compared to non-irradiated internal controls from the same patient harvested during reconstructive free flap surgery.

Recent evidence highlights the role of leukotriene signalling in CVD (107, 124, 128), but another role of leukotriene signalling has also earlier been described in the context of radiation-induced tissue damage (307, 308). The initial hypothesis that the macroscopically observed vasa vasorum expansion could be related to leukotriene-associated inflammation of the adventitia as recently presented by Spanbroek et al. for coronary artery disease is supported by the findings in this study (309). Increased transcriptional levels of 5-LO and the BLT1 receptor were found in irradiated human arterial biopsies at all three layers of the vessel wall compared to non-irradiated internal controls. Irradiation promoted vasa vasorum expansion as measured by vWF expression of ECs within the adventitia. Furthermore, the presence of 5-LO+/CD68+ macrophages surrounding the vasa vasorum indicated that irradiation enabled the recruitment and activation of macrophages into the vessel wall. The presence of macrophages in the adventitial layer, at a median of one year after last radiotherapy treatment, indicated a chronic innate immune response. Monocytes differentiating into macrophages may be recruited through the vasa vasorum in the adventitial layer of the irradiated arteries. 5-LO expression was more prominent in irradiated arteries compared to non-irradiated arteries, and it was mainly confined to the adventitial and medial layer as measured by immunofluorescence staining, which furthermore shows vascular inflammation in both the media and adventitial layer. In order to further evaluate LT-signaling in these two respective layers, LTB₄ levels were analyzed in conditioned media by ELISA, which revealed significantly higher levels in the adventitia compared to the medial layer of irradiated arteries. Radiotherapy probably induces leukotriene-associated inflammation in the adventitia through activation of the LTB₄/BLT1 axis with subsequent promotion of inflammation and immune cell recruitment (111, 126, 127, 310). Thus, the activation of the LTB₄/BLT axis by irradiation may promote further LTB₄ production, which

could contribute to a chronic inflammatory state within the adventitia of the vessel wall. The release of LTB₄ in irradiated human arteries may explain the vasodilatation and leakage of cells into the adventitia by activation of ECs of the vasa vasorum (111).

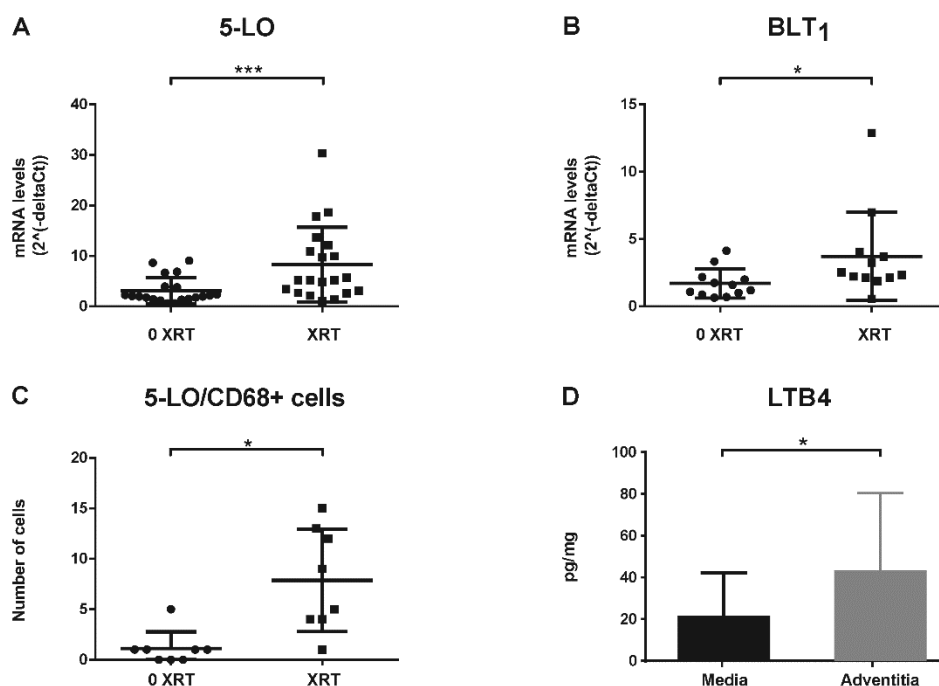


Figure 16. The 5-LO/Leukotriene axis is up-regulated in irradiated human arteries. Increased mRNA levels of 5-LO and BLT₁ in irradiated (XRT) human arteries compared to non-irradiated (0 XRT) internal controls (A-B). The number of CD68+ macrophages that co-localize with 5-LO as semiquantified by immunofluorescence (C). The leukotriene B₄ (LTB₄) was present in both media and adventitia in irradiated human arteries as measured by ELISA. Wilcoxon's sign-rank test was used for statistical analysis (A-C) and student's t-test LTB₄ (D). Reprinted with permission from FASEB J (Paper III)

The observed increase in leukotriene-associated inflammation in irradiated human arteries resembling the findings observed in atherosclerosis may therefore also be able to promote the development of later CVD. The role of the adventitia in the development of CVD disease is further supported by studies implying vessels lacking vasa vasorum are spared from atherosclerosis (311-313).

Anti-leukotriene therapy has shown promising therapeutic effects in the secondary prevention of stroke in humans (314), and furthermore it reduces the mortality rates in rats after thoracic irradiation (315). The previous findings in irradiated tissues (307, 308) and atherosclerosis (107, 124, 128) together with the observational findings in **Paper III** support the role of LT-associated inflammation and implicates a potential role for anti-leukotriene treatment in radiation-induced vascular inflammation, but further interventional studies are needed.

Finally, potential limitations need to be considered. First, the current study does not investigate other 5-LO-derived leukotrienes (e.g., cysteinyl-LTs) due to lack of human

material. Second, the time for radiotherapy exposure to the harvest of the biopsies varies from weeks to several years between patients, while clinical events, on the other hand, are not evident until years after a patient's radiotherapy treatment. Therefore, patients with a shorter duration between radiation and biopsy collection might not yet have developed late adverse effects.

In conclusion, leukotrienes have been found in radiation-induced tissue damage and CVD by promoting a pro-inflammatory state. The present study provides, for the first-time, evidence for a chronic inflammatory state by means of LT-signaling in the adventitia of irradiated human conduit arteries. The involvement of the 5-LO/leukotriene pathway in this process may warrant interventional studies, because drugs targeting this pathway are already available on the market. Future studies on the current topic are therefore required in order to validate and extend the present findings. Our results encourage future *in vivo* studies, and the methodology in **Paper IV** might serve as a base for future studies on anti-leukotriene-based treatments of radiation induced vascular inflammation.

4.4 A role for the IL-1 receptor antagonist anakinra in radiotherapy-induced vascular disease (Paper IV)

In **Paper IV**, there are three major findings. First, we showed an up-regulation of genes associated with inflammasome biology in irradiated human arteries compared to non-irradiated controls after a mean time of three years from last radiotherapy treatment. Secondly, we demonstrated that local irradiation of *ApoE* ^{-/-} mice presented a similar pro-inflammatory phenotype as observed in human-irradiated arteries. Thirdly, we showed that treatment with the recombinant IL-1Ra anakinra reduced the CCL2, CCL5 and I-Ab+ expression in irradiated mice compared to the non-treated mice.

The initial whole transcriptome analysis of radiated and non-irradiated human arteries identified genes in apoptosis pathways enriched after radiation. Cell death and loss of membrane integrity leads to secretion of pro-inflammatory mediators such as IL-1 α and debris from dying cells called DAMPs (316-318). Release of DAMPs and IL-1 α could potentially contribute to the subsequent activation of the NLRP3 inflammasome and the production of IL-1 β (130, 319) (320-322). However, radiotherapy could activate the NLRP3 inflammasome by inducing ROS formation and through the release of DAMPs and IL-1 α from dying and damaged cells triggered by other factors than apoptosis such as mitotic catastrophe and pyroptosis cell death (131, 323-327), but these factors were not further studied in this thesis. The up-regulation of IL-1 β and NLRP3 gene expression may reflect that the irradiation contributes selectively to priming and further to the activating of the NLRP3 inflammasome as demonstrated by the presence of caspase-1 protein in the radiated arteries. With subsequent production of mature IL-1 β in irradiated human arteries is inevitable. IL-1 β could further promote immune cell recruitment and migration by induced expression of adhesion molecules such as VCAM-1 and chemokines such as CCL2 and CCL5 (**Paper IV**) through activation of ECs (135, 136, 320, 328, 329). The increased number of macrophages in irradiated human arteries remained years after the last radiotherapy treatment may suggest

that irradiation injury drives a long-lasting process of IL-1 β generation as seen in **Paper II**. In turn, the chronic production of IL-1 β could promote both immune cell recruitment and further IL-1 β production by the NLRP3 inflammasome and thereby together with the release of IL-1 α from apoptotic cells driving the sterile chronic vascular inflammation in irradiated arteries (Figure 17).

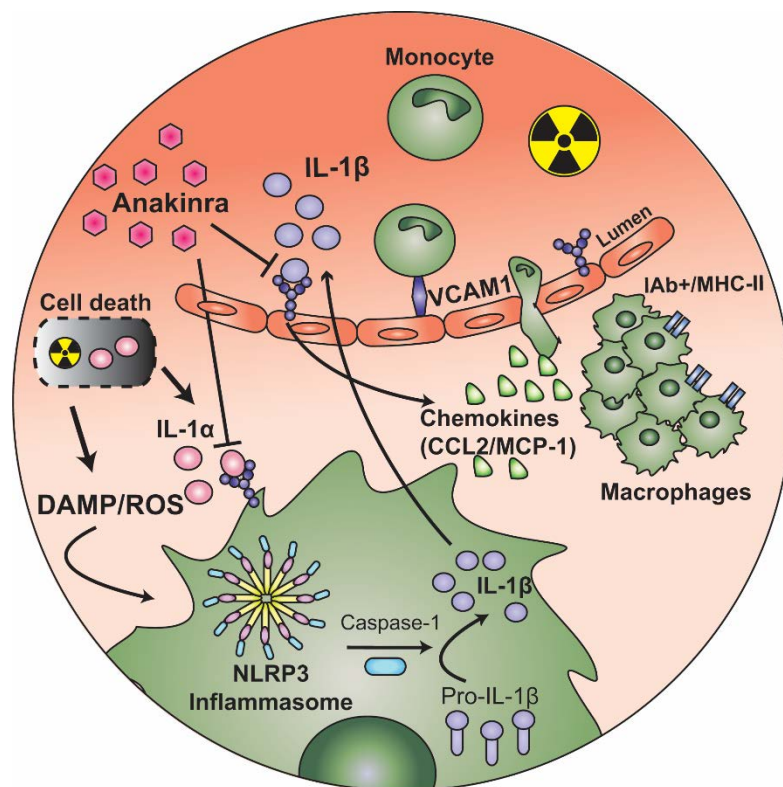


Figure 17. Anakinra treatment dampened the radiotherapy-induced vascular inflammation. Illustration by Tinna Christersdottir, reprinted with permission from European Heart Journal (**Paper IV**). Definitions are in the list of abbreviations.

We therefore came up with the hypothesis that inhibition of both IL-1 α and IL-1 β by the recombinant IL-1Ra anakinra may mediate radiation-induced vascular inflammation. An experimental mouse model was established with localized irradiation toward the neck and upper thorax and demonstrated a similar inflammatory phenotype as observed in irradiated human arteries (Figure 18). Two-week daily treatment with anakinra managed to dampen the long-term radiation-induced vascular inflammation in experimental mice as measured by reduced expression of CCL2, CCL5 in the thoracic aorta and the presence of pro-inflammatory I-Ab-presenting cells in the aortic root lesion. Nevertheless, irradiation did not accelerate atherosclerotic lesion size but rather showed smaller lesion sized in the aortic arch and similar sized between groups in the aortic root (**Paper IV**). Previous studies by others in experimental mice have not clearly showed that irradiation increased lesion size (224, 330). Other factors that are able to affect plaque stability independent of lesion size are circumference and the residual lumen size, however we did not show any differences between groups (**Paper IV**). We and others have found that irradiation may not increase

lesion size but rather seems to predispose the development of less stable atherosclerotic lesions that are characterised by a thinner fibrous cap, which is prone to hemorrhage (223), macrophage accumulation and vascular inflammation (**Paper IV**). Furthermore, plaque size in humans does not *per se* predict clinical events, but increased inflammation is associated with adverse clinical outcomes (331, 332). However, the cause of reduction in lesion size in the aortic arch is not fully clear but may be in line with decreased weight loss in irradiated mice.

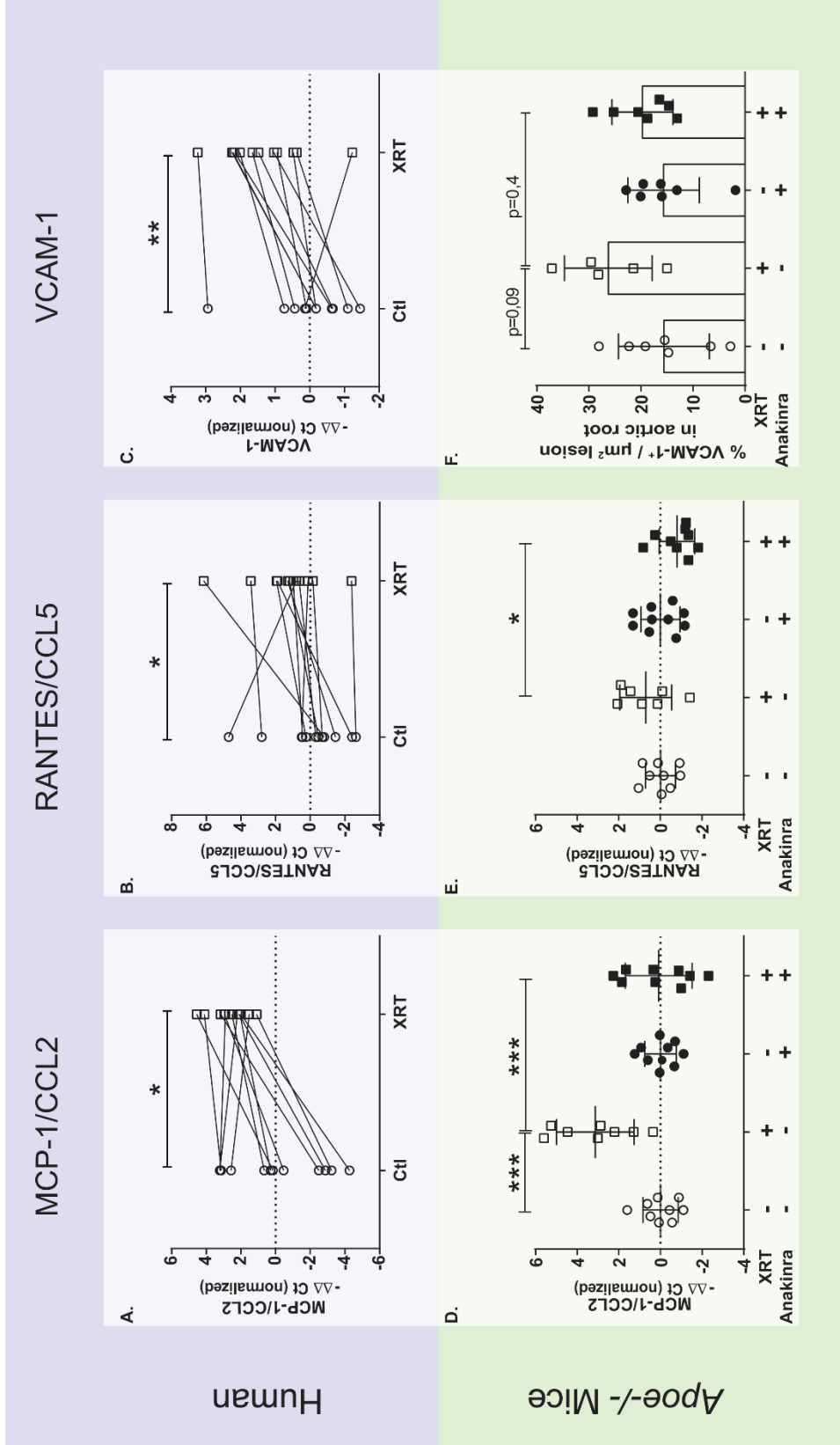


Figure 18. Intervention with IL-blockade in irradiated mice with partly similar vascular inflammatory phenotype as humans. A-E were analyzed using RT-qPCR. A-C human paired arterial biopsies from the BIRKa biobank. (D-E) Thoracic aorta from irradiated or sham treated *Apoe*^{-/-} mice with or without anakinra treatment. Data are presented as $-\Delta\Delta Ct$ normalized to housekeeping genes. (F) Aortic roots were cryosectioned and stained for VCAM-1 in *Apoe*^{-/-} mice with the same treatment groups as above. Differences between groups were analysed using the Wilcoxon signed rank test between paired vessels in humans (A-C) and using one-way ANOVA followed by Tukey post-hoc analysis between treatment groups in mice (D-F). * $p \leq 05$; ** $p \leq 01$; *** $p \leq 001$. Adapted and reprinted with permission from European Heart Journal (**Paper IV**). Definitions are in the list of abbreviations.

One limitation of the study was that it was not specifically designed to investigate the dose-time response of anti-IL-1 treatment in radiation-induced vascular inflammation. In addition, mice at 20-23 weeks of age correspond to a mature adult human but not to the average age for primary CVD event. Another limitation is that human arteries were collected years after radiation exposure, whereas in our mice model, the sample harvest was performed after 10 weeks. However, in terms of age, the two time spans may anyway be comparable, since 10 mouse-weeks may correspond to several human years according to the Jackson laboratory Dr Hagen. Patients exposed to irradiation develop CVD at an earlier age, and the increased risk starts already within the first 5 years after radiotherapy (190). The atherosclerotic lesions in irradiated mice did not correspond to normal lesion development in this atherosclerotic-prone mouse model, which may weaken the validity of this atherosclerotic model. However, atherosclerotic lesion size may not be an appropriate endpoint for radiation-induced vascular disease that seems to be marked by full vessel wall inflammation. Furthermore, there is a limited vessel length that could be harvested in each patient in order to perform the microvascular surgery safely and therefore limits the possibility to evaluate transcript and protein levels from the same patient biopsies. The small sample size is a limitation of the presented WB analysis, but due to ethical and surgical constraints, we have only been able to use a limited number of human arterial biopsies for confirming protein analysis. Therefore, no statistical analysis was performed on protein data. Another limitation was that the human cohort had a clear male dominance, which however, is in accordance with epidemiological data. In contrast, we used only female mice in our study, because, in general, female mice have larger aortic root lesion areas than male mice regardless of diet and genetic background. This study does not take into account any sex differences, and the choice of female mice was based on the requirement to get a significant athero-development within the given 10-week study period (262).

The current study extends previous findings from our biobank (25) in a more chronic cohort and supports the notion that irradiation induced persistent vascular inflammation. This study is a first step towards enhancing our understanding of anti-IL-1 treatment for radiation-induced vascular disease. However, further studies are needed in order to fully understand the role of the NLRP3-IL-1 axis and anti-IL-1 treatment against radiation-induced vascular disease, due to safety aspects for cancer patients.

5 GENERAL DISCUSSION

Vascular inflammation has been linked to both CVD and VTE (1, 2). In this thesis, we show that irradiation induces vascular inflammation in both human arteries and veins (**Paper II-IV**), which may promote occlusion of blood vessel with subsequent clinical events such as flap failure (**Paper I**). Furthermore, we have found that radiotherapy seems to induce a chronic inflammatory response in all three layers of the human arterial vessel wall (**Paper II-III**). Others have described radiation-induced vascular injury to have similarities to atherosclerosis (3). We rather noted general vessel wall inflammation, arteriosclerosis, where anti-inflammatory treatment with anakinra was able to dampen the radiation-induced vascular inflammatory response in mice (**Paper IV**). Results presented in this thesis demonstrate that irradiation induces ECs activation in both the arterial intima and adventitia with an increased number of infiltrating immune cells (**Paper II-IV**) that may explain intima hyperplasia formation in irradiated arteries as shown by others (4). In this thesis, we have extended previous research in our group (5) to include analyses of the whole vessel wall as discussed below. In **Paper IV**, we made transcriptional analyses of human arteries with chronic radiation injury. Our findings in humans led to the development of an experimental murine model of radiation injury, where we further studied the potential to inhibit the vascular inflammation by anti-inflammatory treatment with anakinra.

5.1 Endothelial cell dysfunction and vascular complications

EC inflammation is considered a key component of both atherosclerosis-related CVD and VTE (31, 333). Radiotherapy might be a promotor of EC activation and thrombus formation partly through induced expression of several pro-inflammatory and pro-thrombotic mediators such as PTX3 (**Paper II**) (99, 100), leukotriene receptor BLT1 (**Paper III**), adhesion molecules (VCAM-1) and chemokines (CCL2, CCL5) (**Paper IV**) (Figure 18). The recruitment and transmigration of inflammatory cells (i.e., leukocytes and monocytes) into the vascular wall together with platelet adhesion (334) and thromboxane A2 formation (335) may explain both radiation-induced CVD and vascular complications in free flap anastomosis observed in **Paper I**. Although EC dysfunction may be involved in both arterial and venous disease, the onset of clinical events from radiation exposure seems to differ between the respective vessel type as seen in **Paper I**. Venous ECs, compared to arterial ECs, can rapidly accumulate leukocytes, which, together with subsequent red blood cell aggregation (336), may explain venous thrombosis and the temporal differences between clinical outcomes in arteries and veins (18). Others have associated radiation-induced vascular injury with EC dysfunction by means of increased PAI-1 expression (226, 235), reduced eNOS bioavailability (226) and reduced flow-mediated dilatation (FMD) (337) further supporting the role of EC dysfunction (Figure 19). tPA treatment can counteract an impaired fibrinolysis, which may explain the improved free flap salvage rates observed in **Paper I**, but further prospective studies are needed to confirm this observational finding. Taken together, the

studies in the current thesis (Figure 19, red) together with other (Figure 19, orange) studies on radiation-induced vascular damage shows harmful effects on ECs.

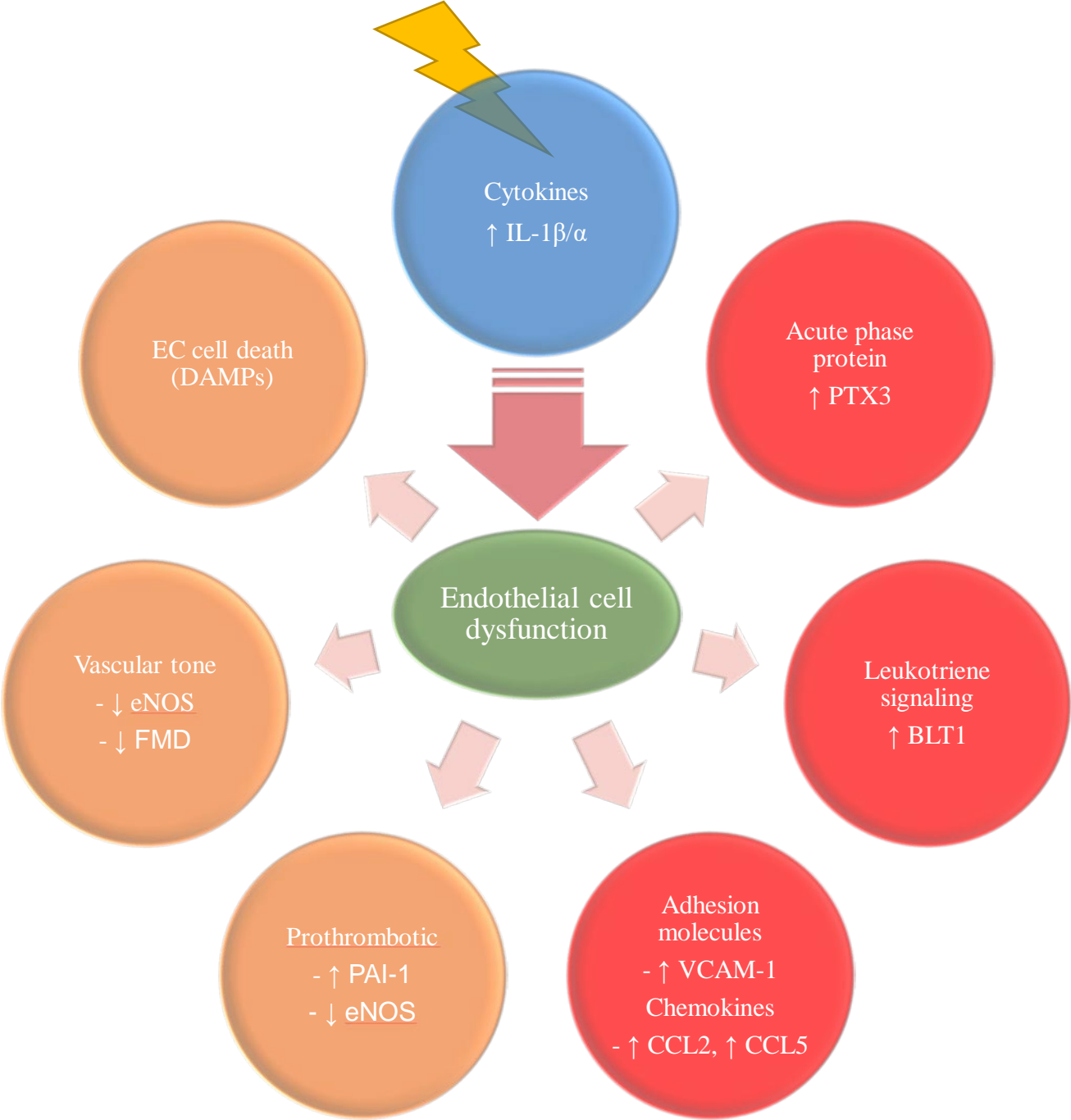


Figure 19. Radiation-induced EC dysfunction. Red = studies from the present paper (Paper II-IV). Orange = From this thesis's references (226, 235, 242, 324, 337-339). Definitions are in the list of abbreviations.

5.2 Smooth muscle cells and vascular remodelling

Irradiation may induce late vascular remodelling of the intima-media layer in response to chronic inflammation. Russel and co-workers showed that radiotherapy contributed to the development of intima hyperplasia in human arteries (340), and intima-media thickening has furthermore been observed in carotid arteries after head and neck irradiation in humans (341). In **Paper II**, we observed increased PTX3 expression in irradiated arteries, which was partly confined to SMCs. However, the development of remodelling has not been further analysed in this thesis. We could also show that irradiated human arterial SMCs express LTB₄ (**Paper III**) and could therefore potentially migrate and proliferate in response to LTB₄/BLT1 signalling as described by Bäck et al. (125). Radiotherapy-induced IL-1 β expression observed in **Paper II** and **IV** could further potentially induce BLT1 expression and thus promote an LTB₄/BLT1 interaction (125).

5.3 Adventitia inflammation and vasa vasorum expansion

The adventitia, as the outermost layer of the arterial wall, has recently drawn attention within atherosclerosis research (124, 128, 311, 342). We showed that radiotherapy drives adventitial inflammation through leukotriene production promoted by the LTB₄/BLT1 axis and 5-LO expression in human arteries (**Paper III**). LTB₄ was present in the adventitia of irradiated human arteries (**Paper III**) and may promote monocyte recruitment into the adventitia through the vasa vasorum (126). Irradiated macrophages expressed 5-LO (**Paper III**) and may thereby contribute to further production of LTB₄ with subsequent monocyte/macrophage recruitment (126, 343). Irradiation induces a pro-inflammatory response that enables the recruitment of immune cells not only from the traditional luminal side, but also through the outer layers possibly through the vasa vasorum. Some recent publications have supported that vascular inflammation is initiated in the adventitia and progresses towards the intima, which is a hypothesis that has been coined the “outside-in” theory (344-346). However, whether radiation-induced cell recruitment is initiated from the vascular lumen or the adventitia or both is still undetermined.

5.4 Macrophages and the vicious cycle of chronic inflammation

Macrophages present years after the last radiotherapy session, in both the subintimal layer and around vasa vasorum of the adventitia (**Paper III, IV**), could potentially have been recruited from either the vascular lumen or through outer layers of the vessel wall where an expansion of the vasa vasorum has been seen. Macrophages in the vascular wall may perpetuate a sustained innate inflammatory response by production of pro-inflammatory cytokines such as IL-1 β that can stimulate NLRP3 inflammasome activation, LTB₄ and CCL2 production by macrophages and VCAM-1 expression by activated ECs, which thereby may lead to further recruitment of macrophages (63, 122, 130, 233). This vicious cycle may even induce cell death (325). Furthermore, LTB₄ has been shown to promote IL-1 β production by activation of NLRP3 inflammasome through ROS production (347, 348). NLRP3 inflammasome activity was observed in irradiated human arteries by gene array analysis, and the activation was further supported by the presence of caspase-1 protein

expression in **Paper IV**. NLRP3 activation may further promote production of the pro-inflammatory cytokine IL-1 β by activated macrophages (323). Radiation may also induce cell death with a loss of membrane integrity and the release of the pro-inflammatory cytokine IL-1 α and DAMPs (349). A hypothetical, radiation-induced IL-1 α and DAMP release might be able to interact with resident macrophages and induce NLRP3 inflammasome activation (323). Others have suggested that radiation might induce NLRP3-dependent pyroptosis (325), ROS formation (327, 350) and mitotic catastrophe (324), but these aspects have not been further studied in this thesis. Taken together our transcriptional findings support priming of the NLRP3 inflammasome where increased protein caspase-1 further could support an activation.

The resolution of inflammation seems to fail even though the initial trigger of radiotherapy is eliminated, and a chronic inflammatory state is, thus, established. This was clearly demonstrated in this thesis by means of PTX3 expression at a median time of one year (**Paper II**), 5-LO at one year (**Paper III**) and NLRP3 activity at three years (**Paper IV**) after radiotherapy exposure. Macrophages are APCs (351) and are therefore together with radiation-induced I-Ab (MHC II) expression (**Paper IV**) able to interact with T-lymphocytes recruited by CCL5 and LTB4 (352, 353) and thus induce an adaptive immune response. However, the presence and role of the adaptive immune system has not been further investigated in this thesis.

5.5 Therapy and future directions

5.5.1.1 Targets

Anakinra neutralized the innate immune response in irradiated mouse arteries by inhibition of IL-1/IL1R signalling (Figure 20) and thereby potentially reduced activation of ECs and their production of CCL2, CCL5, VCAM-1, PTX3 and BLT1 (**Paper II-IV**). Furthermore, it can be speculated if IL-1/IL1R can reduce macrophage production of CCL2, CCL5 and PTX3 with subsequent reduction of infiltrating immune cells. A limitation is that gene expression only have been studied in full arterial wall biopsies and may thus be expressed by ECs and macrophages as well as other cell types. Anakinra can theoretically reduce immune cell recruitment to the irradiated arterial wall, which may enable inflammatory resolution as indicated by the reduced presence of pro-inflammatory I-Ab (MHC II) presenting cells in anakinra-treated irradiated mice (**Paper IV**). Furthermore, inhibition of IL-1/IL1R signalling may reduce SMC migration and proliferation by reduced IL-1 β induced BLT1 expression by SMCs. Hypothetically, anakinra may reduce radiation-induced casp-1 expression (**Paper IV**) and thereby decrease further production of IL-1 β and apoptosis as seen in another study (139). However, this was not further investigated within this thesis.

5.5.1.2 Treatment dose, timing and duration

A two-week inhibition of IL-1/IL1R signalling may be sufficient to inhibit the transition from acute to chronic inflammation by limiting the recruitment of innate immune cells to the site of irradiation in mice as seen in **Paper IV**. The importance of initiating treatment at an early

stage is supported by previous published data showing lack of therapeutic effect of the drug thalidomide, which partly reduces the IL-1 response, when treated at a later stage of radiation-induced heart disease in mice (250).

The inhibition of IL-1/IL1R signalling treatment dose and duration (3.7 years) for secondary CVD prevention presented in the CANTOS in humans differ from our concept of primary prevention in irradiated mice. Smaller pilot studies in humans with short-term 2-week anakinra treatment did not manage to prevent secondary CVD as seen in CANTOS (354, 355). In contrast to humans, lifelong complete inhibition of IL-1 in mice did promote formation of unstable plaques (282). These results suggest there are differences between species. Compared to CVD triggered by irradiation, patients at risk for recurrent CVD due to atherosclerosis already have an established chronic inflammation at treatment start, which may require a longer treatment period. In addition, “the earlier the better” is the concept of anti-inflammatory treatment in RA patients (356). Compared to the slow progression of atherosclerotic disease in traditional CVD (55) RA is a chronic disease with acute or subacute onset (356) and therefore share similarities with disease development in radiation-induced CVD. An early and short treatment period with a high dose anakinra may dampen the acute inflammatory response and therefore reduce the subsequent chronic inflammation when the trigger is evident. Thus, an acute and short treatment period is not likely to work in already established CVD, when the chronic inflammation of atherosclerosis is advanced. A short treatment period in cancer patients is preferable, because it is less likely to interfere with tumour treatment. We, therefore, tested the concept of a short treatment period directly after radiotherapy exposure. In mice, we used anakinra treatment with a relatively high dose compared to the dose used in RA patients, but the dose was in line with other mouse experiments of anakinra treatment against acute MI (139, 141). Nevertheless, lower doses in line with RA treatment may be sufficient in order to dampen radiation-induced vascular inflammation. Therefore, further studies in a dose-dependent manner are needed in order to evaluate optional dose and treatment-duration to prevent radiation-induced CVD.

5.5.1.3 Future directions

The recently published CANTOS contributes to a new era in CVD medicine by promoting anti-inflammatory treatment against secondary CVD. This thesis provides encouraging results for anti-IL-1 treatment also in the context of radiation-induced CVD. Furthermore, radiation-induced vascular disease promotes a more general vascular inflammation that involves the whole vessel wall (**Paper II-IV**) rather than progressive, lipid-rich, large atherosclerotic lesions, which further supports a role for anti-inflammatory treatment in radiation-induced vascular disease.

If a radiation-induced CVD treatment should be introduced, then it should not jeopardize the tumour treatment. Radiotherapy induces genotoxic effects on both tumour and healthy cells and thereby promote apoptosis, cell senescence, pyroptosis, mitotic catastrophe and ROS formation (324, 325, 327, 338, 357). Inhibition of these secondary effects before or during radiotherapy may therefore lead to a risk of hampering the anti-tumour effect. Our concept of

a post-radiotherapy treatment with an anti-inflammatory drug could possibly reduce secondary cell death and inflammatory damage, but the effects on tumour cells must be carefully investigated before any clinical trials can be conducted. Human studies with inhibition of IL-1/IL1R signalling have shown no or decreased cancer incidence, however, none of these studies primarily aimed to investigate cancer incidence and outcome (358-361). It can be speculated that the reduction of CCL2 by anakinra seen in **Paper IV** may reduce tumour formation, progression and metastasis formation as previously described (362), but anakinra could also inhibit the direct cytotoxic effects against tumour cells by decreasing the number of CCL2 recruited macrophages (363).

On one hand, CANTOS highlighted important side-effects by registration of an increased incidence of severe infections and sepsis in the anti-IL-1-treated canakinumab group (49). On the other hand, a reduced risk of all-cause, 28-day mortality rates in patients treated with anakinra during septic shock has been described (358). A benefit of anakinra treatment is that it is a competitive inhibitor and therefore mediates instead of completely blocks IL-1 signalling. Innate immune functions, such as monocyte/macrophage recruitment, could therefore be preserved to a certain extent (364).

Taken together, the studies in the current thesis together with other studies on radiation-induced vascular damage shows detrimental effects on ECs and chronic activation of innate immune functions. If treatment with anti-IL-1 inhibition would be used as standard preventive treatment, then it could potentially have an effect on various surgical complications, normal tissue damage and CVD-related to radiation-induced vascular inflammation. However, further studies are needed before IL-1 inhibition and other targets can be tested in a human cancer setting. We believe that the studied translational model can be modified and used in the search for other new therapies within the field of radiation-induced vascular inflammation (Figure 20).

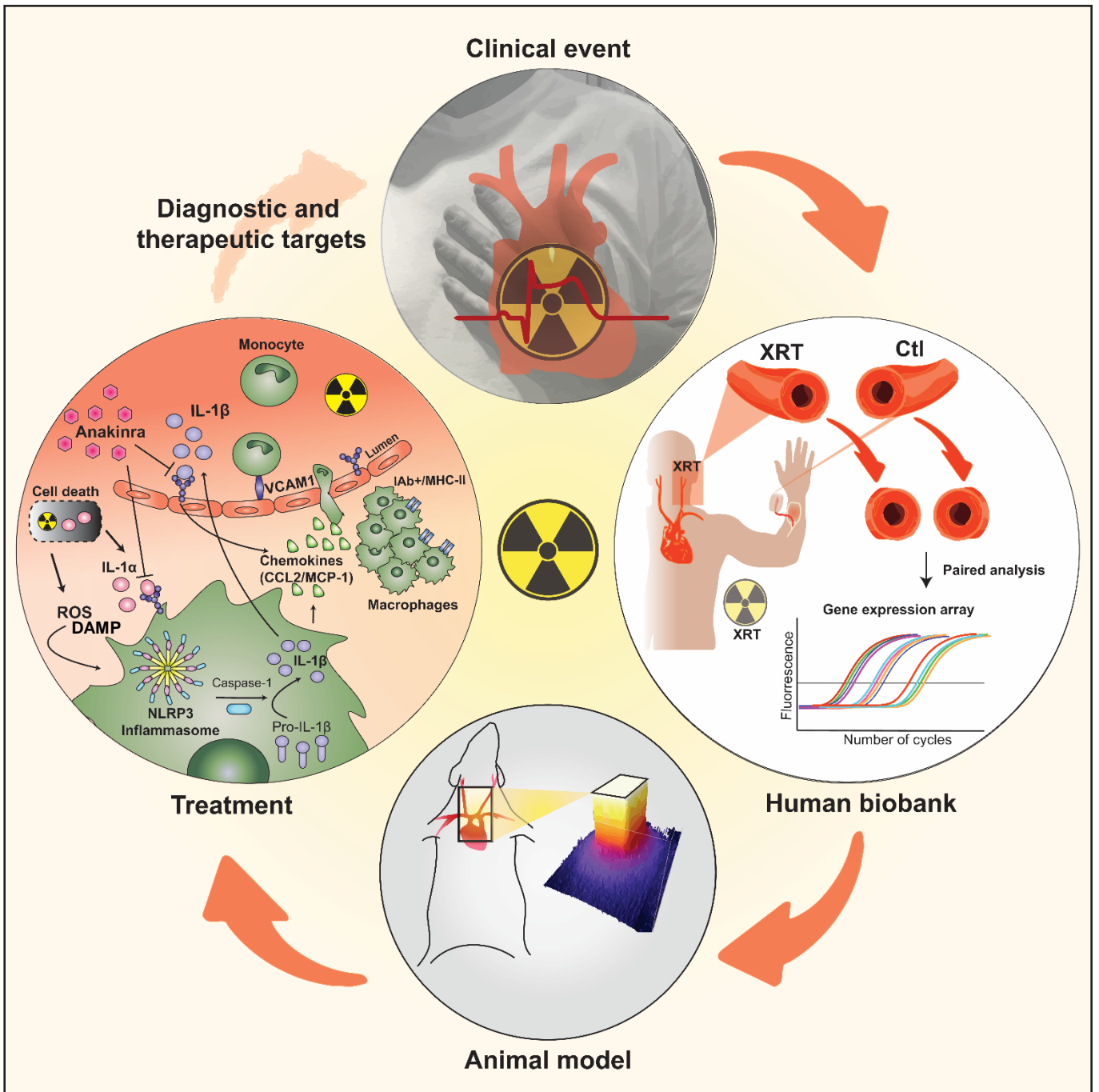


Figure 20. A translational model for therapeutic target discovery in radiotherapy-induced vascular disease. Illustration by Tinna Christersdottir, reprinted with permission from European Heart Journal (**Paper IV**). Definitions are in the list of abbreviations.

6 CONCLUSIONS

The conclusions of the Papers were:

Paper I. Preoperative radiotherapy increases the risk for head and neck free flap necrosis. Venous thrombosis was the dominating vascular complication, most common in delayed reconstructions, 6-15 weeks from last radiotherapy session.

Paper II. *PTX3*, a marker of vascular inflammation and innate immunity, is up-regulated in both arteries and veins years after last radiotherapy session. *PTX3* is expressed by macrophages, ECs and SMC in irradiated arteries.

Paper III. Radiotherapy induces a chronic inflammatory response in the arterial adventitia by means of up-regulation of the pro-inflammatory 5-LO/BLT₄ axis.

Paper IV. Radiotherapy induces the *NLRP3-IL1* axis in human arteries. IL-1 inhibition reduces the vascular inflammatory response in an *ApoE*^{-/-} mouse model of partial body irradiation that mimics the human phenotype.

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