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Late effects on primary murine heart and lung endothelial cells after *in vivo* heart irradiation

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Die wirksamste Medizin
ist die natürliche Heilkraft,
die im Inneren
eines jeden von uns liegt.

Hippokrates

Für meine Mutter

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ABBREVIATIONS

γ-H2AX	γ-Histone 2AX
µm	Micrometer
%	Percent
2D	2 Dimensional
3D	3 Dimensional
5-HT	Serotonin Receptor
5-HT1A	5-Hydroxytryptamine Receptor 1
ADP	Adenosine Di-Phosphate
ALK-1	Anaplastic Lymphoma Kinase 1
Arg	Arginine
Asp	Aspartate
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Tri-Phosphate
CBCT	Cone Beam Computed Tomography
CBD	Cannabidiol
CD	Cluster of Differentiation
cGMP	Cyclic Guanosine Mono-Phosphate
CT	Computed Tomography
CVD	CardioVascular Disease
DIBH	Deep-Inspiration Breath-Hold
DNA	Deoxyribo-Nucleic Acid
DSB-X	Biotin
DSBs	Double Stranded Breaks
EC	Endothelial Cell
ECG	Electrocardiogram
ECs	Endothelial Cells
EDHF	Endothelial Derived Hyperpolarization Factor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmatic Reticulum

FACS	Fluorescence Activated Cell Sorting
FAT	Fatty Acid Transferase
FCS	Fetal Calf Serum
FITC	Fluorescein-5-Isothiocyanate
GP	Glycoprotein
Gly	Glycine
Gy	Gray
H2A	Histone 2A
H2AX	Histone 2AX
HBSS	Hank's Balance Salt Solution
HCAM	Homing-Cell Adhesion Molecule
HDL	High-Density Lipoprotein
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
	β-Hydroxy-β-Methylglutaryl-Coenzyme A
HO-1	Heme Oxygenase-1
ICAM-1	Intercellular Adhesion Molecule-1
ICAM-2	Intercellurlar Adhesion Molecule-2
IFN-γ	Interferon-γ
IL-1	Interleukin-1
kg	Kilogram
kV	Kilo Voltage
LAD	Left-Anterior Descending Artery
LCFA	Long-Chain Fatty Acids
LFA-1	Lymphocyte Function Associated Antigen-1
LDL	Low Density Lipoprotein
LSS	Life Span Study
mA	Milli Ampere
MACS	Magnetic-Activated Cell Separation
mfi	Mean Fluorescence Intensity
MiR-208a	Micro-RNA 208a
mm	Millimeter
mm^2	Square Millimeter
m^2	Square Meter

mSv	MilliSievert
nm	Nanometer
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
NT-pro BNP	N-Terminal per Brain Natriuretic Peptide
ox-LDL	Oxidized-Low Density Lipoprotein
PBS	Phosphate-Buffered Saline
PCSK-9	Proprotein Convertase Subtilisin/Kexin type-9
PE	Phycoerythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PI	Proproium Iodid
PPAR	Peroxisome Proliferator-Activated Receptor
RGD	Arginine (Arg)-Glycine (Gly)-Aspartate (Asp)
RICVD	Radiation-Induced CardioVascular Disease
RILD	Radiation-Induced Lung Disease
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rounds Per Minute
SARRP	Small Animal Radiation Research Platform
SCARB3	Scavenger Receptor B3
SREBP2	Sterol Regulatory Element-Binding Protein 2
sICAM	Soluble Intercellular Adhesion Molecule
t-PA	Tissue-Plasminogen Activator
TF	Tissue Factor
TGF-ß1	Transforming Growth Factor-ß1
THC	Tetrahydrocannabinol
TNF-α	Tumor Necrosis Factor-α
TRPV1	Transient Receptor Potential Cation Channel Subfamily V member 1
u-PA	Urokinase Plasminogen Activator
V10	Volume of Normal Lung Receiving 10 Gy
V16	Volume of Normal Lung Receiving 16 Gy
V20	Volume of Normal Lung Receiving 20 Gy
VCAM-1	Vascular Cell Adhesion Molecule-1

VE-cadherin	Vascular Endothelium-Cadherin
vWF	von Willebrand Factor
x-rays	Energetic High-Frequency Electromagnetic Radiation

SUMMARY

Radiation therapy is an essential component in the fight against cancer in 21st century. More than 50% of all patients with solid tumors are treated with radiation therapy. However, this treatment is also associated with undesirable late side effects, which have now been the subject of numerous studies. For example, when breast cancer patients are irradiated, part of the heart and lungs are in the field of the x-rays. Thus, the standard postoperative therapy for breast conservation treatment according to guidelines can lead to serious long-term side effects: The risk of developing ischemic cardiovascular diseases, such as myocardial infarction is significantly increased decades after radiotherapy.

Therefore, the aim of this study was to investigate the pathomechanism which cause late radiation damages in lung and heart endothelial cells (ECs) in a mouse model.

Previously it has been shown that 5 - 20 weeks after irradiation of the heart with 8 Gy causes damages in ECs of the heart in mice. The goal of my study was to assess very late (20 - 50 weeks after radiotherapy) irradiation effects on primary ECs of the heart and lung of mice after *in vivo* irradiation. Therefore, the heart and one lung of the mice was irradiated with 8 and 16 Gy using the CT-image guided "Small Animal Research Radiation Platform" (SARRP), *in vivo.* At time intervals of 20, 30, 40 and 50 weeks after irradiation primary murine ECs from the heart and the irradiated as well as the unirradiated lung were extracted with the newly developed isolation method for primary ECs and analyzed phenotypically for different cell surface markers by flow cytometry.

The tested cell surface markers for ECs could be assigned into 4 different groups: Markers of proliferation (*Integrin ß3, VE-cadherin, Endoglin*), progenitor cells (*Mucosialin*), inflammation (*PECAM-1, HCAM, ICAM-1/2, VCAM-1*) and lipid metabolism (*CD36)*. The analysis showed a long-term upregulation of inflammatory markers on ECs of the irradiated heart and lung, after *in vivo* irradiation with 8 and 16 Gy. Additionally, *CD36,* which is associated with the development of atherosclerosis, was up-regulated after low (8 Gy) and high radiation doses (16 Gy) at nearly all measured points in time (20 - 50 weeks) in heart ECs. *Endoglin* was the only proliferation marker that showed a significant increase in isolated heart ECs after an irradiation with 16 Gy for 20, 30, 40 and 50 weeks. The stem cell marker *Mucosialin* on the other hand remained unaltered in heart and lung ECs and thus did not show any radiation induced alterations.

In a mouse model the late side effects of radiotherapy were shown. The pathomechanism is based on radiation-induced EC dysfunction. Damage to the vessel wall leads to chronic inflammation and promotes the development of atherosclerotic plaques. As the blood flow is severely reduced by the obstructions, the risk of cardiac ischemia increases over time. However, the irradiated lung ECs also showed chronic inflammatory damages after a high radiation dose. These findings could explain an increased risk for pulmonary fibrosis after irradiation.
In humans, radiation induced cardiovascular diseases mostly occur within the first decades after irradiation. This also affects younger patients without typical cardiac risk factors. Therefore, the first decades after radiation therapy should also include regular inspections of the heart in addition to the tumor control in the follow-up period.

Presently anti-inflammatory drugs are under investigation for the primary prevention of radiation-induced cardiovascular diseases. Cannabidiol, with its anti-inflammatory and anti-oxidative effects, is one of the drugs which has been tested and has shown first promising results in mice.

ZUSAMMENFASSUNG

Die Strahlentherapie ist ein essenzieller Bestandteil bei der Bekämpfung von Tumoren in der Medizin des 21. Jahrhunderts. Sie ist aus der Praxis der Krebsbehandlung nicht mehr wegzudenken und führt bei vielen Patienten zu einer Tumorkontrolle. Mehr als 50% aller Patienten mit soliden Tumoren erhalten im Verlauf ihrer Tumorbehandlung eine Strahlentherapie. Allerdings treten nach einer Behandlung mit ionisierenden Strahlen auch unerwünschte Spätfolgen auf, mit denen sich mittlerweile zahlreiche Studien beschäftigen. So wird beispielsweise bei der Bestrahlung von Brustkrebspatientinnen (speziell bei Patientinnen mit linksseitigem Tumor) zumeist auch ein Teil des Herzens und der Lunge mitbestrahlt. Die leitliniengerechte postoperative Standardtherapie bei brusterhaltender Therapie kann somit zu gravierenden Spätfolgen führen: Das Risiko ischämische kardiovaskuläre Erkrankungen wie beispielsweise einen Herzinfarkt zu entwickeln, steigt 10 Jahre nach einer Strahlentherapie der Brust signifikant an.

Ziel der Dissertation ist es daher, in einem Mausmodell die Pathomechanismen, die späte Strahlenschäden in Herz und Lungenendothelzellen verursachen, zu erforschen.

Es konnten bereits Veränderungen an Lungen und Herzendothelzellen von Mäusen 5 - 20 Wochen nach Bestrahlung des Herzens mit 8 Gy nachgewiesen werden. Für die Analyse der späten Strahlenschäden wurde in dieser Arbeit der Zeitraum von 20 - 50 Wochen nach Strahlenbehandlung ausgewählt. Hierfür wurde mit Hilfe einer "Small Animal Research Radiation Platform" (SARRP) ein CT angefertigt und basierend auf den Scans eine *in vivo* Bestrahlung des Herzens und eines Lungenflügels mit 8 oder 16 Gy durchgeführt. In Zeitabständen von 20, 30, 40 und 50 Wochen nach Bestrahlung wurden die primären Endothelzellen des Herzens sowie der bestrahlten sowie unbestrahlten Lunge mit einer neu entwickelten Isolationsmethode extrahiert. Die isolierten Endothelzellen wurden hinsichtlich unterschiedlicher Zelloberflächenmerkmale durchflusszytometrisch untersucht.

Die analysierten Oberflächenmarker wurden dabei vier unterschiedliche Gruppen zugeordnet: Marker für die Proliferation (*Integrinß3, VE-cadherin und Endoglin*), für Stammzellen (*Mucosialin*), für Entzündungen (*PECAM-1, HCAM, ICAM-1/2, VCAM-1*) und für den Fettstoffwechsel (*FAT*). Bei dieser Analyse konnte eine dauerhafte Hochregulation von Entzündungsmarkern des Herzens und der bestrahlten Lungen nach einer Herzbestrahlung mit 8 und 16 Gy nachgewiesen werden. Zusätzlich war *CD36* für niedrige (8 Gy) und hohen Strahlendosen (16 Gy) an

nahezu allen gemessenen Zeitpunkten in Herzendothelzellen erhöht. Als einziger Proliferationsmarker zeigte *Endoglin* in extrahierten Herzendothelzellen eine signifikante Erhöhung nach 20, 30, 40 und 50 Wochen und einer Bestrahlung von 16 Gy. Der Stammzell-Marker *Mucosialin* hingegen zeigte weder im Herzen noch in den Lungen Veränderungen in der Expressionsstärke.

Anhand eines Mausmodells konnten Spätfolgen der Strahlentherapie erstmals nachgewiesen werden. Grundlegend für den Pathomechanismus ist die strahleninduzierte endotheliale Dysfunktion. Die Schädigung der Gefäßwand führt zu einer chronischen Inflammation und begünstigt die Entstehung von atherosklerotischen Plaques. Da der Blutfluss durch die Obstruktionen dann stark vermindert ist, steigt das Risiko kardialer Ischämien. Doch auch die bestrahlte Lunge zeigt chronisch entzündliche Schäden nach hohen Strahlendosen. Diese gehen mit einem erhöhten Risiko für Lungenfibrosen einher.
Beim Menschen treten strahleninduzierte Herz-Kreislauf-Erkrankungen zumeist innerhalb der ersten Dekaden nach Bestrahlung auf. Hiervon betroffen sind auch jüngere Patientinnen ohne typische kardiale Risikofaktoren. Deshalb sollte die erste Dekade nach einer Bestrahlung nicht nur zur Krebsnachsorge, sondern auch zur Sekundärprävention von kardiovaskulären Risikofaktoren genutzt werden.

Medikamente zur Primärprävention von strahleninduzierten Herz-Kreislauf-Erkrankungen sind derzeit bereits Gegenstand der aktuellen Forschung. Cannabidiol, mit seiner anti-inflammtorischen und anti-oxidativen Wirkung, hat hierbei bereits vielversprechende Ergebnisse in Mäusen gezeigt.

1. INTRODUCTION

1.1. Irradiation induced diseases

The vascularization is key for nutrient support and oxygen supply of humans and mammalian organisms. The blood was considered as one of the main four body juices in ancient Greek history. Hippocrates attributed diseases to an imbalance of the four liquids of the body: blood, mucus, yellow and black bile (Hanson, 2013). Nowadays we know that for the development of a disease not just the four body juices, but also a large variety of different internal and external environmental factors play important roles for health and disease. As the number of different therapeutic interventions are constantly increasing in the medicine of the 21st century, many diseases can be cured. Some treatments help to prolong the life expectancy of the patients but lead to an imbalance of the body homeostasis and thereby cause unfavorable side effects.

The therapy with ionizing irradiation is one example which effectively can kill tumor cells and therefore contributes to the cure of cancer but also can cause normal tissue toxicities (Baskar et al., 2014; "Global cancer incidence in women," 2018; Rakotomalala et al., 2021; Yap et al., 2016). An improved knowledge of the properties of electromagnetic waves in the diagnosis and therapy has increased rapidly, especially in medicine (Milowska et al., 2014). Ionizing radiation is frequently used in medical imaging, isotope diagnostics and radiotherapy. Radiation is applied in numerous diagnostic and therapeutic approaches of modern medicine and is used in the everyday clinical practice (Yap et al., 2016). This aspect is also demonstrated by an increase in radiation exposure: whereas the average effective dose per inhabitant and year in Germany is 2.1 mSv in total, the man-made radiation exposure, including medical x-rays, nuclear medicine and other radioactive products, is 1.8 mSv and thus the biggest subgroup ("Ionisierende Strahlung," 2020). Besides the healing potential of ionizing irradiation, also the harmful properties of electromagnetic radiation should be considered.

The Life Span Study (LSS) about atomic bomb survivors of 1945 in Hiroshima and Nagasaki and their descendants is considered as one of the most reliable sources of evidence to estimate the long-term health risks in humans exposed to radiation (Ozasa et al., 2018). The analysis of the data showed a significantly increased risk for heart disease overall, valvular heart diseases, hypertensive organ damage and heart failure. Only the ischemic heart diseases did not increase over the entire period since 1945 (Takahashi et al., 2017).

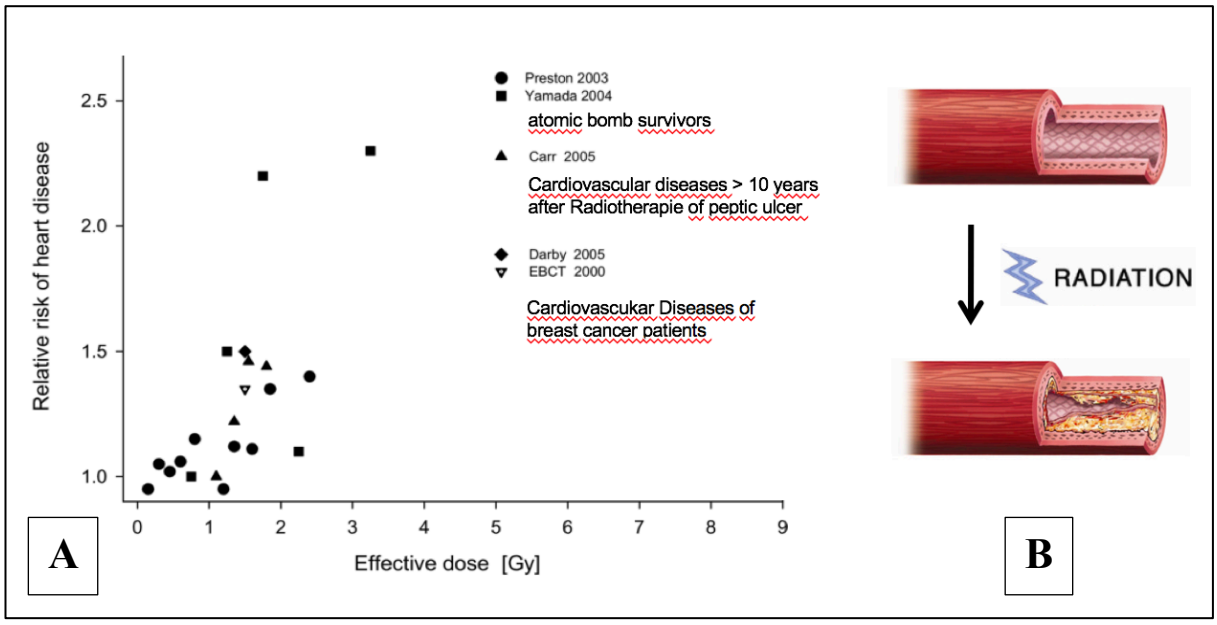

Figure 1: Irradiation induced cardiovascular diseases

(A) *The graphic published in the "International Journal of Radiation Oncology, Biology, Physics" on the first of January 2007 by S. Schultz-Hector and K.-R. Trott compares atomic bomb survivors with breast cancer patients and patients with a peptic ulcer more than 10 years after radiotherapy;(S. Schultz-Hector & K.-R. Trott, 2007)* ***(B)*** *The pictures above show the inside of a vessels before and after radiation. Both were shown in "Frontiers in oncology" performed by Taunk, Haffty, Kostis, and Goyal in the year 2015 (Taunk et al., 2015)*

Schultz-Hector & Trott compared the effects of radiation on atomic bomb survivors with patients who had undergone a radiation therapy after peptic ulcer or breast cancer. The results of their studies illustrate the observed LSS effect: the relative risk of developing heart diseases increases with the radiation dose, irrespectively of the source of the radiation (S. Schultz-Hector & K. R. Trott, 2007). This means that, for example, the standard post-operative-radiotherapy for women, suffering from breast cancer, - the most common cancer in women worldwide ("Global cancer incidence in women," 2018) -, has an underestimated long-term consequence: a higher risk for the development of ischemic heart disease such as myocardial infarction.

This statement is supported by another study of the Early Breast Cancer Trialists Collaborative Group. A review of randomized trials found that women who underwent a postoperative radiotherapy, had an increased risk of developing ischemic myocardial infarction (Early Breast Cancer Trialists' Collaborative, 2000). The follow-up of breast cancer patients shows that the cardiac mortality 10 years after irradiation was around 60% for patients with left-sided breast cancer and 20% for patients with right-sided breast cancer (Darby et al., 2003). One possible explanation for this finding is the different radiation exposure of the heart after irradiation of the left and right breast. Women who get a radiotherapy of the right breast receive an overall

estimated mean dose of 2.9 Gy, whereas patients with cancer in the left breast receive an overall estimated mean dose of 6.6 Gy (S. C. Darby et al., 2013)

Similar results are shown after a reanalyzed surveillance of epidemiology and end-result cancer register data. Of 308.861 women suffering from breast cancer 115.165 got a radiotherapy as a primary treatment. After 10 years 4130 of these women died; 1721 (42%) deaths were caused by recurrent breast cancer, whereas 894 (22%) were caused by different heart diseases. In the group of the 1721 patients who died from cancer no differences were seen whether the tumor was in the left or right breast, whereas in the group of 894 patients who died from heart diseases 176 more patients died who were irradiated on the left breast. Among them, 44 women died from myocardial infarction and 72 from other ischemic heart diseases (Clarke et al., 2005). These numbers show that there is a difference in outcome if the tumor is localized and irradiated on the left or right side of the breast. Furthermore, myocardial infarction is not the only lethal disease after therapy. Also, ischemic heart diseases contribute significantly to the irradiation induced death cases.

The risk of developing ischemic heart diseases was examined: 7.4% per Gray is the linear increase of the risk of a major coronary event starting from 5 years after the radiation up to 20 years (S. C. Darby et al., 2013; Darby et al., 2003) Cardiac risk factors at the time of the radiation do not play a role here (Doyle et al., 2007) .

To estimate the occurrence, the severity, and the progress of radiation-induced heart diseases like myocardial infarction further studies are necessary.

The lung is another organ at risk with respect to radiation of breast cancer patients. Unlike the heart, this organ is spanning over the left and right-hand side of the thorax. Consequently, several lobes of the lungs are exposed to radiation, regardless which side the breast cancer is located. Ionizing irradiation can lead to a secondary lung cancer. The absolute risk to develop lung cancer is approximately 4% in smokers and 0.3% in nonsmokers (Taylor et al., 2017). Hence, the cardiovascular risk factor of smoking changes the preconditions of radiation: The benefit of radiation for breast cancer patients who are smoking is extremely limited.

Furthermore, radiation-induced lung injuries like acute pneumonitis or chronic pulmonary fibrosis could be observed (Hanania et al., 2019). These diseases become manifest with the appearance of symptoms like shortness of breath, chest pain, fever, and even severe respiratory failure and death (Hanania et al., 2019). Similar to the heart the relationship between the dose and the response of the lung is proportional (Moran et al., 2017). The lung volume, which is

irradiated with up to 20 Gy (V20) should be kept under 10% and the mean lung dose below 6 Gy to prevent radiation-induced lung diseases (Blais et al., 2017).
The lungs, the frame of the heart, are the connecting piece between the right and the left heart. With the help of the pulmonary artery the blood is transported from the right ventricle to the lungs to get oxygenated. Then the pulmonary vene transports the blood back to the left atrium and, with the blood ejection of the left ventricle, supplies the whole body with oxygen. Consequently, there is an intense anatomic and functional alliance between the two organs. The radiation induced damage of the lungs could possibly harm the heart. Pulmonary fibrosis, as a radiation-induced lung disease can lead to right ventricular hypertrophy. The induced hypertrophy could be the reason for the progress or onset of severe heart diseases later.
The close relation between the lungs and the heart is also shown in the reciprocal effect. Radiation of one organ leads to lower tolerated doses of the other one and vice versa (Ghobadi et al., 2012). Therefore, a co-irradiation of the heart and the lungs leads to lower tolerated doses of both organs. According to Ghobadi the underlying mechanism has to be the development of a multiorgan damage leading to complications in both organs (Ghobadi et al., 2012).

As recent studies show that radiotherapy halves the rate of relapses and reduces breast cancer death rates by about a sixth after breast conserving surgery, radiotherapy is necessary and not dispensable (Early Breast Cancer Trialists' Collaborative et al., 2011).

1.2. Aim of the study

The aim of this study is to unravel potential pathomechanism which can cause late radiation damages in lung and heart ECs. The basis were studies about irradiation-induced cardiovascular diseases after ionizing irradiation. The analysis of multiple studies by Schultz-Hector and Trott has demonstrated that being exposed to radiation leads to a significant increase in the risk of developing ischemic heart diseases, particularly myocardial infarction (S. Schultz-Hector & K. R. Trott, 2007). Thus, the standard post-operative therapy of breast cancer patients, for example, has an unknown late damage: the cause of myocardial infarction. Now it is not clear, which time-dependent alterations take place in the blood vessels, particularly in the primary ECs. The assumption of Schultz-Hector and Trott that radiation leads to an injury of the capillary network

and furthermore to an ischemic myocardial degeneration was not proven and therefore was the major goal of this study (S. Schultz-Hector & K. R. Trott, 2007).
Furthermore, radiation-induced lesions of lung tissue could contribute to or even increase the risk of developing radiation-induced heart diseases, such as myocardial infarction. Therefore, the radiation-induced damages of the lungs have also been investigated to learn more about potential bystander effects of a damaged lung on the heart.

The long-time changes in heart and lung ECs after a thoracic irradiation needs to be systemically analyzed to prevent patients from long-term irradiation induced micro vessel damages. The best model to investigate long-term radiation-induced effects within an intact organism provides a mouse model. Therefore, we irradiated the heart and part of the lung of mice and studied the effects on isolated primary ECs of these mice 20 to 50 weeks after irradiation. For these experiments the "Small Animal Research Radiation Platform" (SARRP) was used which allows a CT-image guided high precision irradiation of target structures in the sub-mm range. After taking a CT image of the mice an *in vivo* irradiation was planned, like the procedure used for human patients. The whole heart and parts of the lungs, which cannot be excluded due to anatomical reasons, were irradiated (sham 0 Gy, 8 Gy, 16 Gy). Previous studies have indicated that irradiation doses of 8 and 16 Gy on the heart and parts of the lung are tolerated by the mice up to 20 weeks. Moreover, these doses have demonstrated changes in cell surface markers on heart and lung ECs in a dose- and time-dependent manner, 5 and 20 weeks after irradiation (Sievert et al., 2015).

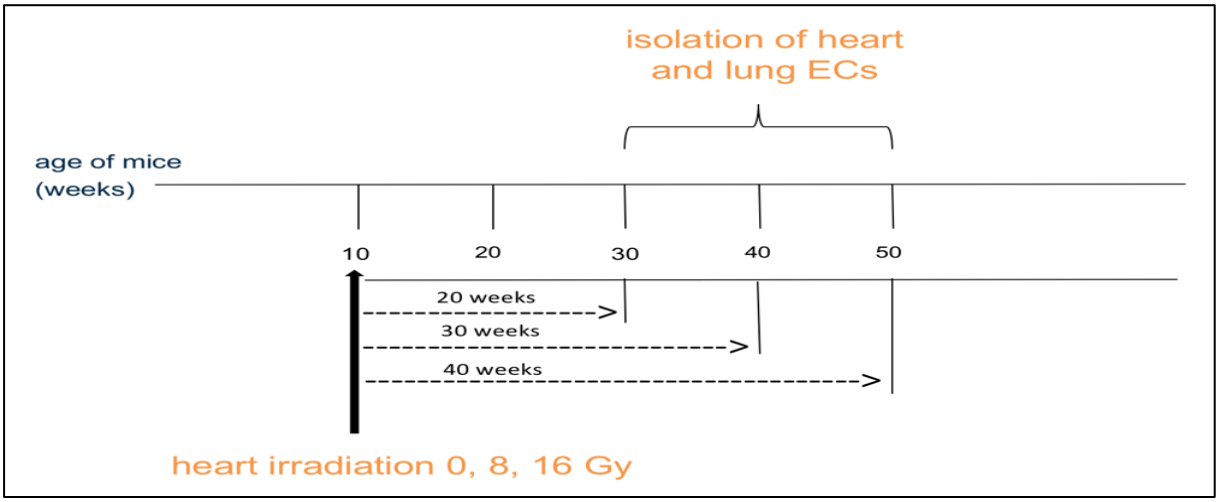

Figure 2: Time schedule of the experiments

In the present study we were interested in very late irradiation effects (20 to 50 weeks after irradiation) with absorbed doses of 8 and 16 Gy. The control group was treated identically and received a sham irradiation with 0 Gy at all tested time points.

To investigate the effect on the irradiated organs, particularly heart and lung ECs, mice were sacrificed under anesthesia 20, 30, 40 and 50 weeks after radiation. Then, primary murine ECs of the heart and lungs were isolated with a newly developed isolation method (Sievert et al., 2014). The isolated ECs were immediately analyzed phenotypically by flow cytometry.
The comparison of the cell surface characteristic in terms of their different surface markers of heart and lung ECs of radiated and not radiated control mice provide information about irradiation-induced tissue alterations in heart and lung ECs.

1.3. Endothelial cells

Alterations on ECs are in the focus of interest in this study. Their structure and functions are described in the following paragraph to understand the importance of these cells for the human body. Also, the cell surface markers of ECs are described in more detail.

1.3.1. Structure of ECs

The blood circulation is defined as the course of the blood from the heart through the arteries, capillaries, and veins back again to the heart (Yamini Durani, 2013). This blood transporting tube system consists of arteries, capillaries and veins (Han et al., 2016). The typical structure of arteries and veins is divided in three layers: Tunica externa, also called adventitia, tunica media or media and tunica interna, also called intima (Tan & Desai, 2005).
Outside the lumen of the arteria or vein adjacent to the adherent tissue, there is the adventitia (also termed tunica externa). The tunica externa contains fibroblasts embedded in an extracellular matrix consisting of collagen fibers (Martinez-Lemus, 2012). For the innervation of the muscular system of the vascular wall, vegetative nerve fibers run in the adventitia (Laine et al., 2000). Furthermore, the tunica externa contains blood supply for the outer vascular wall.

In the media, we have smooth muscle cells (Raines & Ross, 1993), covered by the membrana elastica externa between the media and the adventitia and the membrana elastica interna between media and intima. To resist the high arterial blood pressure this layer is especially strong in arteries, whereas veins have a thinner tunica media (Feletou, 2011).
The innermost layer of blood vessels, lymphatic vessels and heart cavities is formed by an 80 nm thick abluminal basement membrane (Baluk et al., 2003) and a single-shift cell layer of ECs ("Efficient Differentiation of HumanPluripotent Stem Cells to EndothelialCells," 2018; Gu, 2018; Martinez-Lemus, 2012) which forms the tunica intima.

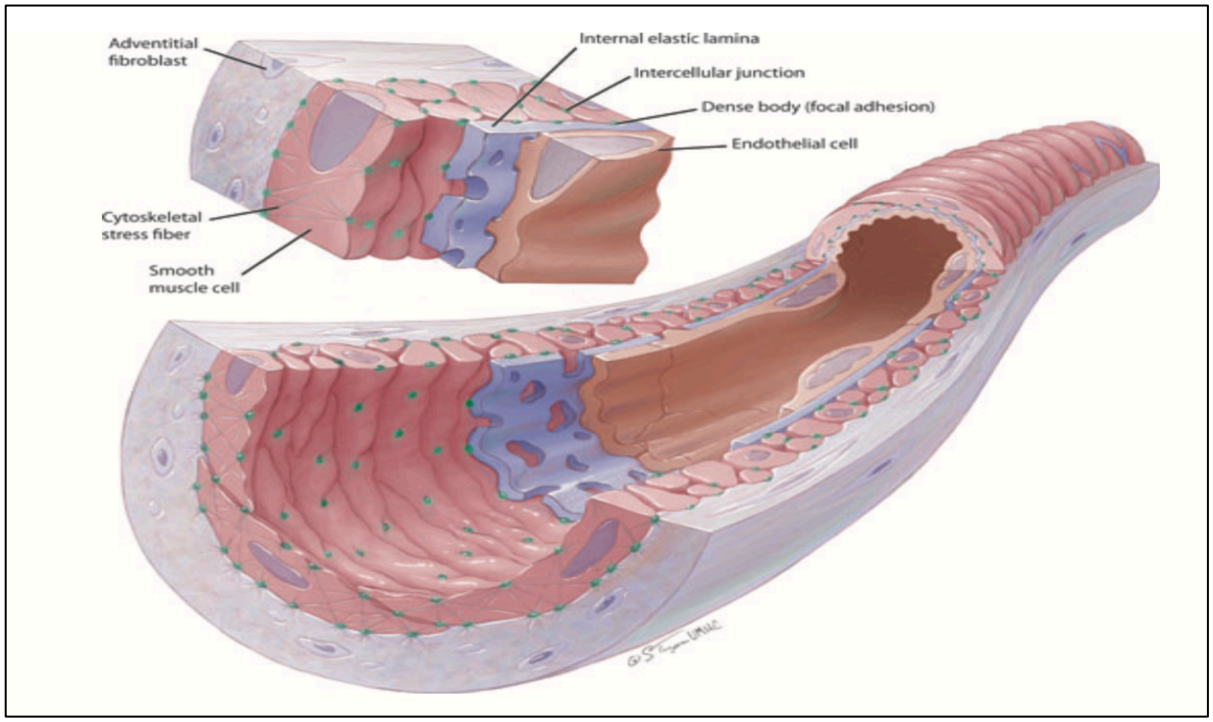

Figure 3: Wall-structure of an arteriole with tone

The picture shown in this figure was perfomed by Martinez-Lemus first published in "Basic & Clinical Pharmacology & Toxicology" in the year 2012. (Martinez-Lemus, 2012; Taunk et al., 2015)

As capillaries are in the terminal vessels to enable the exchange of substances between blood and adherent tissue via diffusion (Landis, 1963), their wall structure is much thinner: only a few squamous ECs, escorted by overlying pericytes, enclosed by a basal membrane, form the wall of the capillaries (Townsley, 2012).

Endothelial cells, from Greek endon = "inside", "within" and thelein = "growing exuberantly" (Jarles Patrick Davis, 2018), stem from the embryonic mesoderm (Tucker & Bhimji, 2018) and have a size of 50 - 70 µm length and 0.1 - 10 µm thickness (Feletou, 2011). With a surface area

of approximately 300 to 1000 m^2 and more than 1 to 6 x 10^{13} cells the endothelium of the blood circulation and make up approximately 1.5 kg of the body weight in an adult human (Beltowski & Jamroz-Wisniewska, 2014). In the circulating system ECs are thin and slightly elongated, aligned to the direction of the blood flow (Galbraith et al., 1998). If the shear stress in the vessels is increasing, ECs elongate in the direction of the flow (Sievert, 2016; Sievert et al., 2014; Tzima et al., 2001).

There are three different types of endothelia: first of all, the continuous endothelium, second the fenestrated endothelium and the discontinuous endothelium (Setyawati et al., 2015). While arteries and veins are lined with continuous endothelium, capillaries can be coated with all three types of endothelia depending on the region or organ.

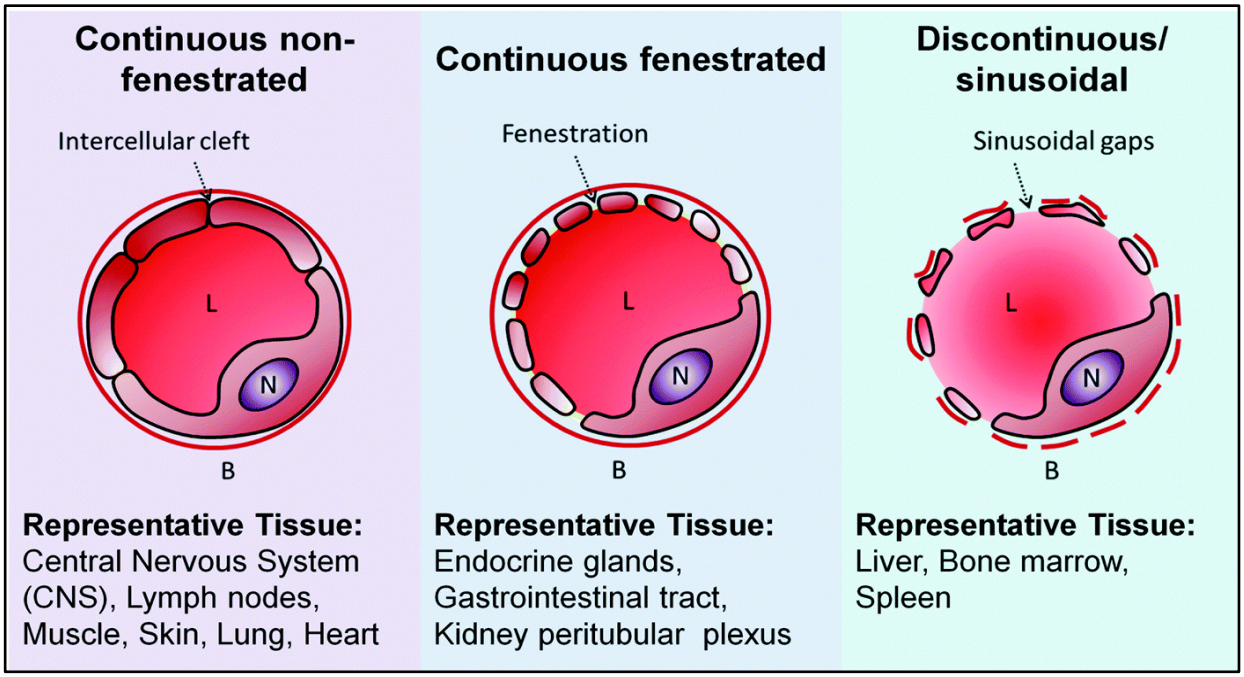

Figure 4: Different types of endothelia

The graphic in this figure was performed by Setyawati, M.I., Tay, C.Y., Docter, D., Stauber, R.H., and Leong, D.T. (Setyawati et al., 2015). They devided endothelium in 3 different types, based upon their wall structure: continuous non fenestrated vs. continuous fenestrated vs. discontinuous sinusoidal endothelium.

1. <u>Continuous endothelium:</u>

   The continuous endothelium forms an uninterrupted cellular layer of ECs and the basement membrane. As cells are connected with tight junctions there is no gap between the cells. This means for the exchange of material that only water, glucose, urea and other hydrophilic substances are able to diffuse in the adherent tissue (Pries & Kuebler,

2006). This kind of endothelium is used in regions of the body where a strict segregation of the intravascular and extravascular space is very important (i.e. the central nervous system). The blood brain barrier, for example, is primarily formed by the continuous endothelium (Zlokovic, 2008).

2. Fenestrated endothelium:

   The difference between continuous and fenestrated endothelium are the 50 - 60 nm wide transcellular pores, which are surrounded by a continuous basal membrane on the abluminal side of the vessel and a 5 - 6 nm thick diaphragm on the luminal side (Pries & Kuebler, 2006). The diaphragm allows just water and hydrophilic low-molar substances to pass the barrier. Hence, fenestrated endothelium is found in the kidneys, endocrine glands, or the mucosa of the gastrointestinal tract, as filtration, secretion and absorption are indispensable in these organs.

3. Discontinuous endothelium:

   The discontinuous endothelium has 100 - 200 nm wide fenestrations between the ECs without a diaphragm. Furthermore, the basal lamina is poorly structured (Pries & Kuebler, 2006). This histological condition leads to a very fast and easy material exchange, which is important in organs like the liver, the spleen or the bone marrow (Feletou, 2011)

1.3.2. Functions of ECs

ECs are not just a semipermeable barrier between blood and tissue, they are highly dynamic and very complex cells. Responsible for many different physiological functions and as a part of many pathophysiological pathways in the cardiovascular system, ECs can be designated as a complex metabolic organ (Aird, 2004). In the following section the most important functions of ECs are mentioned.

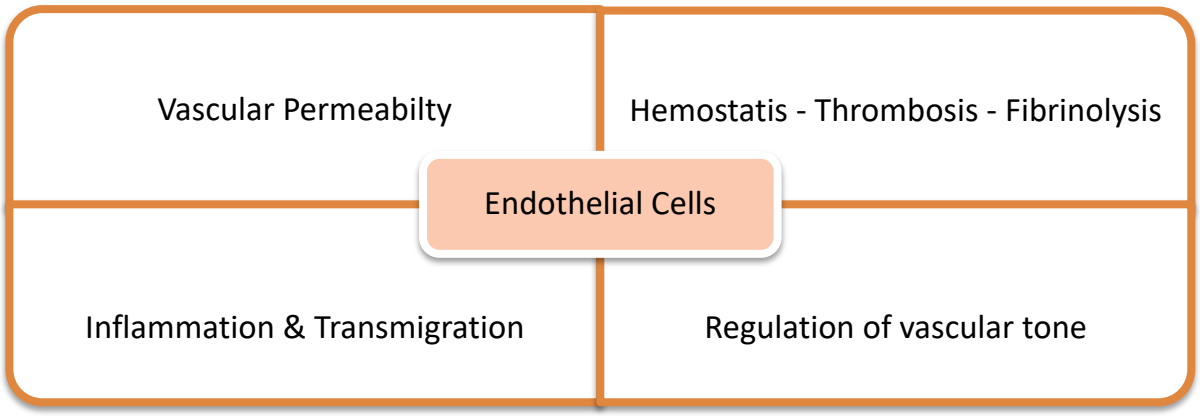

Figure 5: Functions of endothelial cells

Vascular Permeability

As the multifunctional semipermeable barrier between blood and the surrounding tissue ECs are the border between two compartments. Their task is to regulate the passage of molecules like dissolved substances, liquids, ions, macromolecules, hormones and cells under physiological conditions and thus enable the material exchange between the vessels and the organs of the body (Simionescu et al., 2002). While small substances are able to change sides of the bar via passive pathways, bigger molecules have to use either the paracellular or the transcellular way of passing (Minshall & Malik, 2006).

When the barrier function is broken an increased permeability for macromolecules like Albumin and resulting from this to interstitial edema is enabled. This malfunction of the endothelium can be caused by several pathophysiological changes like inflammation, inadequate blood supply, sepsis, burn injuries, etc. (Saraldi et al., 2017). Arteriosclerosis is another example for a disease based on an endothelial dysfunction (Yamaoka-Tojo, 2020). The intact barrier of the ECs leads to an uptake of fatty acids like LDL-cholesterol into subendothelial layers of the vessels, where oxidation, inflammation and plaque formation occur.

Hemostasis – Thrombosis – Fibrinolysis

One of the main functions of ECs is the regulation of hemostasis, thrombosis and fibrinolysis (Lijnen & Collen, 1997). Usually, when the blood flow is uninterrupted, the surface of endothelium is neither thrombogenic nor adhesive for leukocytes or thrombocytes, thanks to the antiadhesive and antiaggregatory characteristic of nitrogen monoxide NO and prostacyclin (Radomski et al., 1990).

However, when the endothelium is injured, the exposure of subendothelial matrix and collagen fibers is starting primary cellular hemostasis. As a first step van Willebrand Factor (vWF) stimulates thrombocytes to cover the damage of the vessel wall by binding to the GP Ib/X/V receptor (Fressinaud & Meyer, 1991). Subsequently they bind to subendothelial collagen by means of the collagen receptor GP VI and transform their shape and are activated. Wall adherent thrombocytes secrete Adenosine diphosphate ADP, Adenosine triphosphate ATP, fibrinogen, arachidonic acid, thromboxane A2, serotonin and van Willebrand Factor (Gawaz, 2001). Thus, the recruitment of other thrombocytes starts, and the aggregation and vasoconstriction stabilize the primary white thrombus.

Parallel to the cellular hemostasis the secondary hemostasis is starting by activating plasmatic coagulation. The essential part of this process are the plasmatic clotting factors, most of them serine proteases, which are cascade-like activated (Gale, 2011). In the end, fibrinogen is split into fibrin, which stabilizes the white thrombus by further cross-linking. The plasmatic hemostasis can be activated through an extrinsic and an intrinsic way (Palta et al., 2014). The extrinsic way, also called exogenous activation, is caused by the release of tissue factor TF, a transmembrane protein (Lasne et al., 2006). This subendothelial located tissue thromboplastin activates factor VII when the TF is in contact with blood. Factor VIIa in turn converts factor IX and X into their active form of coagulation factor (Owens & Mackman, 2010). Clotting factor Xa catalyzes with the help of cofactor Va the transformation of prothrombin (FII) in thrombin (FIIa), which in turn splits fibrinogen into fibrin (Hall, 2015). The intrinsic clotting is activated when blood is in contact with a foreign surface. Thereby the transformation of the coagulation factors is a cascade: factor XII activates factor XI, this one in turn activates IX, factor IX activates factor X (Davie et al., 1991). The rest of the clotting pathway is like the extrinsic activation which ends with fibrin as the last cleavage product.

After a successful hemostasis and a completed reparation fibrinolysis occurs. In this process the thrombus is dissolved, and normal blood flow is restored. Tissue type plasminogen activator (t-PA), produced by the endothelium, and urokinase (u-PA) from the urogenital tract divide plasminogen into plasmin (Collen, 1999). Fibrin is dissolved by hydrolytic splitting from the endopeptidase plasmin.

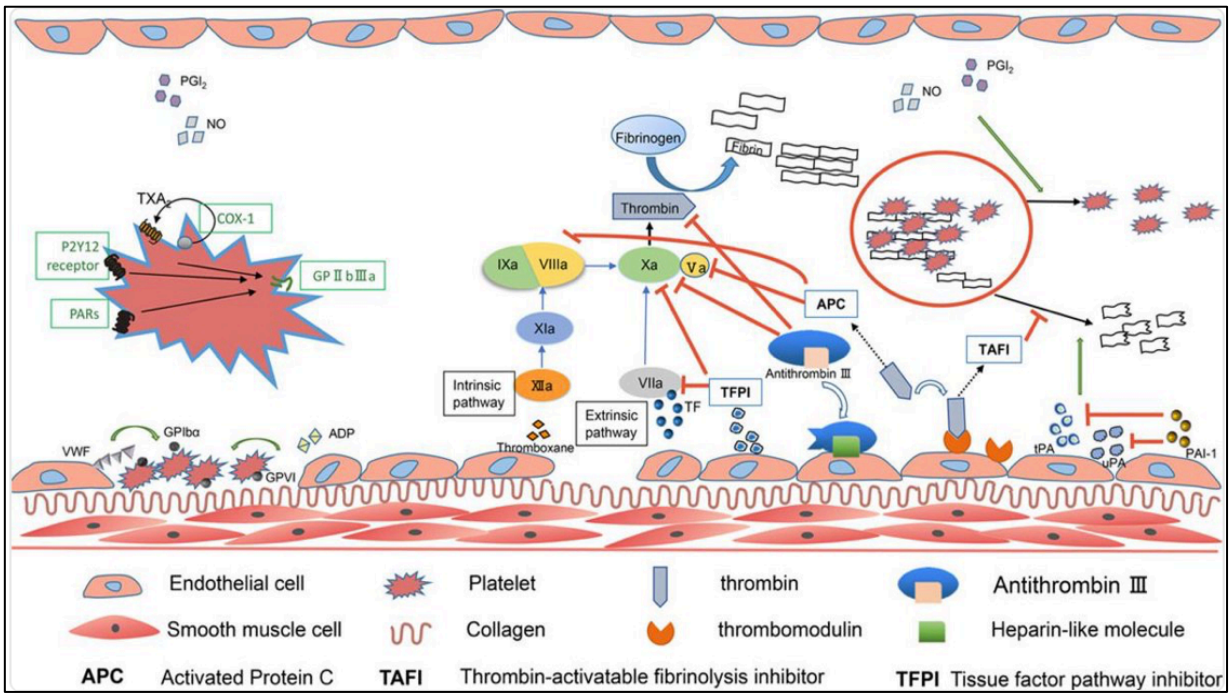

Figure 6: Thrombotic regulation from the endothelial cell perspective

The graphic shown above was published in "Arteriosclerosis, Thrombosis and Vascular Biology" by M. Wang, H. Hao, N.J. Leeper. L. Zhu in the year 2018 (M. Wang et al., 2018).

Inflammation – transendothelial migration of leukocytes

Another important function of ECs is the emigration of mobile cells in immunological and inflammatory processes. Cells like leukocytes are needed for wound healing or local and systemic inflammation. Therefore, they must leave the circulating blood system and migrate to the area of inflammation. This mostly takes part in postcapillary venules (Springer, 1995).

The whole migration process is divided in three parts: *rolling*, *adhesion* and *diapedesis (Lüllmann-Rauch, 2015)*. Stimulated by substances of bacteria and dead cells or cytokines, the endothelium gets activated and exposes certain cell-adhesion molecules like selectins on the luminal surface (McEver, 2015). Appropriate ligands on the surface of leukocytes give a temporary loose liability, which ruptures again because of the shear forces of the circulating blood

stream (Zimmerman et al., 1992). This first process is the so-called, *rolling* of the leukocytes, where cells are slowed down and accumulate on the endothelium.

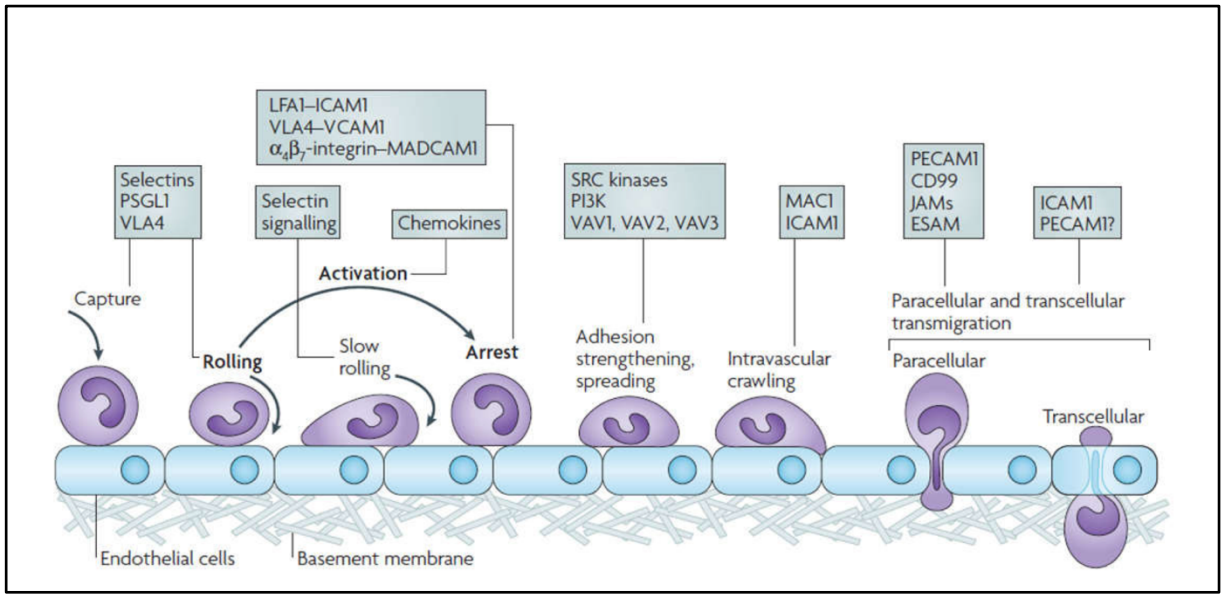

Figure 7: Transmigration of Leukocytes

The steps of Transmigration are mediated through different molecules on the surface of the ECs as well as on the surface of the leukocytes. Tethering of the cells, selectin-mediated rolling, slow rolling, tight adhesion, intravascular crawling, paracellular or transcellular migration are the different steps of diapedesis. The graphic was first published in "Nature Reviews Immunology in the year 2007. The data was performed by K. Ley, C. Laudanna, M. Cybulsky ans S. Nourshargh (Ley et al., 2007) .

Adhesion, the second part of the emigration, is the bond of leukocytes on the ECs. While ECs expose adhesion molecules like ICAM-1, leukocytes expose integrins on their surface, stimulated by chemokines (Carlos et al., 1991). The endothelium and the leukocytes form a bonding and subsequently leukocytes are able to flatten their shape on the endothelium, because of a rearrangement of the cytoskeleton (Chauvière, 2010).

Diapedesis or a walk through the endothelium is the last part of the transmigration. Here the leukocytes are forced through the ECs with the help of more adhesion molecules, for example *PECAM-1*. Another way to leave the vessel is to get absorbed and get dispensed from the ECs, similar to the process of transcytosis (Dietmar Vestweber, 2007). To pass through the basal lamina leukocytes produce enzymes, which locally dissolve this membrane (Kummer et al., 2015). Attracted by chemokines leukocytes migrate through the interstice to the area of injury.

Regulation of the vascular tone

ECs have a huge impact on the regulation of the vascular tone. Via vasodilatation and vasoconstriction, the blood vessels can change their diameter and thus influence the pressure in arteria and veins. This means the perfusion of organs as well as tissue is an essential regulation function of the endothelium.

As ECs have a connection to the innermost layer of the smooth muscle cells in the media of the blood vessel wall (Lüllmann-Rauch, 2015), vasoactive substances can directly affect the muscle in the media and therefore change the tone of the vessel. This means the released vasoactive substances modulate the state of contraction in smooth muscle cells afterwards.

Nitrogen monoxide NO, bradykinin, prostacyclin and endothelial derived hyperpolarization factor EDHF are all examples for substances with a vasodilatory effect (Brandes et al., 2000).

NO plays the most important role in the endothelium derived vasorelaxation (Russo et al., 2002; Vanhoutte, 1993). Separating a nitro group of L-arginine with the help of endothelial membrane bound nitric oxide synthase eNOS (Palmer et al., 1988), the molecule is able to diffuse into smooth muscle cells and activate the guanylate cyclase. With an increase of cGMP and a decrease of the intracellular calcium concentration the vasodilating effect is achieved (Hubert Erich Blum, 2018). If the shear stress in the vessels is increasing, the activity of eNOs and the production of NO is rising. Acetylcholine, bradykinin and serotonin are also humoral factors, which strengthen the vessel relaxing effect (Richard et al., 1990).

Among the vasoconstrictors endothelin-1, thromboxane A2 und prostaglandin H2, endothelin-1 is the most potent vessel constrictor (Noll & Luscher, 1998). Endothelin-1, which is released by ECs under hypoxia or decreased shear stress, binds to the endothelial receptor of smooth muscle cells (Kowalczyk et al., 2015). This leads to a contraction of the muscle and thus, to a consecutive vasoconstriction of the blood vessel. Humoral mediators like angiotensin II, thrombin, vasopressin and noradrenalin strengthen the release of endothelin-1 (Lincoln et al., 1990).

1.3.3. Endothelial surface markers

To identify and characterize ECs different markers on the surface of the endothelium can be analyzed *in vivo* and *in vitro.* To avoid long term culture effects the best time for the analyzing ECs is directly after their isolation process, because the possibility of modifications in the cells and therefore the loss of the specialized properties is occurring very fast, *in vitro*. Most of the time direct monoclonal antibodies against the EC cell surface markers are used for identifying ECs (Garlanda & Dejana, 1997). But the constitutively expressed markers as well as the markers, which are induced by inflammatory cytokines or growth factors are not only to be found on the surface of ECs. Many different other tissues in our body express identical markers. Hence, the combination of different cell surface markers, which are typical for ECs are necessary to prove their origin. Table 1 and 2 show a list of constitutively expressed (1) and inducible markers of the endothelium (2).

Table 1: Constitutive endothelial markers

Constitutive Endothelial Markers		
Surface marker	***Cell Type***	***Reference***
CD31 / PECAM-1	ECs, platelets, megakaryocytes, B and T lymphocyte subsets, monocytes, neutrophils, tumor cells	(DeLisser et al., 1994; Pusztaszeri et al., 2006; Scholz & Schaper, 1997; Tang et al., 1993; Vecchi et al., 1994)
CD34 / Mucosialin	ECs, hemopoietic progenitor cells, tumor cells	(Krause et al., 1996; Lin et al., 1995; Natkunam et al., 2000)
CD36 / FAT	Microvascular ECs, monocyte-macrophages, platelets, megakaryocytes	(Greenwalt et al., 1992)
CD54 / ICAM-1	ECs, leukocytes, fibroblasts	(Dustin et al., 1986; Frohman et al., 1991; Springer, 1990)
CD102 / ICAM-2	ECs, lymphocytes, monocytes, platelets	(de Fougerolles et al., 1991; Springer, 1990)
CD105 / Endoglin	ECs (overexpressed in tumor ECs), monocyte-macrophages, B lymphocytes, syncytiotrophoblasts, tumor cells	(Fonsatti et al., 2010; Gougos & Letarte, 1988; Postiglione et al., 2005)
CD144 / VE-cadherin	ECs, trophoblasts, lymph node sinus macrophages	(Bulla et al., 2005; Lampugnani et al., 1992; D. Vestweber, 2008)

Table 2: Inducible endothelial markers

Inducible Endothelial Markers			
Surface marker	***Cell Type***	***Stimulus***	***Reference***
CD44 / HCAM	ECs, tumor cells,	Radiation-induced marker in ECs	(Al-Othman et al., 2018; Penno et al., 1994; Senbanjo & Chellaiah, 2017; Sievert et al., 2015; Wu et al., 2017)
CD106 / VCAM-1	ECs, macrophages, synovia, dendritic cells, mesothelium	Upregulated in ECs by inflammatory CKs	(Mutsaers, 2002; Springer, 1990)

Based on their different functions the cell surface markers can be divided into four groups: Proliferation, inflammation, progenitor cells and lipid metabolism.

Integrin-ß, *endoglin* and *vascular endothelial cadherin VE-cadherin* belong to the group of the proliferation markers. Proliferation of cells is the process that results in an increase in the number of cells and is defined by the balance between cell divisions and cell loss through cell death and/or differentiation (Nature, 2020). There are many different ways to regulate this process, one of these is mediated via growth factors (R. M. Lyons & Moses, 1990).
These markers are expressed on cells with an increased reproduction capacity of the tissue. *Endoglin*, a proliferation marker is expressed on various cells like monocytes, macrophages, B lymphocytes, syncytiotrophoblasts and tumor cells, it is a transmembrane protein, which can connect cells with each other or with the extracellular matrix. Furthermore, this integrin is also a co-receptor for the receptor complex of TGF-ß. Preferred expression however, is on ECs, especially on microvascular ECs (Torsney et al., 2002). In case of an ongoing inflammation, tissue regeneration or tumor cell growth, ECs are able to upregulate the expression of *Endoglin*, because under this pathophysiological conditions cell proliferation and angiogenesis are increased rapidly (Burrows et al., 1995; Fonsatti & Maio, 2004; Miller et al., 1999; Schimming & Marme, 2002). *VE-cadherin,* controlling the cellular junctions and blood vessel formation is considered to be the major endothelial adhesion molecule (D. Vestweber, 2008). Controlling the cell contacts of the endothelium this proliferation marker regulates the permeability of the blood vessels as well as the extravasation of leukocytes (Gavard, 2014). Furthermore, it plays an important role in cellular processes, such as cell proliferation and apoptosis (Tsuneki & Madri, 2014; D. Vestweber, 2008).

Inflammation markers are increasing during acute and chronic inflammation, which might be indicative for specific diseases (Watson et al., 2012). Examples for inflammation markers on ECs are *platelet endothelial cell adhesion molecule PECAM-1, homing-associated cell adhesin molecule HCAM, intercellular adhesion molecule I ICAM-1, intercellular adhesion molecule II ICAM-2* and *vascular cell adhesion molecule VCAM-1* (Baselet et al., 2019; Grudzińska & Czuba, 2014; Kjærgaard et al., 2016).

PECAM-1 is constitutively expressed on the surface of ECs, platelets, megakaryocytes, B and T lymphocyte subsets, monocytes, neutrophils, and tumor cells (Garlanda & Dejana, 1997; Sievert, 2016). One of the main functions of this inflammation marker is the transduction of signals. Angiogenesis, platelet function and thrombosis are examples for the diverse role as a signaling molecule in vascular biology (Woodfin et al., 2007). Furthermore, this molecule plays an important role in the cell adhesion. (Paddock et al., 2016; Wong et al., 2000). Concentrated at intercellular junctions of confluent endothelium (Paddock et al., 2016) *PECAM-1* regulates the maintenance of EC junctional integrity, the transmigration of leukocytes and, as a mechanosensor, fluid shear stress. (Privratsky & Newman, 2014). By transducing intracellular signals for the functional upregulation of integrins on leukocytes it is one of the main player in the emigration of leukocytes (Delisser et al., 1997). With a number of 10^6 molecules per cell this inflammation marker is constitutively highly expressed on ECs, also intercellular (Newman, 1994). This aspect and the missing expression on non-endothelial cells like fibroblasts or muscle cells (Newman, 1997) lead to the use of *PECAM-1* as a characteristic marker of ECs. Although *PECAM-1* is one of the constitutive surface markers there are publications that indicate a downregulation of this marker under the influence of chemokines (R. J. Stewart et al., 1996).

The surface marker *HCAM*, expressed on leukocytes, and fibroblasts (CD-Marker, 1999) plays an important role in inflammatory processes (Van de Stolpe & Van der Saag, 1996). Under non-stimulating situations *HCAM* is expressed at low levels on the surface of ECs whereas the expression is significantly increasing under the presence of proinflammatory cytokines like TNF-α, IL-1 and IFN-γ or reactive oxygen species (ROS) (Davis et al., 2003). Involved in the "homing" process of lymphocytes the immune cells are attracted to the side of inflammation. The caused immune response depends on the trafficking of lymphocytes (Butcher & Picker, 1996). That is why the surface marker is termed "homing cell adhesion molecule" (*HCAM*). Furthermore, this surface marker is involved in several cell-cell interactions. One example is the interaction between the hyaluronic acids of the extracellular matrix of tumor cells and the multifunctional *HCAM* (Misra et al., 2011). This interaction between these two partners plays a key role in invasion and metastasis of tumor cells (Alaniz et al., 2002).

ICAM-1 is a structurally related member of the immunoglobulin supergene family (Lawson & Wolf, 2009; Witkowska & Borawska, 2004), which is expressed on ECs, leukocytes, and fibroblasts. This adhesion molecule belongs to the constitutive markers on ECs. As a ligand for the β2 integrin molecules, which are present on leukocytes (Meisel et al., 1998), ECs activate several proinflammatory cascades and participate in trafficking of inflammatory cells. That is why the interaction of the leukocyte integrin, lymphocyte function associated antigen 1 (*LFA-1*) and *ICAM-1* is one of the mechanisms for lymphocyte adhesion in the transmigration of leukocytes (Dustin & Springer, 1988). This binding mechanism is also used for the development of vascular diseases. Elevated concentrations of *ICAM-1* have been measured in patients with atherosclerosis, heart-failure, coronary artery disease and transplant vasculopathy (Lawson & Wolf, 2009). Cardiovascular risk factors like hypertension, smoking and frequent alcohol consumption have been associated with the increase of *ICAM-1*(Blann et al., 1997; Rohde et al., 1999). Here *ICAM-1* is the key molecule for the transformation of macrophages into foam cells based on the leukocyte accumulation of inflamed or damaged endothelium (Anbarasan et al., 2015). Furthermore, the increase of the soluble form of *ICAM-1 sICAM-1* is correlated with the severity of diseases such as autoimmune disorder (Coll-Vinent et al., 1997; Nassonov et al., 2000) as well as cancer (Gho et al., 2001). Endothelium, lymphocytes, monocytes, and platelets are carrying *ICAM-2*. The adhesion molecule is a constitutive marker, which is not inducible by

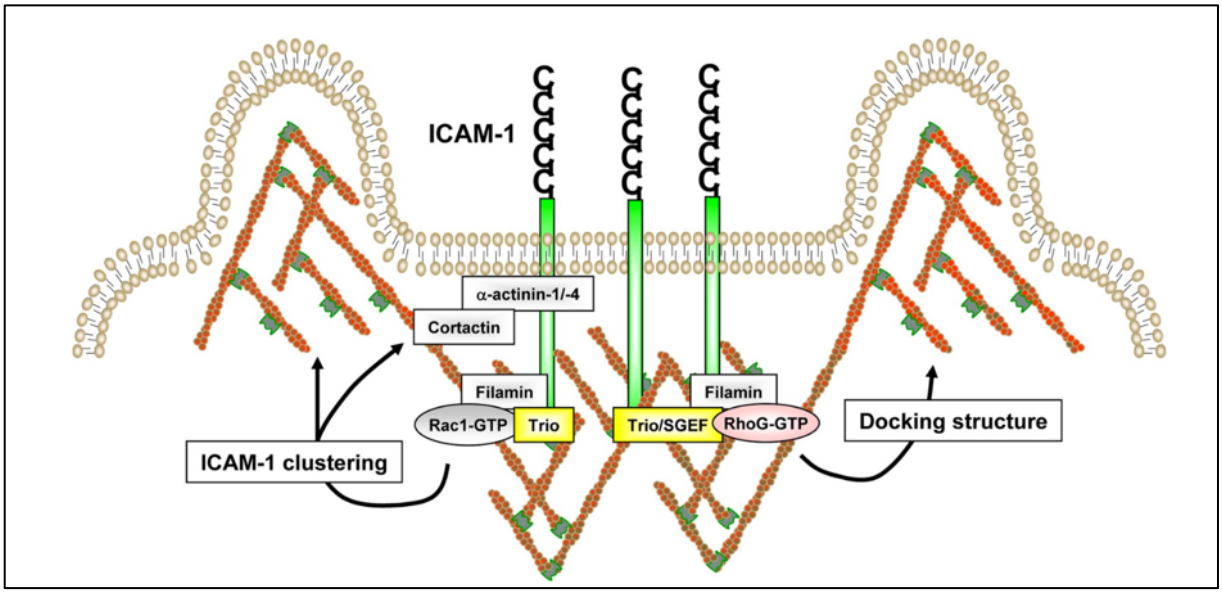

Figure 8: Model for ICAM-1–induced endothelial docking structure formation

The picture was published in "Molecular Biology of the Cell" and analyzed by J.van Rijissel, J.Kroon, M.Hoogenboezem,F.P.van Alphen, R.J. de Jong, E. Kostadinova, D.Geerts, P.L. Hordijk and J.D. van Buul (van Rijssel et al., 2012)

inflammatory reactions with cytokines (Staunton et al., 1989). There are even clues that cytokines, IL-1, and TNF-α decrease the expression of *ICAM-2* (McLaughlin et al., 1998). This molecule is a constitutive marker, which is not inducible by inflammatory reactions with cytokines (Staunton et al., 1989). There are even clues that cytokines, IL-1 and TNF-α decrease the expression of *ICAM-2* (McLaughlin et al., 1998) on cells based on the leukocyte accumulation of inflamed or damaged endothelium (Anbarasan et al., 2015). Furthermore, the increase of the soluble form of *ICAM-1* (*sICAM-1*) is associated with the severity of diseases such as autoimmune disorders (Coll-Vinent et al., 1997; Nassonov et al., 2000), as well as cancer (Gho et al., 2001). Endothelium, lymphocytes, monocytes, and platelets are expressing *ICAM-2*. The adhesion molecule is a constitutive marker, which is not inducible by inflammatory reactions with cytokines (Staunton et al., 1989). There are even clues that cytokines, IL-1, and TNF-α decrease the expression of *ICAM-2* (McLaughlin et al., 1998). This molecule is a constitutive marker, which is not inducible by inflammatory reactions with constitutive markers, which is not inducible by inflammatory reactions with cytokines (Staunton et al., 1989).

VCAM-1 is expressed on macrophages, synovia, dendritic cells, and the mesothelium. Like *HCAM*, *VCAM-1* belongs to the group of inducible endothelial markers. This means that the expression of this adhesion molecule is at a low level and gets upregulated during inflammatory processes via cytokine activation of the endothelium (Mantovani & Dejana, 1998). The main function of the inflammation marker is the signal transduction as well as the adhesion and diapedesis of leukocytes in inflammatory processes (Cook-Mills et al., 2011). Recruiting leukocytes and helping them to transmigrate from the vessels into the adherent tissue is the supporting task of *VCAM-1* (Osborn et al., 1989). Like TNF-α and *ICAM-1*, *VCAM-1* plays also part in the development of cardiovascular diseases, especially in atherosclerosis (Tsalamandris et al., 2018). The upregulation of *VCAM-1* attracts monocytes to atherosclerotic lesions in certain vessel parts (Bailey et al., 2017). The elevation of *VCAM-1* has also clinical relevance in the development of rheumatic diseases, such as rheumatoid arthritis (Davies et al., 2016; N. Zhang et al., 2017).

Progenitor cells are expressing the marker CD34, also called *Mucosialin*. This progenitor cell marker is an early descendant of steam cells, able to differentiate to one or more different tissues, without the ability to divide and reproduce indefinitely.
Mucosialin is constitutively expressed on ECs, hemopoietic progenitor cells and tumor cells.

Lipid metabolism *CD36* is expressed on microvascular ECs, monocyte-macrophages, erythroid cells, platelets, and megakaryocytes. This cluster of differentiation molecule has several roles in our body such as angiogenesis, atherosclerosis, hemostasis, thrombosis, inflammation, malaria, and lipid metabolism (Daviet & McGregor, 1997; Febbraio et al., 2001) This multitude of characteristic functions is based on the different receptor functions of the membrane glycoprotein. Regardless of whether the ligand is a protein or a lipid, the expressed receptors are multivalent and can therefore engage multiple receptors simultaneously. On microvascular ECs the CD36 gene is expressed as receptor for Thrombospondin-1, whereas on phagocytes the marker belongs to the scavenger receptor family (R. L. Silverstein & Febbraio, 2009). Furthermore *CD36* is able to take up long-chain fatty acid with the help of the fatty acid translocase *FAT* (Bonen et al., 2004). This intervention with the energy metabolism and above all the consequences of the dysregulation, namely the obesity-linked diabetes, can contribute to the formation of atherosclerosis (S. Xu et al., 2013). Circulating fatty acids must cross the barrier between the blood and the adherent tissue. This happens through the transfer of the lipids across the endothelium, the gatekeeper for parenchymal fatty acid uptake (N. H. Son et al., 2018). Ligand of the CD36 receptor is oxidized low density lipoprotein LDL. The activation of a CD36 toll like receptor complex leads to the production of atherogenic lipids and stimulates sterile inflammation (C. R. Stewart et al., 2010). This and other studies showed that *CD36* is a key modulator of proinflammatory and oxidative pathways centrally regulating the activation in sterile inflammation (Kennedy et al., 2010; Okamura et al., 2009; Sheedy et al., 2013; Shirai et al., 2018; R. L. Silverstein & Febbraio, 2009; Zhao et al., 2018).

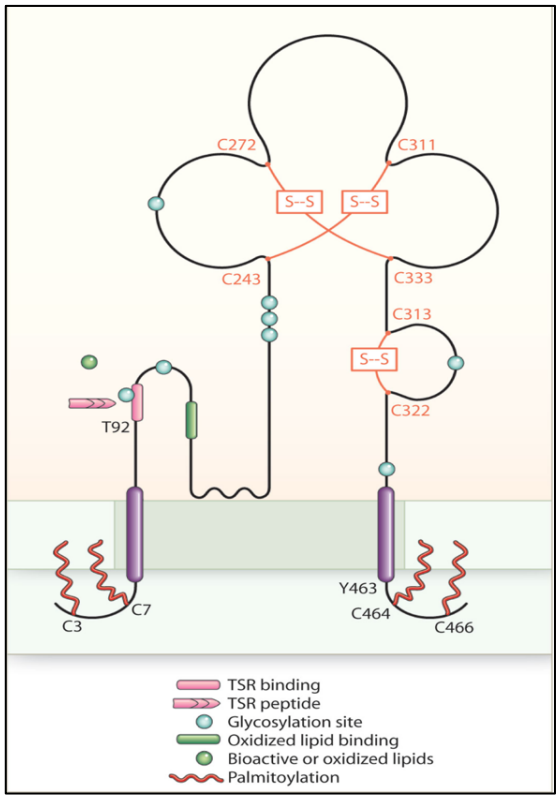

Figure 9: Topology and domains of CD36

The graphic was performed by Silverstein and Febbrraio in the year 2009
(R. L. Silverstein & Febbraio, 2009).

1.3.4. Isolation of endothelial cells

As environmental impact influences the homeostasis of our body (David, 2017; Woods & Ramsay, 2011) it is important to know which external circumstances lead to pathological alterations. Vascular health (Stanhewicz et al., 2018) of the endothelium is affected by risk factors like hypertension, hypercholesterolemia, diabetes and cigarette smoking. These factors cause diseases like atherosclerosis or endothelial dysfunction and often raise queries about the ongoing pathophysiological changes in the vessels of our blood system. Further analysis of ECs is therefore the starting point for more detailed scientific research in the understanding of cardiovascular diseases. As human resources of ECs are strictly limited, alternative cell models are necessary for their examination. Here murine cell models are of utmost significance because of their easy availability. For the isolation of ECs different methods are described in the literature, which mostly do not differentiate between the isolation of macrovascular und microvascular ECs.

Whereas macrovascular ECs can be isolated from human umbilical veins by clamping the vein and enzymatic digestion (Baselet et al., 2016; Gimbrone et al., 1974; Jaffe et al., 1973) or by scraping the intimal surface of arteria (Gajdusek & Schwartz, 1983; Ryan et al., 1980), this process is not possible for the isolation of microvascular ECs. Here the ECs have to be separated from the surrounding tissue.

Enzymatic dissociation of blood vessels as an isolation method for ECs is divided in two parts: First of all, it is important to gain a single cell suspension, which will be separated in a second step to take out all non-endothelial cells (Saraldi et al., 2017). The separation of the endothelium from subendothelial matrix is carried out with enzymes like collagenase and/or trypsin, which release ECs from the basal membrane and the surrounding tissue (Sievert et al., 2014). To obtain a single cell suspension, ECs must be isolated from all non-endothelial cells like smooth muscle cells, collagen fibers or fibroblasts in the second step of the isolation procedure. Magnetic activated cell sorting (MACS) is used here: Murine anti-*PECAM-1* antibodies labeled with DSB-X (biotin) are used to detect and characterize ECs (Sievert, 2016). Adding streptavidin coated magnetic Dynabeads, which are in a non-covalent bond with biotin, a repeating magnetic incubation of the single cell suspension separates the now magnetic ECs from *PECAM-1* negative cells. After this step a single cell suspension with ECs bound to the streptavidin-biotin complex is generated. To eliminate the magnetic Dynabeads a release buffer is added. As the added

release buffer is more strongly competing for streptavidin than biotin the complex of streptavidin and biotin is dissolved and by repeating the magnetic separation ECs and Dynabeads are separated.

The aim of this new isolation technique is to obtain viable primary murine microvascular ECs with a high purity. With this gentle isolation procedure ECs are exposed to very little cellular stress and therefore are reflecting the *in vivo* situation best.

2. MATERIALS AND METHODS

All used materials and methods are listed and explained in the following part. The methods, materials, and devices, which are used in general, are not described below.

2.1. Material

2.1.1. Devices and consumable materials

Table 3: Used devices and their producers

device	producer
4°C refrigerator profi line	Liebherr, Biberach an der Riß, Germany
Autoclav Systex VX-150	Systec, Linden, Germany
FACS Calibur instrument	BD Bioscience, Heidelberg, Germany
ice machine MF22	Scotsman, Milan, Italy
incubator BBD 6220	Thermo Fisher Scientific, Waltham, USA
incubator Heracell 240i	Thermo Fisher Scientific, Waltham, USA
intelli-mixer RM-2L	Elmi, Riga, Latvia
laminar flow safe 2020	Thermo Fisher Scientific, Waltham, USA
Magnetic Particle Concentrator DynaMag	Thermo Fisher Scientific, Waltham, USA
megafuge 16R centrifuge	Thermo Fisher Scientific, Waltham, USA
microscope 40C	Zeiss, München, Germany
microscope observer Z1	Zeiss, München, Germany
microscope Primo Vert	Zeiss, München, Germany
scale Kern ew 420	Kern, Balingen, Germany

Table 4: Consumable material and their producer

consumable material	producer
cell culture plate 6, 12 well	BD Bioscience, Heidelberg, Germany
cell strainer 70 µm	BD Bioscience, Heidelberg, Germany
cover slip (round, 15 x 15 mm, 24 x 50 mm)	Gerhard Menzel, Braunschweig, Germany
cryo tube 20	TPP Techno Plastic Products, Trasadingen, Switzerland
needle 18Gx2	B.Braun, Melsungen, Germany
pipette tips 10, 100, 1000 µl	Sarstedt, Nümbrecht, Germany
reaction tubes 1.5 ml	Sarstedt, Nümbrecht, Germany
safe-lock tubes 0.5 ml	Eppendorf, Hamburg, Germany
single-use pipettes 1, 2, 5, 10, 25, 50 ml	Greiner Bio-One, Frickenhausen, Germany
slide-A-lyzer G2 dialysis cassette	Thermo Fisher Scientific, Waltham, USA
syringes 1, 2, 3, 10 ml	B. Braun, Melsungen, Germany
syringe filter 0.22 µm	TPP Techno Plastic Products, Trasadingen, Switzerland
tissue culture dishes 22.1	TPP Techno Plastic Products, Trasadingen, Switzerland
tissue culture test plates 96U	TPP Techno Plastic Products, Trasadingen, Switzerland
tubes 15, 50 ml	Greiner Bio-One, Frickenhausen, Germany
tubes 5 ml for flow cytometry	Sarstedt, Nümbrecht, Germany

2.1.2. Chemicals

Table 5: Used chemicals and their sources

chemical	source
Aqueous Mount	Zytomed Systems, Berlin, Germany
bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany
collagenase A	Roche Diagnostics, Mannheim, Germany

Dulbecco's Phosphate-Buffered Saline (DPBS)	Thermo Fisher Scientific, Waltham, USA
ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
eosin y-solution	Merck, Darmstadt, Germany
ethanol	Merck, Darmstadt, Germany
fixogum	Marabu, Tamm, Germany
FACS Clean	BD Bioscience, Heidelberg, Germany
FACS Rinse	BD Bioscience, Heidelberg, Germany
FACS Flow	BD Bioscience, Heidelberg, Germany
fetal bovine serum (FSC)	Sigma-Aldrich, Steinheim, Germany
Freund's adjuvant, incomplete	Sigma-Aldrich, Steinheim, Germany
Hanks' Balanced Salt Solution (HBSS)	Thermo Fisher Scientific, Waltham, USA
hematoxylin	Merck, Darmstadt, Germany
methanol	Merck, Darmstadt, Germany
Nonidet P 40	Sigma-Aldrich, Steinheim, Germany
phosphate buffered saline (PBS)	Sigma-Aldrich, Steinheim, Germany
primary antibody diluent	AbD Serotec, Puchheim, Germany
propidium iodide (PI)	Merck, Darmstadt, Germany
pure acetic acid	Merck, Darmstadt, Germany
sodium azide	Merck, Darmstadt, Germany
sodium bicarbonate	Sigma-Aldrich, Steinheim, Germany
sodium chloride (NaCl)	Merck, Darmstadt, Germany
sodium hydrogen phosphate monohydrate	Merck, Darmstadt, Germany
sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
trypan Blue solution	Sigma-Aldrich, Steinheim, Germany

2.1.3. Antibodies

Table 6: Used antibodies (clon, host, isotype, conjugation) and their sources for FACS

antigen	clon	host and isotype	conjugation	source
Isotype	HybIgG2a	mouse IgG2a	FITC	abcam, Cambridge, United Kingdom
Isotype	R35-95	rat IgG2a, κ	PE	BD Bioscience, Heidelberg, Germany
Isoytpe	A95-1	rat IgG2b, κ	APC	BD Bioscience, Heidelberg, Germany
CD14	Sa2-8	rat IgG2a, κ	FITC	eBioscience, Frankfurt am Main, Germany
FAT/ CD36	12-0362-82	hamster IgG	PE	Thermo Fisher Scientific, München, Germany
PECAM-1/ CD31	MEC 13.3	rat IgG2a, κ	PE	BD Bioscience, Heidelberg, Germany
Mucosialin/ CD34	RAM34	rat IgG2a, κ	FITC	eBioscience, Frankfurt am Main, Germany
HCAM/ CD44	Sc-9960	mouse IgG1 κ	FITC	Santa Cruz Biotechnology, Heidelberg, Germany
CD45	30-F11	rat IgG2b,κ	APC	BD Bioscience, Heidelberg, Germany
ICAM-1/ CD54	3E2	hamster IgG1, κ	FITC	BD Bioscience, Heidelberg, Germany
ICAM-2/ CD102	3C4	rat IgG2a,κ	FITC	BD Bioscience, Heidelberg, Germany
Integrin ß3/ CD61	2C9.G2	hamster IgG1,κ	FITC	BD Bioscience, Heidelberg, Germany
Endoglin/ CD105	MJ7/18	rat IgG2a,κ	PE	eBioscience, Frankfurt am Main, Germany
VCAM-1/ CD106	Sc-18864	rat IgG1,κ	FITC	Santa Cruz Biotechnology, Heidelberg, Germany
Prominin-1/ CD133	13A4	rat IgG1,κ	FITC	eBioscience, Frankfurt am Main, Germany
VE-cadherin/ CD144	11D4.1	rat IgG2a,κ	PE	BD Bioscience, Heidelberg, Germany

2.1.4. Kits

Table 7: Used kits and their sources

Kits	source
DSB-X^{TM} Biotin protein labeling kit	Thermo Fisher Scientific, Waltham, USA
Dynabeads FlowComp^{TM}Flexi, part A	Thermo Fisher Scientific, Waltham, USA
PierceTM BCA Protein Assay kit	Thermo Fisher Scientific, Waltham, USA

2.1.5. Software

Table 8: Used software and their sources

software	source
Adobe Photoshop CS4 Version 11	Abose Systems Incorporate, USA
AxioVision	Zeiss, München, Germany
Cell Quest Pro	BD Bioscience, Heidelberg, Germany
ImageJ 1.48v	Wayne Rasband, National Institutes of Health, USa
Microsoft Office	Microsoft Corporation, Redmmond, USA
Muriplan	Xstrahl – Medical & Life Sciences
SARRP control	Xstrahl – Medical & Life Sciences
SigmaPlot 11	Systat Software, wpcubed, Germany

2.1.6. Primary cells

Table 9: Used primary cells and their sources

primary cells	source	references
heart endothelial cells	this laboratory from mouse heart	(Sievert et al. 2014)
lung endothelial cells	this laboratory from mouse lung	(Sievert et al. 2014)

2.1.7. Laboratory animals

Female C57Bl/6 mice (Charles River) within an age range **of 10 to 14 weeks** were used for the experiments. Mice were housed in single, ventilated cages under pathogen-free conditions. Experiments were in agreement with German law on animal experiments and welfare (TVA number: 55.2-1-54-2532-191-14).

Table 10: Used pharmaceuticals and their sources

pharmaceutical	**source**
Dexpanthenol	Bayer, Leverkusen, Germany
Isoflurane	Piramal Critical Care, Hallbergmoos, Germany

For the anesthesia of the mice a cocktail of isoflurane and oxygen was used. They were inhaling the gas during the whole treatment (shooting of the CT-scans and irradiation).

2.2. Methods

2.2.1. Local heart irradiation with CT guided imaging

For a high precision irradiation of the heart a "Small Animal Radiation Research Platform" (SARRP, X-strahl, Camberley, UK) was used. The machine enables a CT-image guided high precision irradiation of structures in the sub mm range in small animals such as mice. Based on the images the irradiation of a specific region can be planned and carried out similar to that in the clinical radiation therapy.

For the experiment 10 -14-week-old female C57Bl/6 mice (Charles River Laboratories, Sulzfeld, Germany) were used, and different treatment groups were formed randomly. The mice were put under anesthesia with an inhalation of 3% Isoflurane/Oxygen in a box room. To prevent the eyes from drying out dexpanthenol was applied to both eyes. As soon as the mouse was anesthetized, it was placed on the mouse bed of the SARRP instrument. To ensure the mouse was in the correct position during the CT scans and radiation, the ventilation with Isoflurane was continued with an anesthesia mask. For the radiation a concentration of 1 - 2% of isoflurane was used. During the whole process the respiratory rate of the mouse was monitored. When the inhaled concentration of the anesthetic is too low, the respiratory rate is increasing, and the mouse can wake up. If the respiratory rate decreases, the Isoflurane concentration is too high, and the mouse develops gasping or respiratory arrest. Therefore, it is obligatory to monitor the respiratory rate during the whole process and to react with an increased or decreased concentration of isoflurane, when necessary. When the respiration problem of the mouse cannot be solved the anesthesia and the experiment must be stopped immediately.

To visualize the anatomy of the heart and the lungs of each mouse a CT cone beam computed tomography (CBCT) was taken with the SARRP machine. As settings for the CBCT 60 kV and 0.8 mA photons were used, which were filtered with aluminum (1 mm). 720 scans in two-dimensional projections over 360° were obtained in a transverse, sagittal, and frontal view. Based on this CT scans, the radiation therapy was planned, using *Muriplan*, the fitting image registration and dose planning system for SARRP. Heart and lung tissue was drawn in, in all obtained CT scans. For the irradiation of the whole heart and the surrounding parts of the lungs, the size of the beam and different collimators had to be chosen wisely. As there was no perfect collimator for the shape of the heart, different collimators were used, available in the sizes: 1 x

1, 3 x 3,5 x 5, 10 x 10, 9 x 13 mm^2. Three single beams with one 5 x 5 mm^2 collimator and two beams with a 3 x 3 mm^2 collimator were used to cover the area of the heart tissue. For the high precision heart irradiation, the central axis of the beam was set in the isocenter of the heart, using 220 kV and 13 mA, filtered with copper (0.15 mm), as settings for the x-rays. Mice, which were in the control group received a sham irradiation (0 Gy).

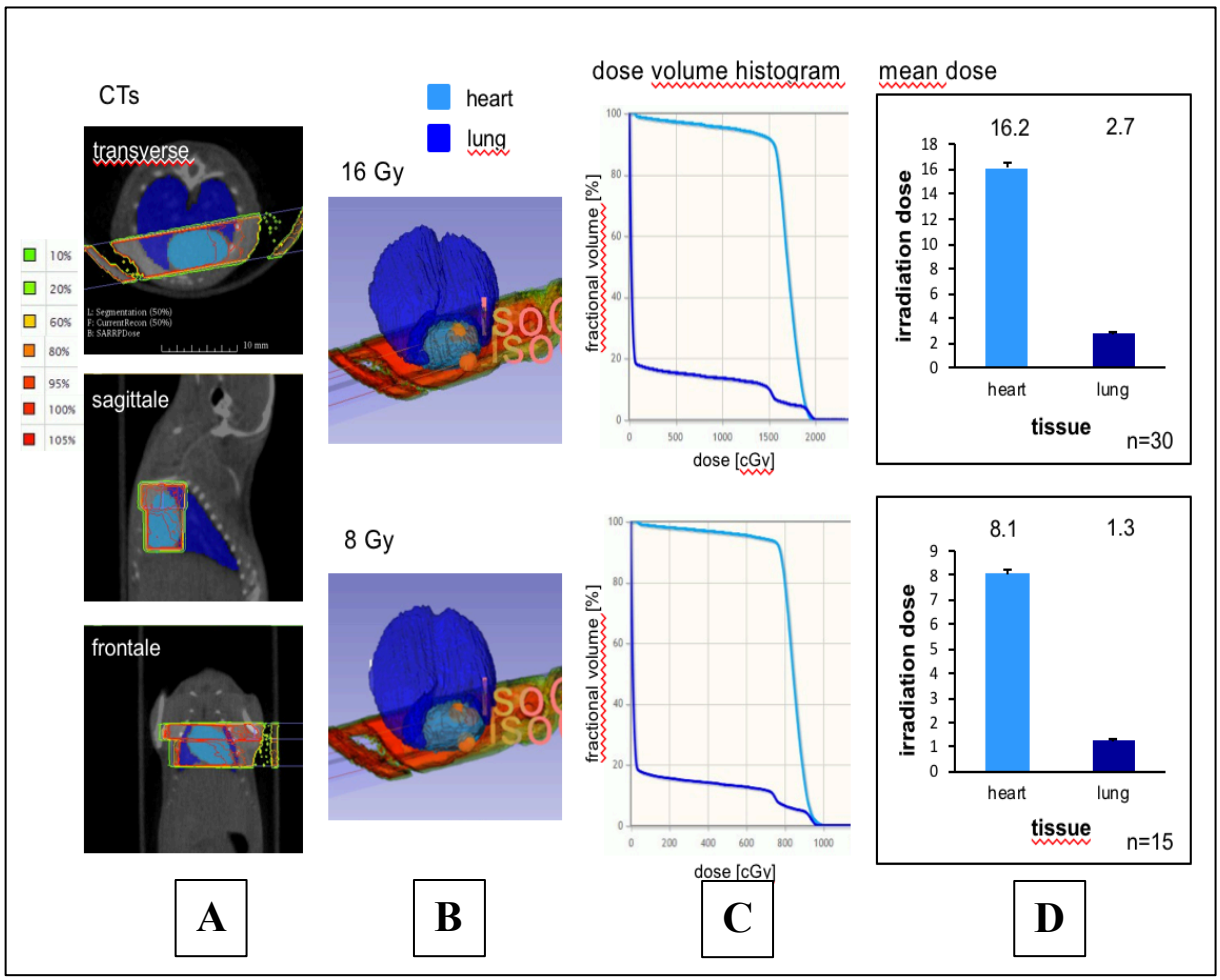

Figure 10: Local heart irradiation with CT guided imaging (SARRP)

*The graphic was published in the "International Journal of Radiation Oncology*Biology*Physics" by W. Sievert, S. Stangl, K. Steiger and G. Multhoff in the year 2018* ***(Sievert et al., 2018)****.* ***A)*** *Cone beam computed tomography (CT) of mouse thorax in transverse, sagittal and frontal view with drawn in heart tissue in lighter blue and drawn in lung tissue in darker blue. Beam of planned radiation is shown in green, orange and red rectangles.* ***(B)*** *3D reconstruction of the heart and lung tissue, established of the CT scans, with the beam of planned radiation, in red and orange for 16 Gy and 8 Gy.* ***(C)*** *Dose-Volume Histogram for whole heart irradiation with 16 Gy and 8 Gy. More than 95% of the heart volume was irradiated, whereas less than 20% of the lung tissue was exposed to radiation. 7% of the lung was in the field of the beam with an intensity of 16Gy or more, whereas 14% of the lung volume was irradiated with 10 Gy or more.* ***(D)*** *Tissue irradiation dose bar chart for a mean dose of 16 Gy and 8 Gy. If the heart was treated with a dose of 16.2 Gy the lung was irradiated with 2.7 Gy. For an irradiation of the heart with 8.1 Gy the lung was in the field of irradiation with 1.3 Gy.*

Radiation-induced lung diseases (RILD), such as pneumonitis, are confounding factors in clinical therapy and should be at a minimum level. Therefore, it was important to avoid the radiation of lung tissue. To optimize this irradiation plan V10 and V16 had to be as small as possible. V10 describes the percentage of lung volume, which is irradiated with 10 or more Gy. V16 describes the percentage of lung volume, which is irradiated with 16 Gy or more Gy. Using this high precision irradiation, it was possible to reduce the lung tissue irradiated with 10 Gy or more to 14% (= V10), whereas only 7% (= V16) of the lungs were irradiated with 16 Gy or more. As only 18% of the whole lung tissue was exposed to radiation, 82% of the lung tissue did not get a radiotherapy at all. In contrast to the lung volume, over 95% of the heart volume was in the field of radiation with measured mean irradiation doses of 16.2 ± 0.3 and 8.1 ± 0.1 Gy. To simplify the mean doses were rounded to 8 and 16 Gy.

For each of the 4 points (20, 30, 40 and 50 weeks after irradiation) the experiment was performed 3 times (n = 3), in which 2 mice were sacrificed for one run. Since all mice survived the irradiation with 16 Gy, 3 instead of 2 mice were included into each irradiation group. In total 84 mice were sacrificed in the whole study.

Table 11: Treatment groups after 20, 30, 40 and 50 weeks

	n*	**animals/n***	**points in time* =**	**Sacrificed mice**
Sham group **(0 Gy) =**	3*	2*	4 =	**24**
Irradiation group **(8 Gy) =**	3*	2*	4 =	**24**
Irradiation group **(16 Gy) =**	3*	3*	4 =	**36**
In total				**84 sacrificed mice**

2.2.2. γ-H2AX-staining

Irradiating the heart of mice leads to cell death. The exogenous factor of radiation cause base damage, sugar damage, single stranded or even double stranded breaks (DSBs), which result in apoptotic cell death (Kuo & Yang, 2008). The produced DSBs and their activated repair mechanism can also be used for visualizing the severity of damage. Therefore, the physiological modification of H2AX, a Histone of the H2A family, was used for visualization of DSBs. H2AX is phosphorylated at serin 139 by ataxia telangiectasia mutated (ATM) in the presence of a DNA damage (Kuo & Yang, 2008). The γ-H2AX, is a sensitive DNA damage repair

marker. It can be detected by using an γ-H2AX-antibody which is coupled to a dye. This staining method is used for the distinction between tissue with irradiation-induced DNA damage and non-damaged tissue. It is very important to know, which parts of the lungs that are in the field of irradiation on the CT scans, were irradiated and therefore stained positively for γ-H2AX.

For this γ-H2AX (Cell Signaling Technology, Danvers, MA) on a Bond Rx staining machine (Leica Biosystems, Nussloch, Germany), using a Polymer Refine detection system without post primary reagent, was applied to visualize the DNA repair after DSBs. As heart and lung tissue was needed for this investigation, three mice were sacrificed one hour after *in vivo* heart irradiation with 16 Gy. To detect irradiation-induced damages, heart and lung tissue was extracted at each time point and after each individual irradiation dose (n = 3).

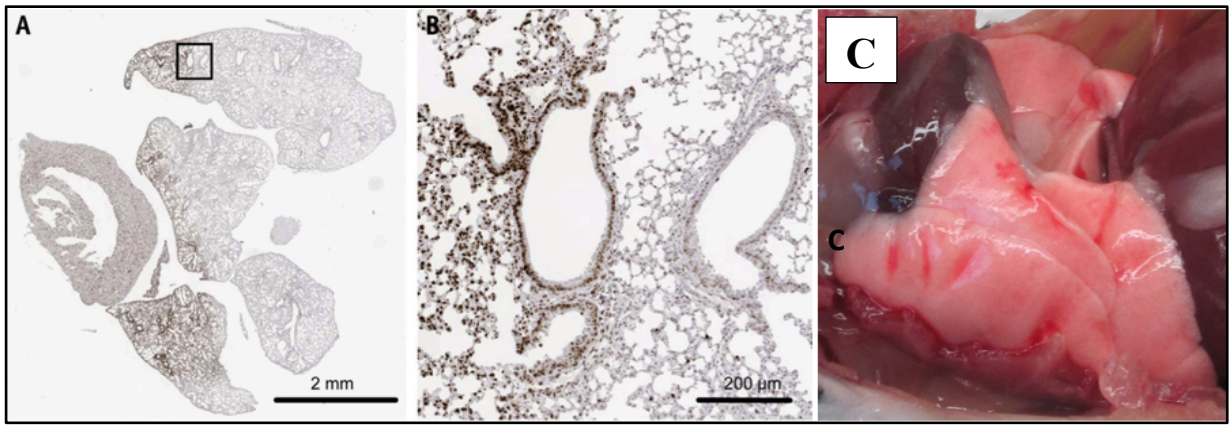

Figure 11: Immunohistochemical γ-H2AX staining of heart and lung and photo of the used heart and lung tissue for the staining in vivo

(A) In the whole heart and the surrounding lung, especially the cranial lung lobes, a positive staining is detectable. **(B)** Very sharp beam edge visualized by γ-H2AX immunohistochemistry. (A) and (B) were published by W. Sievert, S. Stangl, K. Steiger and G. Multhoff in the International Journal of Radiation Oncology*Biology*Physics (*Sievert et al., 2018)* **(C)** Heart of a mouse surrounded by the lung tissue in vivo, which was used for the γ-H2AX staining.

After one night in formalin the tissue was embedded in paraffin and cut into 2 µm slices. The sections were stained with eosin (eosin y-solution 0.5% aqueous) and hematoxylin (Mayer's hematoxylin). Therefore, the slices were dried at room temperature and fixed in a solution of cold acetone for 10 minutes at 4°C (Sievert, 2016). In a next step the sections were washed in PBS for 10 min. Hematoxylin was added for one minute and the slices were washed under warm flowing tap water for 10 min. After that, 200 µl eosin with 2 drops of acetic acid (100%) were put on the slices for 2 min. For the following 5 minutes the slices were washed under

warm flowing tap water. To embed the stained tissue aqueous mount was used. To keep dust and any contamination away from the slides, cover slips without air bubbles were applied.
For the quantitative analysis a digital slide scanner (Leica AT2), which creates digital scans, was used to compare the cell density. ImageJ was the used program for the evaluation of the sections.

2.2.3. Isolation of primary ECs

In the first 2 -3 weeks after the irradiation acute inflammation occurred (Himburg et al., 2016). Thereafter, the mice were sacrificed 20, 30, 40 and 50 weeks after the irradiation. Searching for long irradiation damages or chronic modifications of the irradiated tissue, primary heart and lung ECs were isolated. The used procedure is a newly established isolation method (Sievert et al., 2014), which is enables the isolation of viable primary microvascular ECs of heart and lung tissue at a high purity.
To obtain sufficient ECs for further analysis two to three mice were sacrificed by cranio-cervical dislocation for each time point. Heart and lung tissues were collected under aseptic

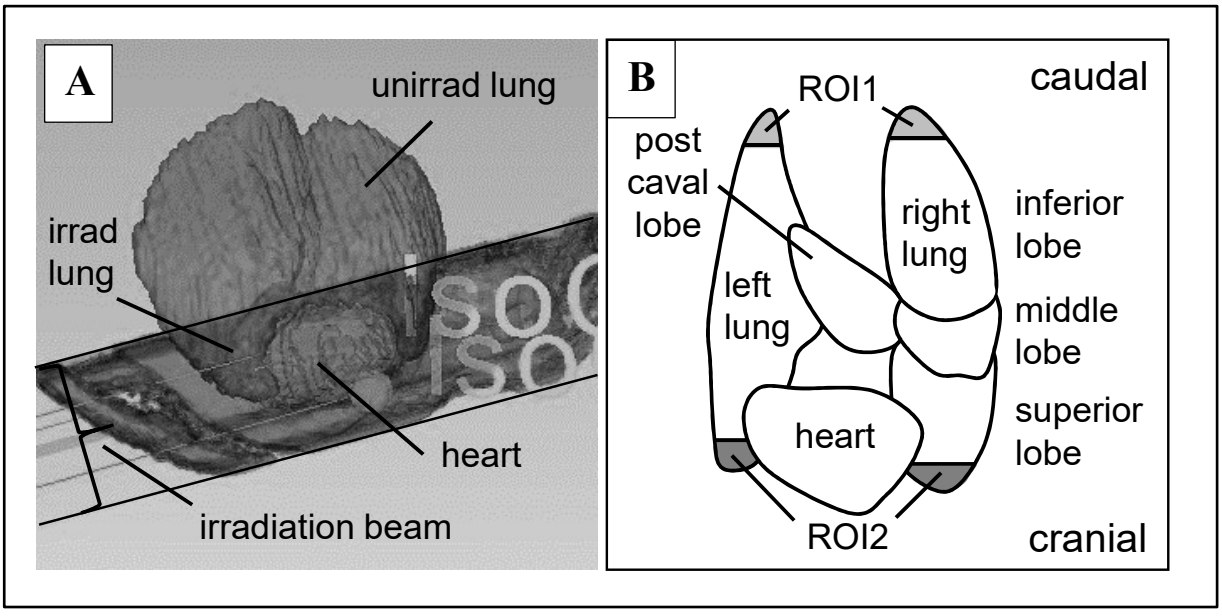

Figure 12: Way of the x-rays through the heart and the lung shown in an CT-reconstruction and a schematic illustration

(A) 3D reconstruction of the heart and lung tissue*, established of the CT scans, the beam of radiation is shown in this image (see Figure 10B);* ***(B) schematic illustration of the anatomical situation in the thorax of mice;*** *the illustration is based on the position of the mouse lying on her spine, the caudal lain parts of the right and left lung are outside the field of radiation (= Region of Interest 1), the parts close to the heart are in the field of radiation (Region of Interest 2).*

conditions and the right and left atria of the heart were removed to avoid contamination of the cell samples with macrovascular ECs. Two different samples of the removed lungs were isolated: on the one hand the lung tissue, which was in the field of radiation (= Region of Interest 2 (ROI2), see Figure 12B), on the other hand the part of the lung tissue, which was unirradiated (= Region of Interest 1 (ROI1), see Figure 12B). To know which parts of the lungs were irradiated, the CT image guided irradiation plan as well as the γ-H2AX staining were used for confirmation. Each sample (heart and lung) was placed in wells, filled with phosphate-buffered

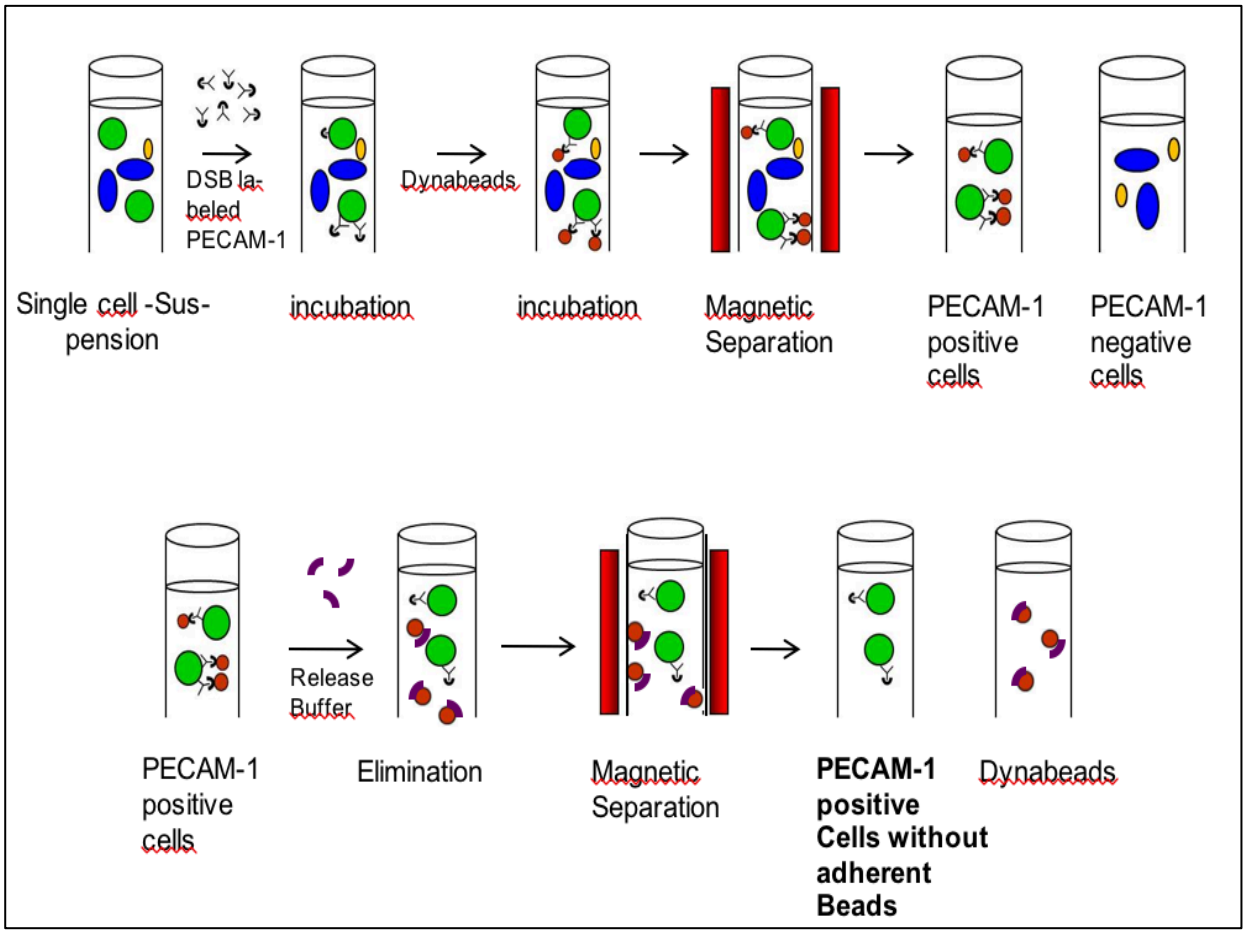

Figure 13: Schematic representation of the isolation procedure of primary, bead-free ECs

After the mechanic and enzymatic dissolution of the heart and lung tissue, the viable ECs (green) were collected in a single cell suspension. After the incubation of the added DSB-X (biotin) labeled anti mouse PECAM1-antiobody, they bound to the PECAM-1-antigen of the ECs. In the next step streptavidin coated magnetic micro-Dynabeads (red) were given to the suspension, which interacted with biotin and formed a DSB-X-streptavidin-bead-complex. Using a magnet, PECAM-1 positive cells und PECAM-1 negative cells (blue and yellow) could be separated, because of the magnetic character of the Dynabeads. PECAM-1 negative cells were pipetted into another tube, whereas PECMA-1 positive cells remained on the wall of the tube. In the last step PECAM-1 positive cells were washed several times and release buffer was added. The buffer was used to lose the magnetic Dynabeads, which were in a noncovalent interaction with streptavidin. Magnetic separation was used for the second time and as a result PECAM-1 positive ECs without adherent Beads could be gained, whereas the beads remained in the tube. The figure was performed by W.Sievert (Sievert, 2016; Sievert et al., 2014).

saline (PBS) and cooled on ice and rinsed. As PBS is an isotonic buffer solution the pH-value remained constant with the same osmolarity of the body, which provides a non-toxic environment for the tissue during the washing steps.

In a next step the heart and lung tissues were cut into 1 mm pieces with a sterile scalpel blade. Each sample was transferred into a 15 ml tube filled with pre-warmed (37 °C) 10 ml sterile Collagenase A (0.15 U/mg), which was dissolved in Hank's Balance Salt Solution HBSS/10% fetal calf serum (FCS). After 45 minutes at 37°C under rotation with two rounds per minute the tissue was pre-digested. To disperse the sample a 10 ml syringe with an 18 G needle was used and the tissue was taken trough 10 times. Afterwards the single cell suspension was filtered through a 70 µm cell strainer (BD Bioscience) and washed with 50 ml HBSS/10%FCS (Sievert, 2016). The next step was to centrifuge the sample at 1500 rpm for 10 minutes. To prevent the loss of ECs, the last washing procedure was repeated once. To transfer the final cell pellet in 1,5 ml tubes it was resuspended with 400 µl ice-cold isolation buffer and the 50 ml tube was washed with 200 µl of isolation buffer (Sievert et al., 2014).

In the last step of the whole isolation process 25 µl of DSB-X labeled anti mouse PECAM1-antiobody was added und incubated for 10 minutes at 4 °C under 3 rotations per minute. Next, the cell suspension was transferred into a new 15 ml tube and the 1,5 ml tube, in which the sample was before, was washed twice with 1ml of isolation buffer each time. The cell suspension was centrifuged for 8 minutes at 1400 rpm. After centrifugation the cells were resuspended in 700 µl ice-cold isolation buffer and transferred to a new 1.5 ml tube, afterwards the tubes were washed with 300 µl to reduce the loss of primary cells (Sievert et al., 2014).

As a next step 75 µl of streptavidin coated magnetic Dynabeads were added and taken under rotation (3 rpm) for 15 minutes. As streptavidin and biotin (DSB-X) are in a noncovalent biological interaction, the Dynabeads bind to the ECs. After this step we got a DSB-X-streptavidin-bead-complex, which could be separated by a magnet from *PECAM-1* negative cells. After an incubation time of 2 minutes in the magnetic separator, it was possible to remove *PECAM-1* negative cells from the cell suspension using a pipette and resuspend the bead bound *PECAM-1* positive cells, which remained on the wall of the tube in 1ml of isolation buffer. To get off the other cells this washing process was repeated at least five times. After the last washing process 1 ml of flow comp release buffer was added and mixed with the cells by pipetting up and down 10 times. For 5 minutes the buffer was incubated under rotation. After the mixing of the cells by pipetting 10 times again, the *PECAM-1* positive cells and Dynabeads were separated during the stay of 1 minute in the magnetic field. The release buffer removed the streptavidin biotin complex from the anti-mouse CD31 antibody. The magnetic streptavidin biotin

complex was on the wall of the tube and the *PECAM-1* positive ECs in the supernatant, which was transferred to a new 1.5 ml tube (Sievert, 2016). To prevent losing ECs magnetic separation was repeated with the streptavidin biotin complex. As a last step the supernatant was transferred in a new 15 ml tube with 2 ml cold isolation buffer and centrifuged for 8 minutes at 350 g.

For counting the obtained ECs in the suspension, the trypan-blue exclusion test and a Neubauer chamber were used. Trypan blue is a dye to distinguish viable from non-viable cells. As stained cells do not have an intact cell membrane trypan blue is able to get into dead cells and stain them. With this technique it is possible to count and differentiate dead from viable cells.
In this experiment a 1:2 dilution of the sample, the unstained viable isolated ECs, and the trypan blue dye was filled under a Neubauer chamber. Viable cells could now be counted in the big squares under the microscope. It is important to count just the upper and left limit or the lower and right limit of cells, touching the lines.
With the following formula the total number of ECs can be calculated:

$$total\ cells = \frac{counted\ cells\ x\ 10^4}{number\ quadrants}\ dilution$$

Approximately 0.1 x 10^6 viable *PECAM-1* positive ECs can be isolated from one heart or one lung.

2.2.4. Flow cytometry analysis

2.2.4.1. Theory

The flow cytometry is a method to quantify cell surface markers (Sievert, 2016). For this technique diffuse light and fluorescence property is used to judge the examined cell types.
The instrument for flow cytometry is a *FACS Calibur*. To distinguish the different cell types, all cells are passed through a laser beam one at a time. The light of the laser scatters is registered when the cells have to pass through. This scattered light creates two signals: on the one hand the signal of the forward scatter, which can be compared to the size and surface of the cell, on the other hand the signal of the sideward scatter. The sideward scatter examines the granularity of the cell. The forward scatter detector is in the same plane as the laser, whereas the side scatter

detector is in a right angle to the beam. Combining these two pieces of information it is possible to make a 2-D reconstruction of the cells in the sample and assign them to different types (Suring et al., 2016).

In addition, it is also possible to examine the cells by using immunofluorescence. At the extrinsic part of the cell membrane there are a lot of anchored surface proteins. These are defining membrane-bound glycoproteins for different cell types, which are also called cluster of differentiation (CD) molecules (Engel et al., 2015). To make the cell defining surface proteins visible specific fluorescence labelled antibodies were added to the single cell suspension. To detect the unspecific interactions of the immunoglobulins, isotype-matched antibodies, which were labelled with fluorescence molecules, were added as a negative control. These are important to take the fluorescence signal of non-specific binding into consideration and, furthermore, to confirm the specificity of the primary antibody (A. B. Lyons & Parish, 1994). For this the fluorescence molecules, such as FITC (Fluorescein-5-isothiocyanat), PE (Phycoerythrin) or APC (Allophycocyanin), of the labelled antibodies are necessary (Jung et al., 1993).

When a cell passes the beam of the laser, it will change the level of energy from the ground state to an excited state. By returning to the ground state, the transmitted energy is emitted as a photon with a certain wavelength. The FACS instrument has particular filters, mirrors and detectors to separate the different colors of the emitted photons and it measures them as a voltage signal. In the used *FACS Calibur* we had a blue argon-laser beam with a wavelength of 480 nm and a Helium-laser with a wavelength of 630 nm. The filter, which is used for FITC is at 530 nm and detects green light, the one used for PE is at 585 nm and detects yellow light (Mei et al., 2015). In the end all the collected data was evaluated with the program *Cell Quest Pro.*

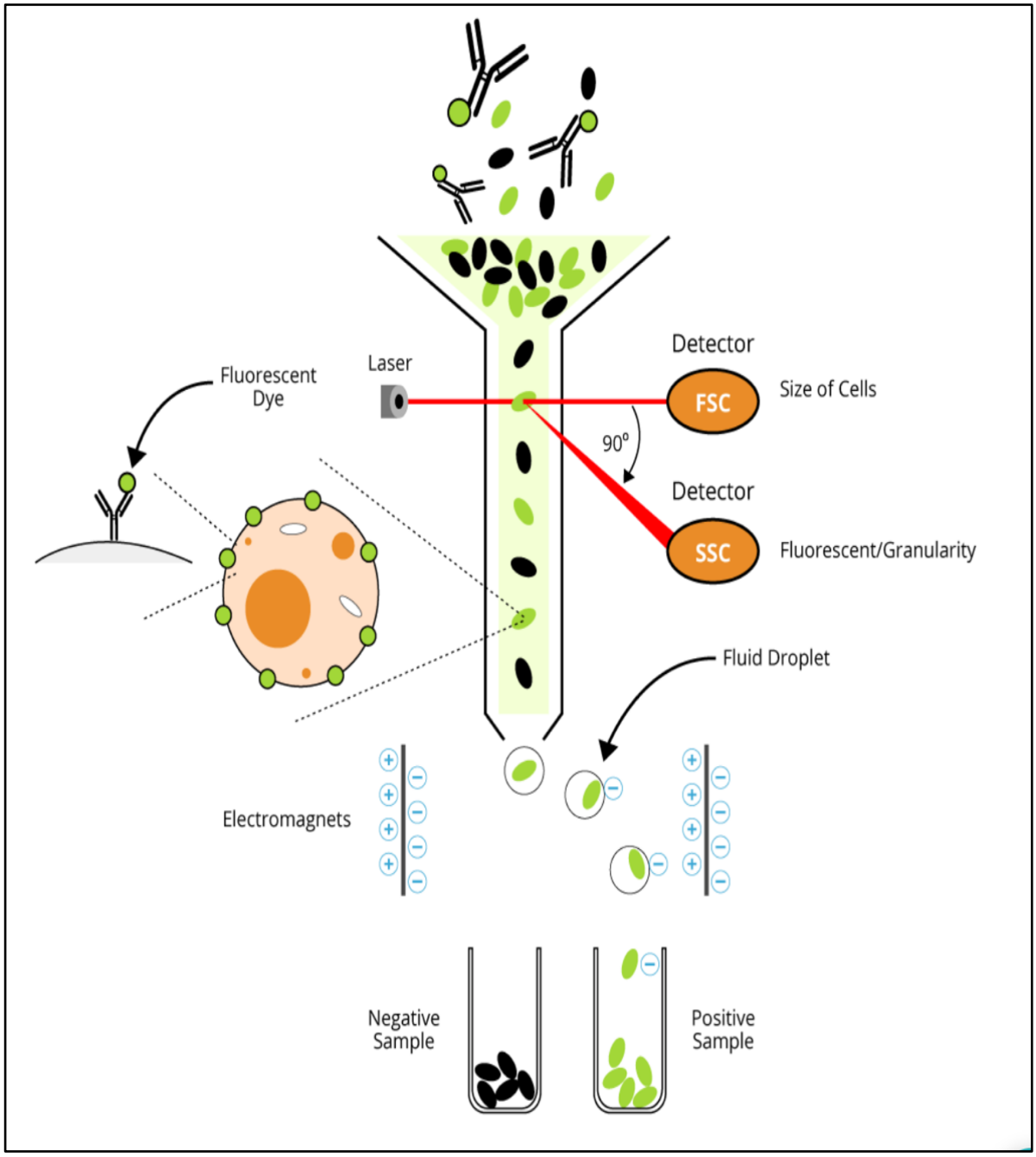

Figure 14: The mechanism of Fluorescence Activated Cell Sorting (FACS)

The laser beam hits the single cell suspension in a right angle and scatters the light. The forward scatter detector (FSC), in the same plane as the laser, gets signals, which can be compared to cell size and surface. The signal of the scattered light in the side scatter detector (SSC), located in a right angle to the beam, examines the granularity of the cell. The fluorescent channels are able to receive photons, produced by FITC, APC or PE. With the help of electromagnets, the cells are sorted in negative and positive samples. In the end all the signals are transferred to the PC software program Cell Quest Pro. The figure was shown on the webside of bosterbio in an article about "Flow Cytometry principles" (Principle, n.y.).

2.2.4.2. Examination of heart and lung endothelial cells

For examining primary heart and lung ECs, the laser-based, fluorescence assisted cell sorting (FACS) technique was used. Therefore, the single cell suspensions had to be prepared with the immunofluorescence labelled antibodies.

As a first step the suspension of the obtained primary heart or lung cells (0.1 x 10^6 cells/tube) was washed with 10 ml PBS/FCS 10% in 15 ml Eppendorf tubes and centrifuged for 8 minutes with 1400 rpm at 4°C. The supernatant was decanted, and the cells were resuspended with 1ml of PBS/FCS 10%. All cell suspensions were transferred in 1.5 ml tubes with a content of 1 ml per tube and they were centrifuged for 5 minutes with 500 g at 4°C. The supernatant was drawn off afterwards.

In the next step the fluorescence labelled antibodies were added to the samples. In each tube there should be at least 1x10^5 living cells. After an incubation time of 30 minutes on ice in the dark the samples were washed with PBS/FCS 10% again. The cells were resuspended in the tubes and PI was added.

After the examination with *FACS Calibur*, the software program *Cell Quest Pro* was used for the evaluation. It was very important to exclude cells from the evaluation, who bound the fluorescence labelled antibodies non-specifically. These were, on the one hand, the primary heart or lung ECs, that died during the editing process. For eliminating these, a co-staining and gating strategy with proprium-iodide (PI) was used, because of the characteristic of this molecule to penetrate the cell membrane of dead cells (Tung et al., 2007). On the other hand, unspecific binding of living cells should be removed. Therefore, the number of cells, which have been stained unspecifically, were subtracted from the number of ECs, which have been stained specifically. In the end the outcome was the percentage of positive cells. For the mean fluorescence intensity (MFI) a calculation also had to be done: the mean for specific fluorescence intensity minus the mean for unspecific fluorescence intensity (Sievert, 2016).

2.2.5. Statistics

The analysis of the numbers of positively stained cells (proportion of cells) and the mean fluorescence intensity (mfi), the expression density of protein per cell, was performed by *Cell Quest Pro* software. A comparative analysis of the data was carried out by using the Tukey test. The significance levels were $p^* < 0.05$ (5 %); $p^{**} < 0.01$ (1 %) and $p^{***} < 0.001$ (0.1 %). The data are presented as means of the number (n) of indicated experiments (Sievert, 2016).

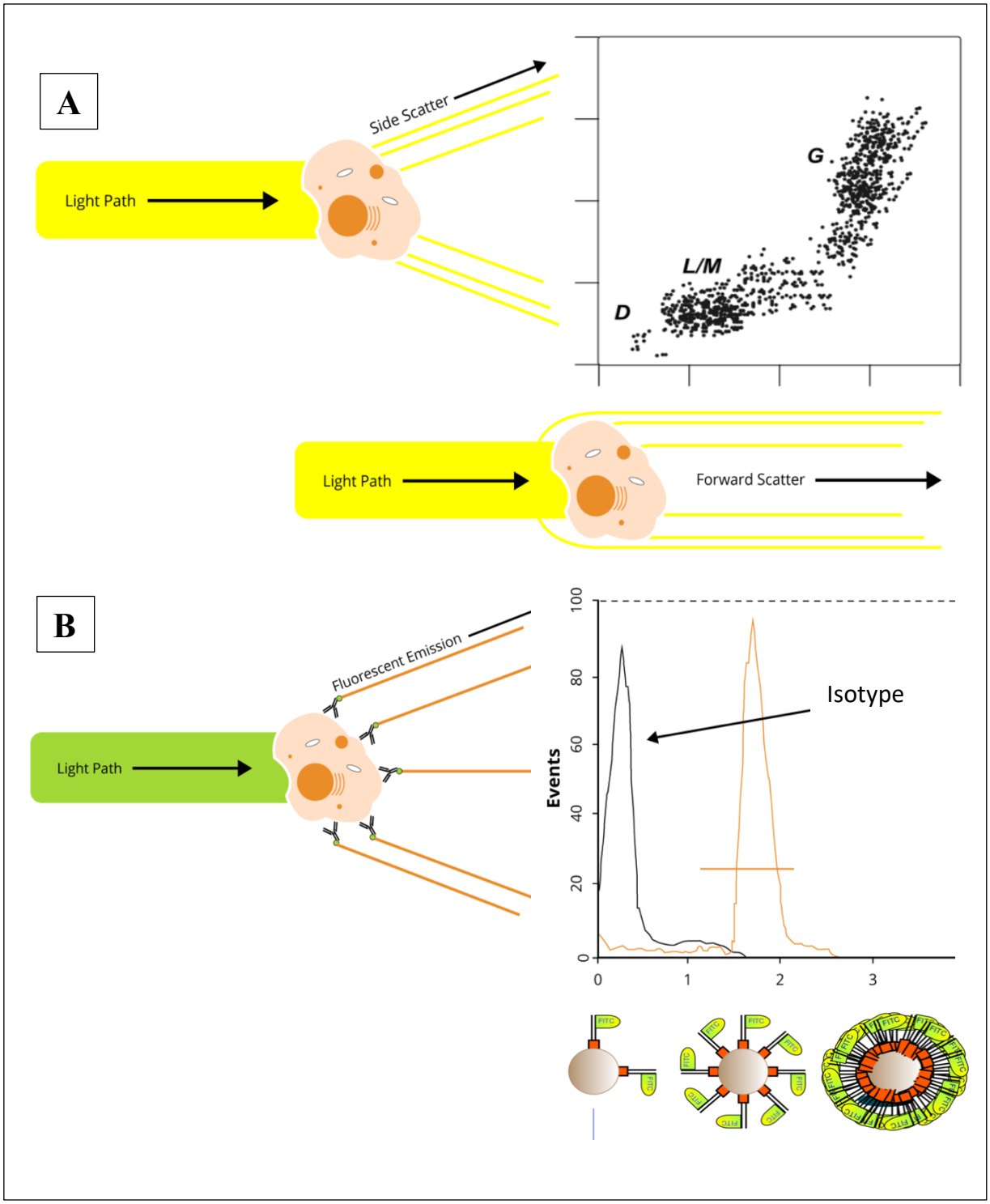

Figure 15: Density and Histogram Plot of a FACS-Analysis

(A) Density Plot: *The measured results of the forward scatter on the x-ray are put against the results of the Side Scatter on the y-ray. Hence, the different crossing points can be assigned to different cell types;* ***(B) Histogram Plot:*** *The measured results for the intensity of the fluorescence molecules like FITC on the x-ray are put against the measured events of the fluorescence emission signal on the y-ray. The first peak is the negative control of the Isotype, the second peak are the cells, expressing the antibody. The pictures and graphics shown above were published by M.Holschbach in the year 2013 and on the website of boster-bio.com (Martin Holschbach, 2013; Principle, n.y.).*

3. RESULTS

To analyze late irradiation damages or chronic modifications after heart irradiation, primary ECs of the heart and the lungs were isolated with a high purity. They were screened for different surface markers using flow cytometry. By analyzing the expression pattern of the markers, it was possible to prove that the isolated cells were indeed ECs. Afterwards the markers were grouped into i) markers of proliferation, ii) progenitor cells, iii) fatty acid metabolism and iv) inflammation. Since we were interested especially in late-stage effects of irradiation the acquisition of the data was carried out 20, 30, 40 and 50 weeks after irradiation.

Comparing the ECs of hearts, after *in vivo* radiation therapy with 8 and 16 Gy, with ECs of the control group, which obtained a sham irradiation, different alterations were detected.

As the heart is surrounded by lung tissue, directly adjacent parts of the lungs are in the field of radiation. To determine whether parts of the lungs in the field of irradiation are also damaged, irradiated lung tissue was examined and compared to parts of the lungs which received no irradiation.

Although the task of the lungs and the heart is widely divergent, both organ systems are interacting and rely on each other. Possible bystander effects of the connected heart and lung tissue were studied by comparing the lung tissue in the field of radiation with the unirradiated parts of the lungs after irradiation of the heart with a dose of 8 and 16 Gy. Hence, the same endothelial surface markers were applied for the detection of the irradiation-induced alterations of the heart and the lung ECs. The lung EC´s were also isolated and screened 20, 30, 40 and 50 weeks after irradiation.

3.1. Proportion of cell surface markers for heart and lung endothelial cells after irradiation

After the isolation of primary heart and lung ECs their surface was screened for different markers. Therefore, EC specific antibodies were used to trace the surface markers ***HCAM, Integrin ß3, Endoglin VE-cadherin, Mucosialin, Prominin-1, PECAM-1, ICAM-1, ICAM-2, VCAM-1 and CD36.*** By using flow cytometry, ECs were characterized directly after the isolation procedure. CD45**,** the surface marker for leukocytes, was not measurable in all isolated ECs. This marker was used as a negative control marker.

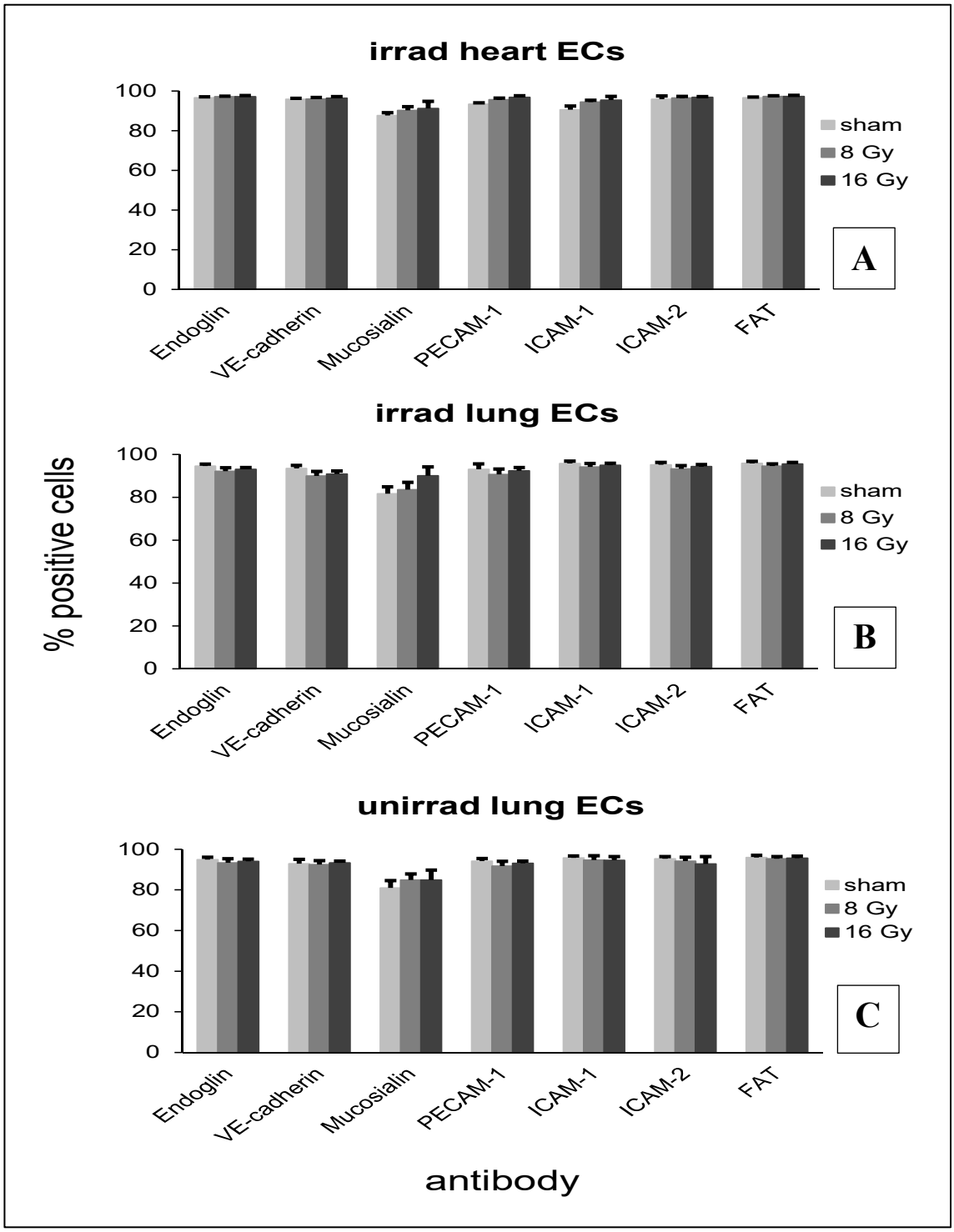

Figure 16: Percentage of positively stained cells for the identification and characterization of endothelial cells (ECs) derived from different tissue

(A) irradiated heart ECs *(n = 3);* ***(B) irradiated lung ECs*** *(n = 3);* ***(C) unirradiated lung EC´s*** *(n = 3) Using flow cytometry analysis ECs were identified with specific antibodies against Endoglin, VE-cadherin, Mucosialin, PECAM-1, ICAM-1, ICAM-2 and FAT on irradiated heart and irradiated lung ECs as well as on unirradiated lung ECs. The bar charts show the different surface markers on the x-ray and the % positive cells on the y-ray.*

Asterisk represents significantly different values ($p^{*} < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$).

In figure 16 all positively stained cells for heart and lung tissue are shown. Irrespectively of the irradiation of the heart or lung tissue, there is no difference between the surface markers. The same applies to unirradiated and irradiated lung tissue. The percentage of positively stained cells is very high for *Endoglin* (95 ± 1%), *VE-cadherin* (93 ± 1%)**,** *Mucosialin* (86 ± 3%)**,** *PECAM-1* (93 ± 1%), *ICAM-1* (95 ± 1%), *ICAM-2* (95 ± 1%) and *FAT* (96 ± 1%). The combination of surface markers is characteristic for ECs. Therefore, it can be assumed that the analyzed cells are indeed ECs.

The proportion of positively stained ECs was low for the proliferation marker *Integrin ß3* in irradiated heart and lungs (29 ± 4%) as well as on unirradiated lung tissue (27 ± 4%). For the inflammation markers *HCAM* and *VCAM-1* significant differences are measurable in heart ECs. The originally low percentage of positive stained cells for *HCAM* is significantly increasing after 20 (11 vs. 71%, p = 0.005), 30 (11 vs. 74%; p = 0.03), 40 (6 vs. 49%; p = 0.042) and 50 (5 vs. 38%; p = 0.005) weeks after heart irradiation with a dose of 16 Gy. Similar results could be shown for *VCAM-1*: At all time points (20 – 50 weeks after irradiation) the percentage of positively stained ECs of the heart increased with an irradiation dose of 16 Gy (51% and 49% and 52% and 53% vs. 33%; p = 0.015, p = 0.038, p = 0.036 and p = 0.033). The values for irradiated und unirradiated lung tissue did not show any differences compared to the sham group. The inflammation marker *HCAM* (54 ± 8%) and *VCAM-1*(18 ± 0.3%) remained unaltered for both radiation doses from 20 up to 50 weeks after irradiation.

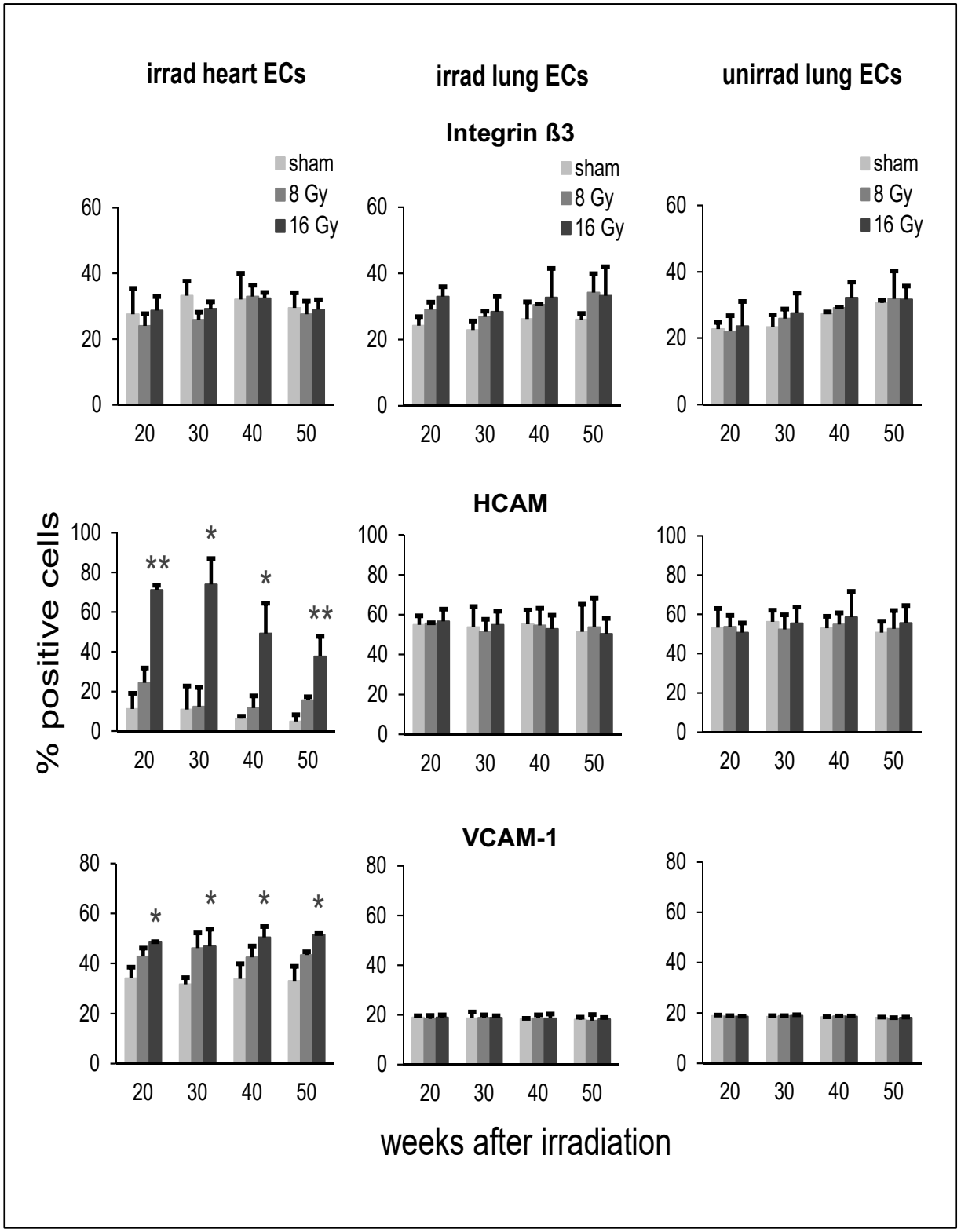

Figure 17: Positive stained endothelial cells (ECs) from irradiated heart and lung as well as unirradiated lung tissue after different points in time

Proportion of positively stained ECs (Integrin b-3, HCAM and VCAM-1) isolated from irradiated heart and irradiated lung as well as unirradiated lungs 20, 30, 40 and 50 weeks after irradiation with 8 and 16 Gy.

Asterisk represents significantly different values ($p^{*} < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$).

3.2. Irradiation induced alterations of heart and lung endothelial cell surface markers

3.2.1. Proliferation markers

For the analysis of the proliferation markers after *in vivo* irradiation special antibodies, which bind directly to the endothelial surface markers, were used. The investigated markers of the cell surface of ECs were *Integrin ß3 (CD61), Endoglin (CD105) and VE-cadherin (CD144).*
Compared to the sham group, which did not get a radiotherapy session, for 8 Gy no significant measurable difference was detectable after 20, 30, 40 or 50 weeks. For an irradiation with 16 Gy *Integrin ß3* and VE cadherin stayed at the same levels as the control group. Only *Endoglin* showed an increase of the mean fluorescence intensity (mfi) for the whole observation time. This means we measured a higher level of the mfi for 20 (216 vs. 159; $p = 0.033$), 30 (198 vs. 136; $p = 0.007$), 40 (238 vs. 157; $p = 0.009$) and 50 (214 vs. 151; $p = 0.006$) weeks after the irradiation. Furthermore, it was conspicuous that the mean fluorescence intensity of *VE-cadherin* decreased nearly by half from the initial value within the observed period.
For the investigation of the lungs the same proliferation markers were used. Like before *Integrin ß3 (CD61), Endoglin (CD105) and VE-cadherin (CD144)* were examined, but this time irradiated and unirradiated lung tissue was compared. Fluorescence marked antibodies were used to detect the proliferation markers, which are expressed on the cell surface of ECs. These are markers for the growth and division of cells.
For the surface markers *Integrin ß3*, *Endoglin* and *VE-cadherin* no significant differences were shown, neither for the lung tissue in the radiation field nor for the unirradiated lung tissue, because the analyzed values remained at the same levels as in the control group for a heart irradiation with 8 and 16 Gy over the whole period (20, 30, 40 and 50 weeks).

3.2.2. Progenitor cells

The marker of interest in the group of progenitor cells was *Mucosialin* (CD34). The surface of ECs was screened for this membrane protein, with the help of their specific binding antibody.
The expression density of *Mucosialin* on heart ECs showed no significant changes for an irradiation with 8 and 16 Gy over the observed period (20 - 50 weeks). Like the measurements on heart ECs *Mucosialin* showed no changes for lung ECs after a heart irradiation with 8 and 16 Gy, no matter if the analyzed lung tissue was in the field of radiation or not.

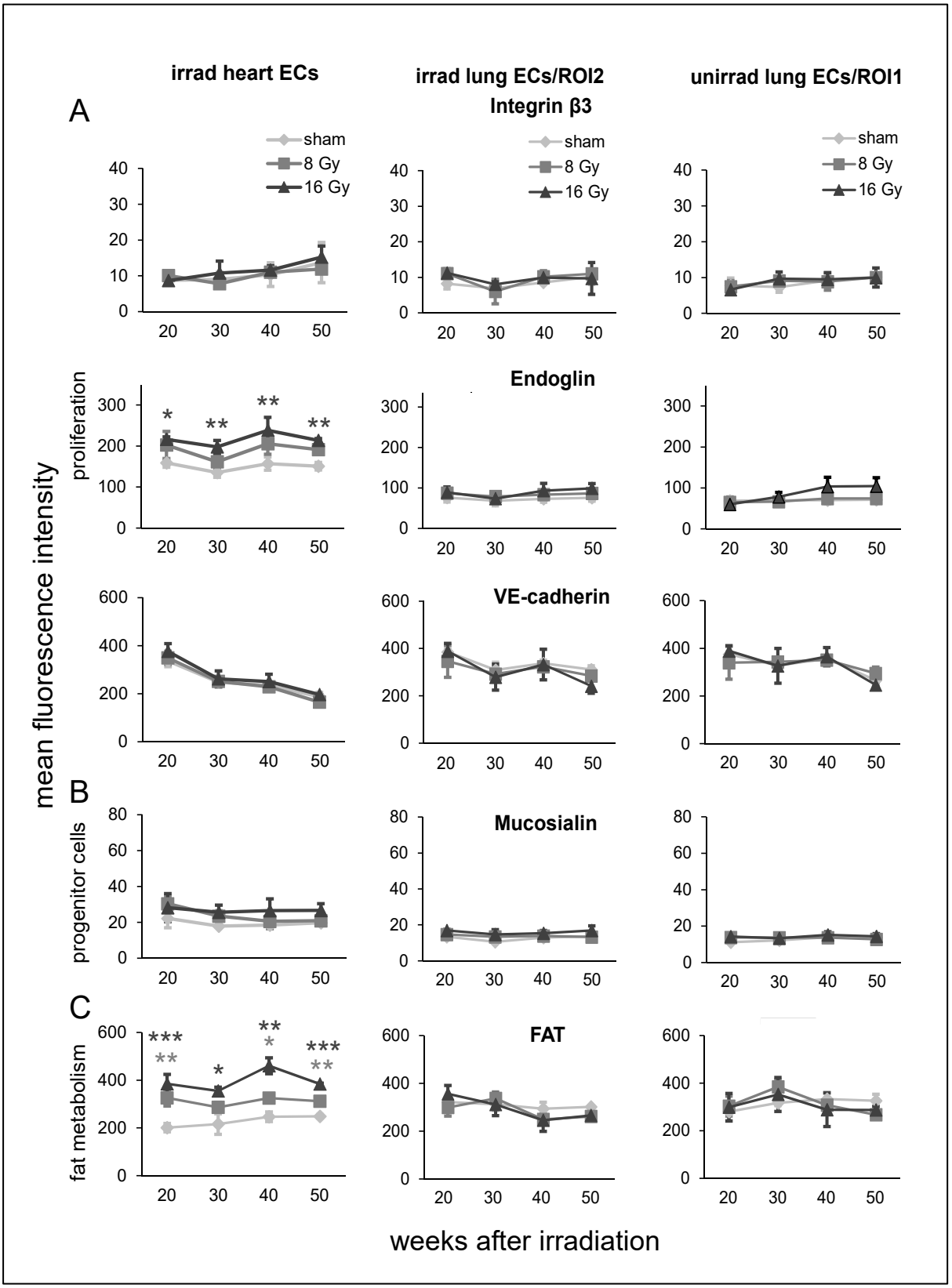

***Figure 18**: Endothelial cell (EC) expression density of antibodies at different points in time divided into groups of markers for (A) Proliferation (B) Progenitor cells and (C) Fat Metabolism from irradiated hearts and irradiated lungs (ROI2) as well as unirradiated lungs (ROI1)*

(A) Proliferation markers: *Expression density (mean fluorescence intensity) of* ***Integrin ß3, Endoglin, VE-cadherin*** *on ECs isolated from irradiated hearts and irradiated lungs (ROI2) as well as unirradiated lungs (ROI1) 20, 30, 40 and 50 weeks after irradiation with 8 and 16 Gy.* ***(B) Progenitor cells:*** *Expression density (mean fluorescence intensity) of* ***Mucosialin*** *on ECs isolated from irradiated hearts and irradiated lungs (ROI2) as well as unirradiated lungs (ROI1) 20, 30, 40 and 50 weeks after irradiation with 8 and 16 Gy.* ***(C) Fat metabolism:*** *Expression density (mean fluorescence intensity) of* ***FAT*** *on ECs isolated from irradiated hearts and irradiated lungs (ROI2) as well as unirradiated lungs (ROI1) 20, 30, 40 and 50 weeks after irradiation with 8 and 16 Gy.*

Asterisk represents significantly different values ($p^* < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$).

3.2.3. Fat metabolism

Taking up fatty acids is one of the main tasks of *CD36*, also termed scavenger receptor (Pepino et al., 2014). As this process can be the beginning of atherosclerosis (Boord et al., 2002), it was very important to determine this endothelial surface marker.

After 20 weeks a significant up-regulation for 8 and 16 Gy took place (mfi: 326 and 385 vs. 201; $p = 0.007$ and $p = 0.0009$). Although we did not measure any alterations after 30 weeks for 8 Gy, we had an increase of the expression density after 30 weeks and 16 Gy (mfi: 354 vs. 216, $p = 0.036$). For 8 and 16 Gy the scavenger receptor was elevated after 40 weeks (mfi: 325 and 460 vs. 247; $p = 0.043$ and $p = 0.001$) and 50 weeks (mfi: 312 and 384 vs. 248; $p = 0.002$ and $p = 0.0001$).

Compared to the sham group, which did not get a radiotherapy session, for the whole period of the experiments (20, 30, 40 and 50 weeks) no significant differences were measured for *CD36* on irradiated and unirradiated lung ECs.

3.2.4. Inflammation markers

Acute local and systemic inflammation, as a side effect of irradiation, plays an important role in the acute post-radiotherapeutic treatment. To examine the long-term modifications of radiotherapy several inflammation markers had to be analyzed. Therefore, we searched for *PECAM-1 (CD31), HCAM (CD44), ICAM-1 (CD54), ICAM-2 (CD102)* and *VCAM-1 (CD106),* with the specific binding antibody. These markers get elevated during an inflammatory response.

ICAM-2 was the only inflammation marker, which neither increased nor decreased for an irradiation with 8 or 16 Gy on heart and lung ECs.

PECAM-1, which stayed at the same level as the control group for a radiotherapy with 8 Gy on heart ECs, showed a significant increase for 16 Gy on heart ECs. For 20 and 30 weeks we got an upregulation of this inflammation marker on heart ECs (mfi: 168 and 170 vs. 106; p = 0.002 and p = 0.003). For irradiated lung ECs the expression of *PECAM-1* did not show any significant changes after a radiation with 8 or 16 Gy over the observed period.

For an irradiation with 16 Gy *HCAM* as an inflammation marker increased on irradiated heart ECs over the whole observed time. The mfi for 20, 30, 40 and 50 weeks of the measured values (16 and 15 and 13 and 14 vs. 7; p = 0.01; p = 0.02; p = 0.02 and p = 0.001) showed a significant difference to the control group. For lower radiation doses and irradiated as well as unirradiated lung ECs no significant changes in the mfi could be measured.

The expression density for *ICAM-1* permanently increased for an irradiation with 16 Gy on heart ECs. (mfi 20 weeks: 184 vs. 109; p = 0.007; 30 weeks: 172 vs. 103; p = 0.008; 40 weeks: 177 vs. 113; p = 0.003 and 50 weeks: 160 vs. 111; p = 0.004). After 20 and 50 weeks we also had a significant difference for an irradiation dose of 8 Gy (mfi 20 weeks: 167 vs. 109; p = 0.023 and 50 weeks: 146 vs. 111; p = 0.01). Considerable differences could be measured for *ICAM-1* on irradiated lung tissue, too. For an irradiation intensity of 8 and 16 Gy a constant upregulation of the inflammation marker in irradiated lung tissue was detected for 20, 30 40 and 50 weeks (mfi 20 weeks; 315 and 358 vs. 238; p = 0.0011 and p = 0.00015; 30 weeks: 332 and 354 vs. 227, p = 0.015 and p = 0.004; 40 weeks; 314 and 328 vs. 236, p = 0.02 and p = 0.008 and 50 weeks: 367 and 334 vs. 235, p = 0.005 and p =0.02).

We also had an up-regulation for the inflammation marker *VCAM-1* for heart and irradiated lung ECs. After 20, 30 and 50 weeks *VCAM-1* increased for 8 Gy on heart ECs (mfi 45 and 38 and 37 vs. 29; p = 0.0009, p = 0.05 and p = 0.009) and for 16 Gy after 20 – 50 weeks (mfi: 46 and 50 and 51 and 49 vs. 29; p = 0.001, p=0.0009, p = 0.001 and p = 0.0006) after heart irradiation.

The expression density for the inflammation maker *VCAM-1* increased for 8 Gy after 20 and 50 weeks in irradiated lung ECs (mfi: 14 and 15 vs. 10, p = 0.018 and p = 0.025). For 20 - 50 weeks the mean fluorescence intensity of *VCAM-1* (13 and 14 and 14 and 14 vs. 10; p = 0.043, p = 0.025, p = 0.03 and p = 0.048) was enhanced on the surface of irradiated lung EC for 16 Gy.

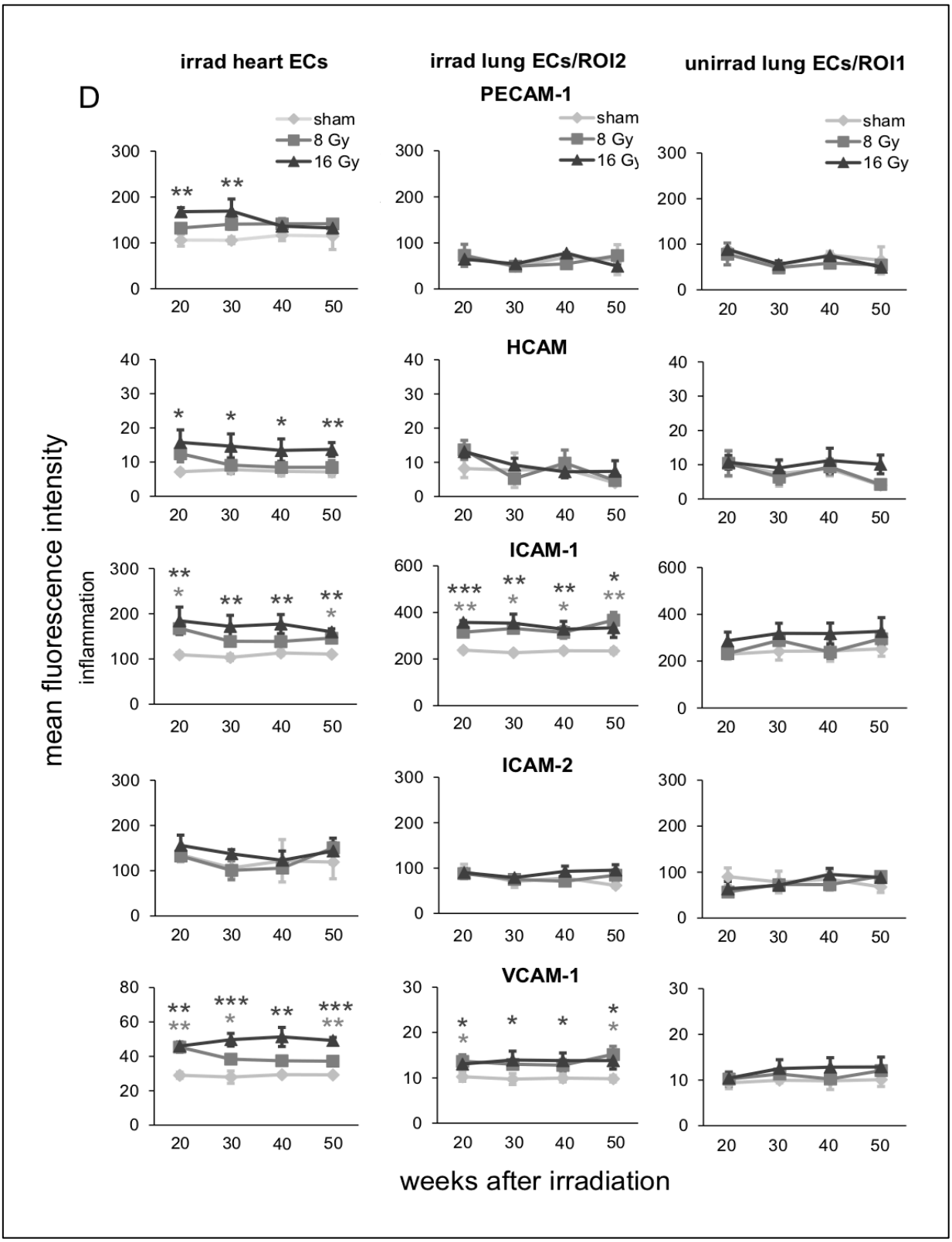

Figure 19: Endothelial cell (EC) expression density of antibodies of (D) inflammation markers at different points in time from irradiated hearts and irradiated lungs (ROI2) as well as unirradiated lungs (ROI1)

(D) Inflammation markers: Expression density (mean fluorescence intensity) of **PECAM-1, HCAM, ICAM-1, ICAM-2 and VCAM-1** on ECs isolated from irradiated hearts and irradiated lungs (ROI2) as well as unirradiated lungs (ROI1) 20, 30, 40 and 50 weeks after irradiation with 8 and 16 Gy.

Asterisk represents significantly different values (p* < 0.05; p** < 0.01; p*** < 0.001).

4. DISCUSSION

The aim of the thesis was the systematic analysis of very late irradiation effects of primary murine heart and lung ECs after *in vivo* heart and part lung irradiation. A CT image guided *in vivo* radiation of the heart and parts of the lung were performed with 8 and 16 Gy. After acute inflammatory effects have subsided, primary ECs were isolated 20, 30, 40 and 50 weeks after irradiation from the heart and the lung. As the lung tissue, which is near the heart gets also partially radiated, ECs from irradiated and non-irradiated lung tissues were also investigated with respect to potential abscopal or bystander effects. For the examination of late term effects, a new isolation procedure was used which enables the isolation of viable ECs from old mice at a high purity. The proportion and expression density of markers was analyzed which were involved in proliferation, inflammation, lipid metabolism and stemness of ECs by flow cytometry. The results were compared to a control group, which received a sham irradiation with 0 Gy. The investigation of time- and dose-dependent alterations in heart and lung ECs after a local radiation may provide evidence to better understand long-term effects of a radiation therapy on the blood vessels of breast cancer patients with an increased risk for myocardial diseases. Furthermore, the formation of atherosclerosis as well as the progression of radiotherapy-induced diseases on the microvasculature was analyzed. The evaluation of the kinetics of chronic inflammation, as a permanent alteration of the microvasculature of heart and lung vessels, was also a main goal of the study.
All examined parameters contribute to a better understanding of the mechanisms which are responsible for an increased risk to develop myocardial infarction, as a long-term damage caused by radiotherapy in patients (S. Schultz-Hector & K. R. Trott, 2007).

The interaction of the organs heart and lung influences the tolerated radiation dose: The radiation of one organ leads to a lower tolerated doses of the other organ (Ghobadi et al., 2012). This effect is responsible for tachypnoea and right ventricular hypertrophy for radiation doses above 20 Gy for the heart and 5 Gy for the lungs (Gabriels et al., 2012; van Luijk et al., 2007; van Luijk et al., 2005). As the highest radiation dose in the described experiments was 16 Gy for the heart and 3 Gy for the lung the reciprocal effect between heart and lung tissue after radiation appears to be less relevant. Furthermore, no abscopal effect was detected after irradiation with 16 Gy, as non-irradiated ECs remained unaffected up to 50 weeks after irradiation.

4.1. Identification of primary endothelial cells

The presence of all typical cell surface markers *Endoglin*, *VE-cadherin*, *Mucosialin, PECAM-1, ICAM-1, ICAM-2 and FAT* indicated that the isolated primary cells were indeed ECs (Griffioen et al., 1997; Klar et al., 2016; Konradt et al., 2016; Lertkiatmongkol et al., 2016; Y. Liu et al., 2019; N.-H. Son et al., 2018; Torres et al., 2020; J. Wang et al., 2016). As no other cell types shows the combined expression of all markers, it was proven that the EC isolation procedure was successful.

The expression of the markers mentioned above was nearly 100% on the analysed heart and lung ECs irrespective of the age of the mice (20 - 50 weeks after irradiation), the origin of the radiated tissue (heart or lung, irradiated or non-irradiated), or the dose (8 or 16 Gy).

4.2. Late radiation effects on heart and lung endothelial cells

4.2.1. Proliferation markers

The analysed proliferation markers for heart and lung irradiation with 8 and 16 Gy were **Integrin-ß3**, ***VE-cadherin*** and ***Endoglin***.

The markers *Integrin-ß3 and VE-cadherin* did not show any significant changes after heart irradiation with 8 and 16 Gy compared to the control group. Since the value for *VE-cadherin* in irradiated mice as well as in the control group is downregulated, this effect could be assigned to an aging process of the mice.

Endoglin, which is highly expressed on the surface of ECs (Dai et al., 2019), is involved in proliferation, migration as well as angiogenesis (Goumans et al., 2002). It is an accessory receptor for the transforming growth factor beta system (Cheifetz et al., 1992; Teama et al., 2016) using the TGF-ß/ALK-1 signal cascade in ECs (Oxmann, 2007). The expression of *Endoglin* on ECs is increased during wound healing, tumor vascularization and in inflammation (guide, 2018; Zhu et al., 2017). The results of the thesis showed a permanent up-regulation of the proliferation marker *Endoglin* in heart ECs 20, 30, 40 and 50 weeks after local heart irradiation with 16 Gy. Consequently, we conclude that a permanent damage of the heart tissue increases the wound healing capacity in terms of tissue regeneration even 50 weeks after irradiation. The structural and functional damage of the microvasculature has also been proven in other studies

(Seemann et al., 2012). It was shown, that the microvascular density in the left ventricle decreased 40 weeks after heart irradiation with 16 Gy (Seemann et al., 2012). The radiation led to a continuous damage of the ECs. Therefore, the body tries to replace the cells by increasing their proliferative capacity. Despite all efforts the organism doesn't manage to increase the number of vessels up to a normal level.

Furthermore, *Endoglin* plays an important role in myocardial fibrosis. The cardiac remodelling after acute myocardial infarction leads to a MiR208a expression which is induced by an increase of the *Endoglin* expression. The result of the elevated *Endoglin* levels induces myocardial fibrosis (Shyu et al., 2015). But also, endogenous molecules can lead to an increase of *Endoglin*: Angiotensin II activates TGF-ß1 which induces the production of *Endoglin* in cardiac fibroblasts. *Endoglin*, also strengthens the production of collagen I (Shyu, 2017). This increase in the deposition of collagen in activated fibroblast which is characteristic for cardiac fibrosis (Dai et al., 2019; Hinderer & Schenke-Layland, 2019). As the upregulation of *Endoglin* after acute myocardial infarction as well as after the stimulation with Angiotensin II leads to fibrotic changes in the tissue, it seems obvious that the radiation induced upregulation of *Endoglin* also causes fibrotic changes in heart ECs. Hence radiotherapy damages ECs and leads to a dysfunction of the microvascular resulting in fibrosis of the cardiac vessel (Taunk et al., 2015). This fact may lead to the assumption, that radiation strengthens the fibrotic remodelling changes of ECs.

On the other hand, it seems as if the proliferation is increased in ECs by a continuous inflammation after irradiation. *Endoglin* functions as an integrin based transmembrane glycoprotein in vascular EC interaction with its extracellular RGD (Arg-Gly-Asp) sequence. This tripeptide is recognized as a key structure for regulating adhesion molecules (Gallardo-Vara et al., 2018). Recent studies showed that *Endoglin* also induces leukocytes transendothelial migration (Rossi et al., 2013). Hence the infiltration of leukocytes is probably a consequence of an increased *Endoglin* expression. This assumption would mean that *Endoglin* is involved in late radiation-induced inflammation in the heart tissue. In addition the highest expression of *Endoglin* was detected in capillaries, where leukocyte transmigration occurs (Jonker & Arthur, 2002). This aspect matches to the data derived from immunohistochemical tissue analysis of the microvasculature.

Temporary proliferation after radiation therapy already occurs after a low radiation dose with 2 or 8 Gy after 10 or 15 weeks (Sievert et al., 2015). As this process is considered as an acute reaction, the proliferation stops finished after 20 weeks, and the proliferation markers are no

longer increased. The results of this study showed a transient (up to 20 weeks) elevation of one out of three proliferation markers.
The increase in *Endoglin* may be interpreted as a stimulator of inflammation, as *Endoglin* is also involved in the migration of leukocytes (Rossi et al., 2019). The implication is that radiation with 16 Gy activates an *Endoglin*-induced transmigration and thus leads to chronical inflammation in the microvessels. Hence, *Endoglin* can be considered as an inflammation marker for high radiation doses.

Although the proliferation markers *Integrin-ß3*, *VE-cadherin* and *Endoglin* were found to be elevated 5 – 15 weeks after lung irradiation with 8 Gy in earlier studies (Sievert, 2016; Sievert et al., 2015), the investigated markers in the irradiated parts of the lungs after 20 weeks and later did not show any differences. As the percentage (< 20%) and the dose of the irradiated lung tissue (1.3/2.7 Gy for heart irradiation with 8/16 Gy) was kept as low as possible, it seems that there were no measurable late time damages concerning the proliferation of lung ECs. This means that the unirradiated parts of the lungs compensated the accumulated damage of the irradiated lung tissue already 20 weeks after radiation.
Furthermore, no significant difference in the unirradiated lung tissue was measured. Not surprisingly it could be shown that there are no late time changes in the unirradiated parts of the lung.

4.2.2. Progenitor cells

CD34, also known as *Mucosialin*, is a marker for progenitor cells. Being able to differentiate several tissues, progenitor cells showed no significant differences, neither in irradiated hearts nor lung tissues nor in unirradiated lung tissues.
Thus, no changes of progenitor cells could be detected as a long-term consequence of irradiation. Therefore, the question about the radiation sensitivity of progenitor cells could not be answered in this study. At least our analysis gives a hint that there are no measurable changes concerning the marker CD34.

4.2.3. Evolution of atherosclerosis as a late time effect of radiation

Atherosclerosis, known as hardening of the arteries in the vernacular, develops into a chronical inflammatory disease which can lead to vascular cell death (Herrington et al., 2016).

The start of this disease is an endothelial dysfunction, leading to the uptake of monocytes which differentiate into tissue resident macrophages (Zimmer, 2013). This macrophages phagocytose accumulated lipoprotein particles like ox-LDL and migrate into the intima of arteries (Daiber et al., 2017). For this a specific transmembrane receptor is necessary: the surface marker *CD36*, which is expressed on ECs and monocytes/macrophages. In this study the membrane receptor *CD36* was found to be upregulated in heart ECs after *in vivo* heart irradiation with 8 and 16 Gy after 20, 30, 40 and 50 weeks. *CD36*, in its role as an integral membrane glycoprotein, has an extracellular part for binding of exogenous and endogenous ligands like long chain fatty acids (LCFA), pathogen-associated molecular patterns (PAMPS) or endogenous molecules like ox-LDL (Zhao et al., 2018). Studies have shown that the uptake of oxidized LDL via *CD36* leads to a foam cell formation (Roy L Silverstein, 2017; Staels, 2005). Therefore, monocytes activate the c-Jun N-terminal kinase, which in turn increases the expression density of *CD36* (Katayama et al., 2008). As *CD36* is mainly responsible for the uptake of cholesterol by macrophages, they are transformed into lipid loaded foam cells (Yu et al., 2013). Hence the expression of the scavenger receptor *CD36* and the production of foam cells implicate atherosclerosis-like lesions in the microvasculature (Lim et al., 2006; Rahaman et al., 2006).

Another important factor involved in the development of atherosclerosis are the peroxisome proliferator activated receptors PPARs (Li & Glass, 2004). These ligand dependent transcription factors regulate the lipid transport and metabolism in the blood flow (Kersten et al., 2000). Radiation therapy of the heart with 16 Gy leads to a decrease of PPAR-α: after 8 (Azimzadeh et al., 2013) and 40 weeks (Subramanian et al., 2016). This means that one of the main lipid metabolism regulators is working insufficiently and consequently makes it easier for fatty acids to create a metabolic chaos. PPAR- γ also regulates the expression of *CD36* (Sato et al., 2002) which is involved in the uptake of ox-LDL and foam cell formation (Lim et al., 2006). Furthermore, PPAR-α and PPAR- γ induce the removal of cholesterol in foam cells (Chinetti et al., 2001). As the activity of PPAR-α after radiotherapy is decreased, this process may be reduced or even stopped. In summary this means that low levels of PPAR-α support the uptake of fatty acids and prevent the reverse cholesterol removal. Hence, this may increase the formation of foam cells (Azimzadeh et al., 2021).

Apart from that, PPAR-α is also involved in inflammatory processes (Pontis et al., 2016). High levels of fibrinogen, a key regulator of inflammation (Davalos & Akassoglou, 2012), result in a downregulation of PPAR-α and the related protein expression (S. Wang et al., 2015). Thus, PPARs and fibrinogen reinforce each other. As PPAR-α is decreased after radiotherapy (Azimzadeh et al., 2013; Subramanian et al., 2016), high levels of the pro-atherosclerotic proteins fibrinogen or C-reactive cause or strengthen an inflammatory response (Bouhlel et al., 2008). The arisen inflammation in combination with the formation of foam cells could consequently be the onset of atherosclerosis (Katayama et al., 2008; Yazgan et al., 2018).

As atherosclerosis is a chronical disease, this aspect is also reflected in our analyzed data. The expression density of the scavenger receptor *CD36* is upregulated from the first point of measurement after 20 weeks as well as after the last point in time after 50 weeks. That is another reason why we should presume that atherosclerosis is a long-term consequence of radiation therapy.

This consideration was elaborated in several studies. For example, it has been shown that patients after radiotherapy of the neck have a higher risk of developing stroke. The basis for this is a radiation-induced atherosclerosis of the carotid arteries (Gujral et al., 2014; Kim et al., 2018). Hence, it may seem obvious that radiation of the heart leads to an increased risk for cardiovascular diseases such as myocardial infarction because of radiation-induced atherosclerotic lesions in the coronaries. As atherosclerosis is a chronical disease it may take decades for cardiovascular diseases to become symptomatic (S. C. ; Darby et al., 2013; F. Stewart et al., 2013). For example, for breast cancer patients treated with radiotherapy, there is the risk of developing cardiovascular diseases (CVD) and thus a higher probability of CVD-mortality approximately 7 years after therapy (Bradshaw et al., 2016).

Furthermore, it was noticed that the internal thoracic artery, which is used as a recipient for the deep inferior epigastric perforator flap (DIEP) in autologous breast reconstruction, was not suitable in late reconstructive surgery. There are two different reasons for this: either the arteria was destroyed in previous surgery, or it has been damaged by postoperative radiotherapy. Caliber differences and insufficient vascular quality caused by atherosclerosis were mentioned by surgeons as a reason for the failure of the arteria (Munhoz, 2008). A more satisfying and better aesthetic outcome was detected by patients with an immediate DIEP, avoiding irradiation (O'Connell et al., 2018).This can be considered as another clinical indication for the late time damage of vessels of the chest after radiotherapy in humans.

4.2.4. Inflammation markers

Significant differences after radiation of the heart could be shown for the inflammation markers ***PECAM-1*, *HCAM, ICAM-1*** and ***VCAM-1*** on **irradiated heart ECs.**

Whereas the expressed inflammatory surface markers *PECAM-1* and *HCAM* only reacted after high doses of radiation to the heart (= 16 Gy) and a certain time after the treatment *(PECAM-1:* 20 & 30 weeks; *HCAM:* 20 -5 0 weeks), *ICAM-1* and *VCAM-1* reacted more rapid and sensitive to radiation. Those two markers were already upregulated after a heart irradiation with 8 Gy for 20 and 50 weeks *(ICAM-1)* and 20, 30 and 50 weeks (*VCAM-1)*. For higher radiation doses (= 16 Gy) *ICAM-1 and VCAM-1* showed an upregulation at all measured time points (20 - 50 weeks).

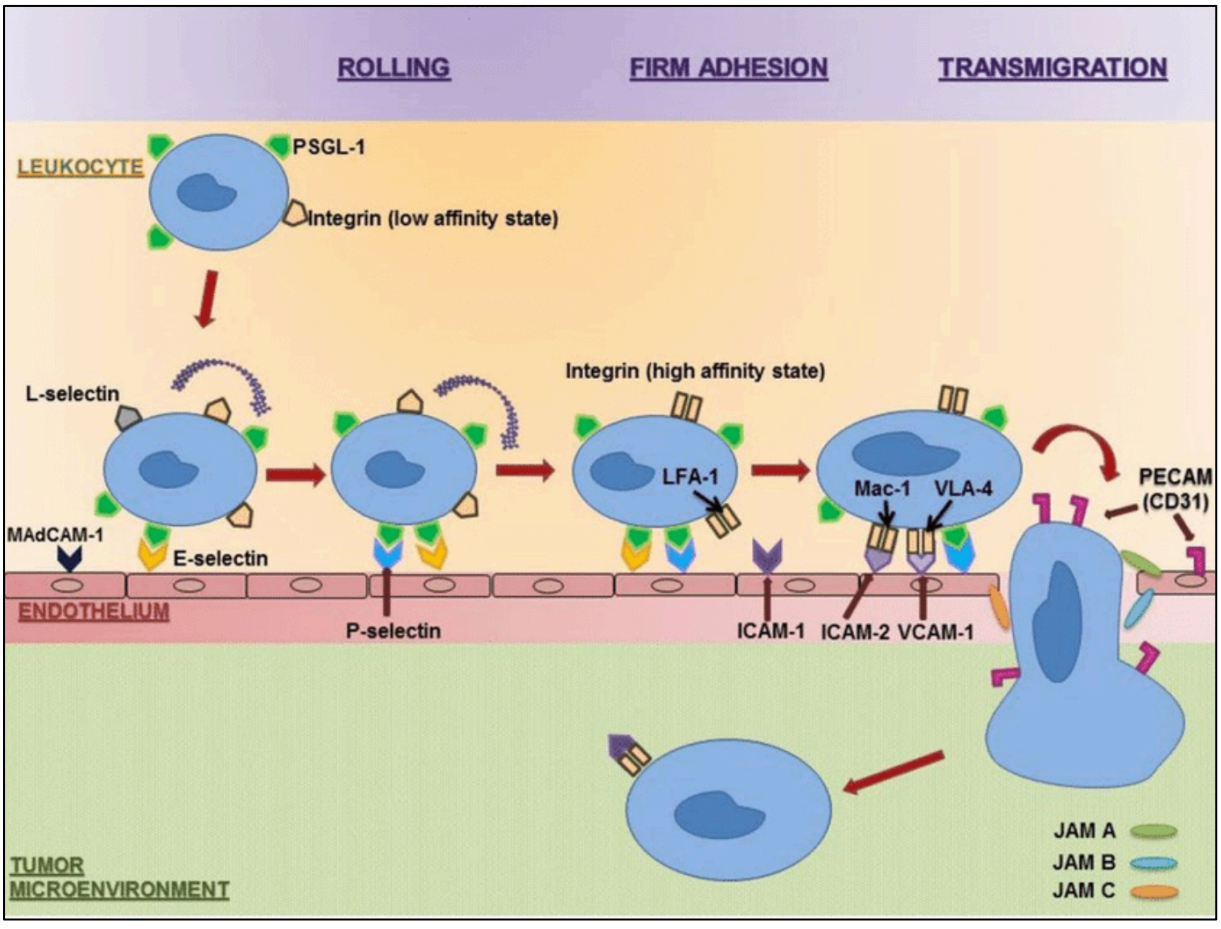

***Figure 20**: Leukodiapedesis*

The emigration of leukocytes is shown in this figure, divided into rolling, firm-adhesion, and transmigration. Selectins and integrins as well as *ICAM-1/2, VCAM-1 and PECAM* are involved. The figure was published in "Scientific reports" by Chae, Y.K., Choi, W.M., Bae, W.H., Anker, J., Davis, A.A., Agte, S., Iams, W.T., Cruz, M., Matsangou, M., and Giles,in the year 2018 *(Chae et al., 2018)*.

The only significant upregulation for the analyzed lung ECs was measured for the inflammation markers *ICAM-1* and *VCAM-1*. For 8 and 16 Gy a significant difference was measured for *ICAM-1* at all tested time points, starting at 20 weeks and ending at 50 weeks. *VCAM-1,* however, was not that sensitive to radiation. Hence the increased values for 8 Gy were detectable after 20 and 50 weeks whereas, for 16 Gy an upregulation of the marker were detected 20, 30, 40 and 50 weeks after irradiation.

The emigration of leukocytes through ECs into the adherent tissue depend on different adhesion molecules. The process of leukocyte extravasation is divided in different parts: First of all, leukocytes get captured and start rolling on the wall of the vessel, slowing down through the interaction of P- and E-selectin-receptor expressed on ECs and the ligand for this receptor, a oligosaccharide of the glycoproteins of the leukocytes (Velázquez et al., 2016). CD44, also called home-cell-adhesion-molecule *HCAM*, and the ligand hyaluronan cause a rolling process between leukocytes and ECs (Misra et al., 2015). Once the leukocytes are activated via chemokine dependent or independent mechanism they start expressing integrins and connect with key adhesion molecules of the ECs like *ICAM-1* and *VCAM-1* (Golias et al., 2007; Johnson et al., 2006). Furthermore *PECAM-1* has several extracellular domains for the attraction and adhesion of leukocytes to ECs (Chistiakov et al., 2016). The process of adhesion leads to a strong and firm bond between ECs and the activated leukocytes. Hence, the leukocytes are stuck on the wall of the vessel. As *HCAM, ICAM-1, VCAM-1* and *PECAM-1* were upregulated in the experiments this results in the attachment of leukocytes to the blood vessels. The last part of transmigration is mediated via *PECAM-1* which is also involved as a regulator of EC junctional integrity (Privratsky & Newman, 2014).

Inflammatory processes in radiated tissue, like the heart ECs in the described experiments have been described several times as an acute reaction of normal tissues (Najafi et al., 2018). The fact that the inflammation markers are upregulated for at least up to 50 weeks is a hint that this process is continuing. Hence, the assertion of a chronic inflammation as a late side effect of radiation can be supported with the data presented.

The inflammation process is an indication that the heart ECs are damaged. Even after a certain period they have not recovered from the radiation damage. The reason for that is the continuous dysfunction of the vascular endothelium (Raghunathan et al., 2017). The microvascular damage causes problems with the vascular tone, the blood hemostasis or can lead to an inflammatory process (Bonetti et al., 2003). The dysfunction of the endothelium after radiation has already been shown in several studies. For example, patients with a free flap reconstruction in head and

neck reconstructive surgery more often have complications with their flaps if the skin was exposed to radiation before. The reason for severe complications with the flaps is the radiation-induced endothelial dysfunction caused by the increase of *ICAM-1* and *VCAM-1* even after years (Preidl et al., 2019).

Microvascular damages caused by radiation lead to a chronic permanent inflammatory response of the endothelium. The combination of these aspects are the basis for the development of radiation-induced cardiovascular diseases (Taunk et al., 2015). Ischemic heart disease is the most common cause of cardiac death after radiotherapy (Donnellan et al., 2016).

The permanent upregulation of the inflammation markers *PECAM-1*, *HCAM*, *ICAM-1* and *VCAM-1* lead to the development of a chronic inflammatory milieu. This long-term change makes it easier for other diseases like atherosclerotic lesions in coronary vessels to spread. *ICAM-1* (Plotkin et al., 2017; Yao et al., 2016) as well as *VCAM-1*(Blankenberg et al., 2003; Cybulsky et al., 2001; Peng et al., 2017) also support the development of these chronic inflammatory diseases.

The inflammation markers *ICAM-1* and *VCAM-1* were also upregulated on irradiated lung ECs. This shows that the irradiation of the heart also damages the lung tissue in the field of irradiation. As the markers were upregulated at all observed points in time a chronic inflammatory milieu was created in this specific part of the lungs. As far as chronic inflammation and injured ECs are concerned a severe side effect in lung cancer patients after radiotherapy is the radiation-induced lung fibrosis (Ding et al., 2013). Fibrosis of the lungs results in loss of life quality, because of a respiratory insufficiency and is a major complication after radiation (Oh et al., 2012). As the added damage to the lungs is severe, the treatment of the thorax with radiation was limited by fibrosis (Giridhar et al., 2015). The radiation of the heart and the lungs together with the natural aging process increases the risk of developing radiation-induced lung fibrosis (Cella et al., 2015). The clinical manifestations of loss of weight, mobility and breathing as well as an increased mortality (Plathow et al., 2004) has not been observed in the control or the radiation group. As the radiation dose, harming the lungs, is a bit smaller in the described experiments than the dose used for lung irradiation and the parts of the lungs in the field of irradiation are always under 20 percent, lung fibrosis has not been detected in the analysis.

4.3. Radiation-induced cardiovascular diseases (RICVD)

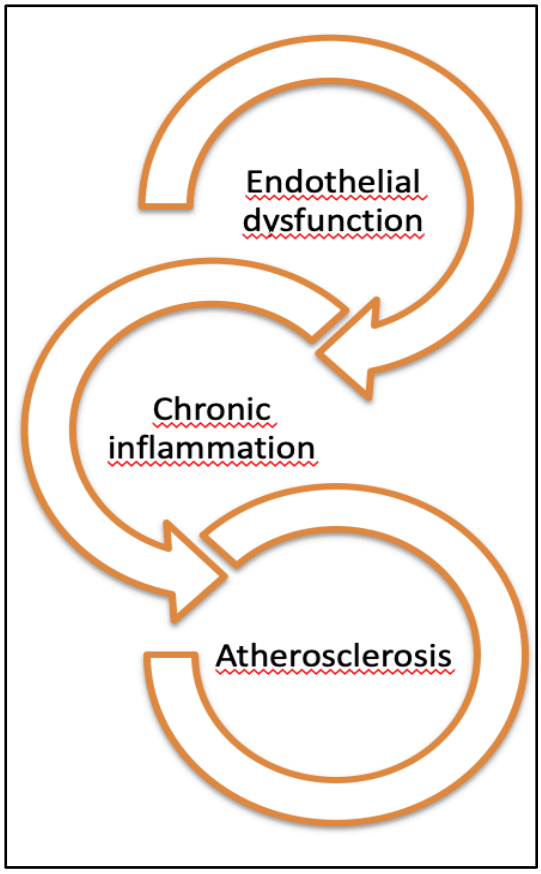

Figure 21: Development of RICVD

Summarizing the discussed results, the conclusion can be drawn that radiotherapy causes a permanent upregulation of inflammation markers, even after lower doses (8 Gy) of irradiation. This means that after 50 weeks there is still an inflammatory process in the damaged microvasculature. Additionally, *CD36*, a marker for lipid metabolism involved in the development of atherosclerosis, is elevated for low (8 Gy) and high radiation doses (16 Gy) and nearly at all measured points in time (20 - 50 weeks). Both processes potentiate by each other: on the one hand, chronic inflammation supports the development of atherosclerosis, on the other hand, atherosclerosis is a chronic inflammatory disease, which increases the inflammation. Fundamental is the radiation-induced damage of the endothelium which ends in endothelial dysfunction.

With the analyzed data the late side effects of radiotherapy could be shown in a mouse model: endothelial dysfunction leading to chronic inflammation and the development of atherosclerosis ending in radiation-induced heart diseases. This implies several diseases like obstructive coronary artery disease, myocardial fibrosis, pericardial disease, arrhythmias and valvular abnormalities, related to radiotherapy (Podlesnikar et al., 2022; Taunk et al., 2015).

The radiation-induced damages weaken the heart's own supply system, the coronary vessels, the essential working muscle of our blood systems. Without a continuous oxygen supply the heart itself cannot pump properly and thus cannot maintain the oxygenation of the body (H. Wang et al., 2019). Radiation-induced coronary artery diseases appear frequently in the clinic. One third of the screened patients even have stenosis of 70% or more in two or three vessels (Anderson et al., 2007; Kupeli et al., 2010). This obstructive disease of the coronary vessels reduces the blood flow to

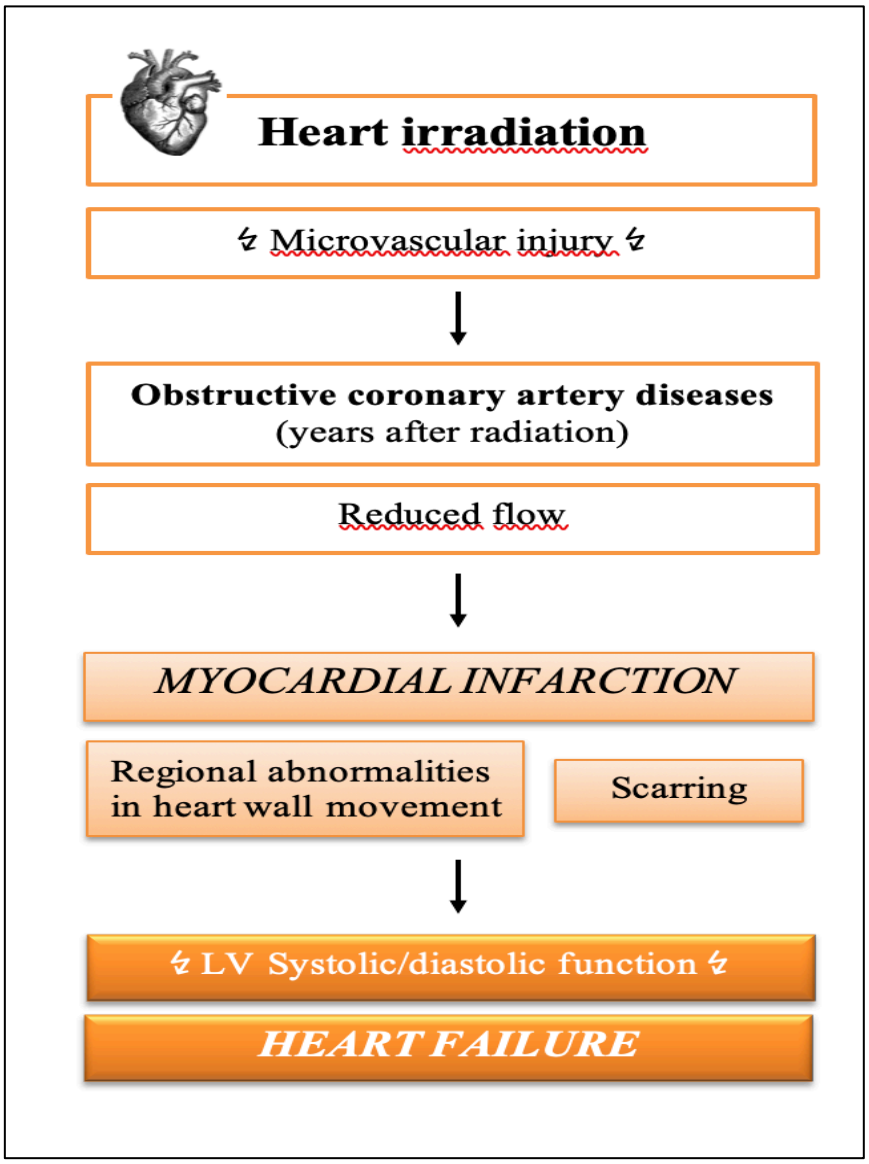

***Figure 22**: Pathogenesis of heart failure after heart irradiation*

a specific territory of the heart years after the radiotherapy. Finally, the risk of developing myocardial infarction, even in younger patients without typical cardiac risk factors, is increasing (Letsas et al., 2006). The anterior wall as well as the apex of the heart are the areas where myocardial infarction is most likely to happen years after irradiation. The supplying blood vessel for this area is the left anterior descending artery LAD (Lind et al., 2003). Subsequently the consequence after myocardial infarction can be regional abnormalities in the movements of the heart wall as well as a scarring process in the affected part of the myocardium (Di Bella et al., 2013).

Furthermore, nonischemic myocardial fibrosis caused by radiation hinders the heart in its contractility. Thus, the loss of left ventricular systolic and diastolic function is the result of the added damage to the heart leading to arrhythmias and disorders in the impulse formation or conduction system (L. K. Liu et al., 2017).

In summary, radiation-induced heart diseases like obstructive coronary artery diseases increase the risk of developing a cardiovascular event such as myocardial infarction. The wall movement abnormalities, the scarring process and the radiation-induced nonischemic myocardial fibrosis are leading to a left ventricular dysfunction, whose finale stage is the destruction of the heart tissue ending in heart failure.

The time between the exposition of radiation and the following radiation-induced heart diseases is about 10 to 20 years (Gujral et al., 2016). Retrospective studies show that approximately one out of ten Hodgkin lymphoma patients has developed a coronary artery disease at a median of nine years after radiotherapy (Hull et al., 2003).
With the expansion of cardiovascular diseases, the risk of developing ischemic myocardial infarction is increasing as well. For breast cancer patients with radiotherapy meta-analyses showed that the number of deaths not related to the breast cancer but due to vascular causes was going up (Abe et al., 2005). Furthermore the patients undergoing a radiotherapy before or after surgery have a higher death rate due to coronary artery diseases compared to patients without it (Rutqvist et al., 1992).
Depending on the dose and the duration, the risk of developing myocardial infarction and thus the death rates are increasing proportionally. With doses of values above 7.4% per Gy the risk of coronary artery diseases increases extremely (S. C. ; Darby et al., 2013).
In the radiotherapy of left-sided breast cancer patients the apex and the anterior wall of the heart, are the anatomic areas with the highest radiation doses and hence the most endothelial damage. Therefore, the risk of developing radiation-induced diseases such as atherosclerosis leading to myocardial infarction years later is very high in these parts of the heart (Lind et al., 2003; Paszat et al., 2007). Decreasing the mean whole heart dose also reduces the radiation related damage and thus improves the cardiovascular situation for patients after radiation (Jacobse et al., 2019).
Comparing the radiation of breast cancer patients in the clinic with the radiation in the mouse model a few differences could be noticed: Whereas the patients in the clinic received radiotherapy five days a week for about six weeks, the mice just got one radiation. Unfortunately, it was not possible to fractionate the dose for the mice in contrast to the procedure in the clinic. Normally, the irradiation dose of 60 Gy is the sum of the fractionated doses of 1 - 2 Gy in 30 sessions. The mice in the experiments got 8 or 16 Gy in one radiotherapeutic session. Despite these fundamental differences in the realization of the radiation treatments to save money and resources, the analyzed results are comparable to the therapies carried out in the clinic.

4.4. Prevention strategies

Many patients nowadays are concerned about possible late damaging effects of radiotherapy. Although radiotherapy is necessary to reduce and destroy tumor cells, the late side effects of this treatment are in the focus of ongoing research. On the one hand ionizing radiation, is definitive necessary to prolong the life of tumor patients or even cure them, on the other hand we know now that it also causing harm to normal tissues such as heart and lung many years after the treatment.

In the future the development and dissemination of radiation-induced cardiovascular diseases such as chronic inflammation leading to atherosclerotic lesions and cardiovascular events should be stopped. The easiest way would be to treat patients without using radiation. As this is not possible, especially with cancer patients, modern scientific research combined with the clinical expertise must find ways to minimize the side effects.

Patients suffering from breast cancer or peptic ulcer are patients who run a risk of developing radiation-induced cardiovascular diseases, as the radiation of the chest damages especially the heart (S. Schultz-Hector & K.-R. Trott, 2007). Therefore their risk of cardiovascular diseases increases within the first ten years after radiation (Chandra et al., 2021) and the risk of cardiac mortality from the second decade on (Cheng et al., 2017). The median time between the radiation and the induced myocardial infarction is 13.6 years (Jacobse et al., 2019). To prevent and contain the radiation- induced damages and diseases physicians in clinic are trying to implement prevention programs for the patients at risk. The first ten years after irradiation are very important to examine the progression of the radiation-related damages to the heart (Andratschke et al., 2011).

It is advisable for patients to have clinical examination focused on the heart in regular intervals. As breast cancer patients, for example, have periodic aftercare check-ups, the easiest way would be to expand these. A simple non-invasive way to evaluate the cardiovascular state besides the clinical examination is to write a 12-channel-electrocardiogramm (ECG). Comparing the ECG with previous ones, it is possible to recognize changes in the heart rhythm, frequencies, position of the cardiac vector as well as the impulse formation and regression of the heart. Since ECG are available in all clinics and most medical practices this is an easily available and performable option of primary prevention.

In addition, sonography of the heart also supplements the primary and secondary prevention of radiation-induced cardiovascular diseases. This imaging presentation of the heart and its four

chambers enables the physicians to assess the heart action, the size of the atriums and the chambers, the blood flow between the right and the left side, the pressure gradients over the heart valves and so on. Used as a diagnostic tool in cardiology, it monitors patients with cardiovascular diseases like hypertension, auricular fibrillation or after myocardial infarction. This heart-imaging examination method is the best and cheapest way to show alterations of the heart, without using ionizing radiation. Besides the ultrasound device, it requires an experienced doctor to perform the investigation properly.

Taking blood of patients with a risk of developing radiation-induced cardiovascular diseases is another important part of secondary prevention. The blood samples can be used to control the status of cardiac cells in a patient. Values of interest here are biomarkers like NT-proBNP or cardiac troponin. The first protein is a progression parameter for acute or chronic heart insufficiency showing the dilatation of the left atrium. This biomarker is also increasing if the impulse formation is not working properly, for instance for patients with auricular fibrillation. Cardiac-derived troponin instead shows elevated values when the heart muscle is damaged. It is the first elevated marker in the blood showing a myocardial infarction about three hours after ischemia (Bang et al., 2019). Furthermore, it can be increased in patients with heart valve diseases or heart failure. So, both markers can be used for tertiary prevention.

4.4.1. Control of blood lipids and medication

Another aspect of interest are the blood lipids. As *CD36*, a marker of atherosclerosis, is upregulated after heart irradiation, obstructive coronary heart disease is one of the late time damages caused by radiation. The increase of blood lipids like LDL-cholesterol as well as the decrease of HDL play an important role in the development of atherosclerosis. A strict control of these measurable blood values is absolute necessary if the start and progression of this chronic disease should be prevented. An important aspect in discussion presently is the range of values appropriate of the mentioned blood lipids. In clinical practice healthy patients are differentiated from patients with cardiovascular risk factors or events. For the secondary prevention, the normal range of values is lower than for patients without cardiovascular history. Whereas LDL-cholesterol values up to 115 are normal for patients with a low cardiovascular risk, 55mg/dl is the upper limit for patients with a very high risk (Mach et al., 2020). If radiation is a risk factor and which section it may be associated to, is a topic of discussion in the European society of cardiology, presently. So far, the experts have not reached a common conclusion.

Patients suffering from high cholesterol values should change their lifestyle (Kopin & Lowenstein, 2017). Conducive modification would be to stop smoking, to reduce the alcohol consumption to a minimum, to lose weight or to start doing sports (Srikanth & Deedwania, 2016). If these alterations do not lead to the desired result of lowering the blood lipids, especially the LDL-cholesterol, taking medication against the dyslipidemia will be the next step. As atherosclerosis is a widespread disease, different opportunities are possible:

First line therapy will involve *Statins*. This medicament reduces LDL by inhibiting HMG-CoA-Reductase, the key enzyme of the cholesterol biosynthesis (Jiang et al., 2018). Furthermore, it has a second effect mechanism: The reduction of cholesterol leads to an increase of cytoplasmatic LDL-receptors via the sterol response element-binding protein 2 (SREBP2). If the cholesterol level is lowered in the endoplasmic reticulum (ER), this regulator protein induces an upregulation of the receptor for the uptake of LDL-cholesterol (Yang et al., 2020). The first inhibition process initiates and intensifies the reduction of cholesterol via the second mechanism. The most potent drug is *Atorvastatin*, a pill which can be taken at any time of the day (Laufs et al., 2016). As this medicament group has already been used for many years for patients with dyslipidemia, the side effects are well-known and the application is common in clinic and medical practices, moreover the payment by health insurance is confirmed.

If the highest tolerated dose of *Statins* is not able to decrease the LDL-cholesterol values to the desired level, *Ezetimib*, as a medicament of second line, can be added to the oral therapy regime (Bajaj et al., 2020). Additionally, it is the medicament in line if there is an intolerance to *Statins*. The mode of action of this specific antibody is the inhibition of Niemann-Pick C1-Like 1 (NPC1L1), a transport protein on the apical membrane of enterocytes (Huang et al., 2020). Thus, the absorption of endogenous and exogenous cholesterol in the intestines is inhibited.

It is only recently that *PCSK-9-inhibitors* have been used for the reduction of LDL cholesterol as well. This monoclonal antibody inhibits the protein *PCSK-9,* which is involved in the depletion of LDL-receptors (Sabatine, 2019). Consequently, the uptake of LDL into the cells is increasing and the LDL levels in the blood are decreasing. The medicament is given as a subcutaneous injection every two or four weeks. Although the intensity of the lipid downregulation of *PCSK-9-inhibitors* is far superior to the one of *Statins* (60% vs. 30%), it has been used reservedly in clinic (Mach et al., 2020). The reason for that is, that the costs for the injection for example in the U.S. (>$ 14.500/year) are a hundred times higher compared to the generic *Statins* (Hlatky & Kazi, 2017). Consequently, the therapy is only cost-effective, if the risk for developing an atherosclerotic cardiovascular disease is very high (Hlatky & Kazi, 2017). Worth mentioning is the fact that the number of prescriptions of this medicament is increasing for patients

with coronary artery or heart diseases as a medicament of tertiary prevention. The use for patients with dyslipidemia as a medicament of secondary prevention is quite rare (Chamberlain et al., 2019).

Another very interesting medicament for the prevention of radiation-induced cardiovascular diseases is *Fenofibrates*. Although this drug is not mentioned in the first- or second-line therapy in the dyslipidemia guideline of the European Society of Cardiology (Mach et al., 2020), the target of this medicament fits perfectly to the radiation-induced alterations. *Fenofibrate* activates the nuclear receptors of PPAR-α (Keating & Croom, 2007) , which is downregulated after radiation. Hence, the radiation-induced alteration of PPAR-α is compensated with this drug. The reduction of LDL-cholesterol values as well as the uptake of fatty acids are not reduced and continue normally. But *Fenofibrates* have targets besides the lipid metabolism as well. This pill reduces fibrinogen (Koh et al., 2004). In inflammatory tissue fibrinogen leads to a downregulation of PPAR-α (S. Wang et al., 2015). Thus, this second working mechanism strengthens the first one: the activation of PPAR-α Already used for the prevention of cognitive impairment (Greene-Schloesser et al., 2014) or the decrease of hippocampal neurons (Ramanan et al., 2009) after fractionated whole brain irradiation, *Fenofibrates* have shown its protective influence on radiation-induced alterations. Furthermore this drug also sensitizes cancer cells for radiation in head and neck squamous cells (J. Liu et al., 2014).

In conclusion, a strict control of cholesterol levels for patients after radiotherapy is advisable. If the values of the blood concentration are above the range, a lifestyle intervention in combination with drug treatment should be initiated. Appropriate available medicaments are *Statins, Ezetimib* and *PCSK-9 inhibitors* for high-risk patients. The role of *Fenofibrate*, protecting the heart from radiation-induced alterations, must be analyzed next. With these preventive measures the beginning and progress of atherosclerosis and its lesions in the coronaries should be stopped.

4.4.2. Anti-inflammatory drug treatment

Many treatments and medications have already been found for inflammatory processes, appearing as acute side effects after radiotherapy. Now the late side effects of chronic inflammation after radiation should be the focus of new treatment approaches. One imaginable way is the use of anti-inflammatory medicaments.

Glucocorticoids, used for the treatment of many chronic diseases such as inflammatory bowel diseases, bronchial asthma or rheumatic diseases, are effective in an anti-inflammatory and even immunosuppressive way (Vandewalle et al., 2018). A serious problem of steroidal medicaments are the numerous side effects, appearing in musculoskeletal, gastrointestinal, cardiovascular, endocrine, neuropsychiatric, dermatologic, ocular, and immunologic organ systems (Oray et al., 2016) The cause of glucocorticoid resistance is another severe side effect (Rodriguez et al., 2016). High doses over a long period of time increase the risk of developing side effects (Alan & Alan, 2018). As the inflammation after radiotherapy is an acute as well as a chronic side effect, this causes many difficulties.

NSAIDs, also known as non-steroidal anti-inflammatory drugs, are another medicament group, that fight inflammation. This medicament group is divided into selective and non-selective inhibitors of the enzyme Cyclooxygenase, which produces prostaglandins as a representative marker of inflammation (Grosser et al., 2017). The problem here are the side effects as well. The medicament damages the stomach or the kidneys if it is taken permanently (X. Zhang et al., 2017). *Diclofenac* even increases the risk of developing a cardiovascular event and is consequently contraindicated in cases of cardiovascular history (Al-Lawati et al., 2020). Consequently, the medication with *Diclofenac* would even increase the risk of developing myocardial infarction instead of reducing the late time damages of radiation like chronic inflammation.

The best solution would be a medicament, which specifically inhibits the inflammation process in the ECs of the heart. Like most of the antibody therapies this is an expensive option, which is often not paid by health insurance companies. It would be easier to use specific inhibitors of inflammatory processes already used in daily clinic routine. The off-label use of *Pioglitaz*one would be imaginable. This second-line antidiabetic drug is an agonist of PPAR-γ (Legchenko et al., 2018). Already investigated in several studies, the anti-inflammatory and endothelium protective effects have been shown for patients with coronary heart diseases (Byelan et al., 2017). Furthermore, anti-arteriosclerotic effects have been detected. This medicament prevents coronary arteriosclerosis (Ishibashi et al., 2002), fights against early vascular microcalcification

(J. Xu et al., 2018), and stabilizes the atherosclerotic lesions, when the disease has already broken out (Tian et al., 2017). Hence, this drug perfectly fits to fight against chronic inflammation and the onset of atherosclerosis caused by radiation. Through the combination of different target mechanism one pill can prevent and inhibit the progress of two diseases.

Since the new law "Cannabis als Medizin" has come into force in March 2017 in Germany, medicaments containing *Cannabis* can be prescribed for patients with matching indications (Cremer-Schaeffer & Knöss, 2019). Unlike the active substance *Tetrahydrocannabinol (THC), Cannabidiol (CBD)* has no psychoactive or euphoretic effects (Abu-Sawwa & Stehling, 2020). The considerable anti-inflammatory mechanism of this drug is in the focus of interest, concerning radiation-induced chronic inflammation (Atalay et al., 2020). In studies for oral mucositis after radiation (Cuba et al., 2017) the medicament showed positive effects on oxidative stress and pain, too. This multi-target drug works by using several systems of the body, like the activation of PPAR-γ an agonistic effect at 5HT1A, a Serotonin receptor (Linge et al., 2016) or an increase of activated TRPV1, the transient receptor potential vanilloid 1 (Nichols & Kaplan, 2020). Furthermore, *Cannabidiol* plays an important role in protective autophagy in ECs (Böckmann & Hinz, 2020). This recycling program of our body uses misfolded proteins or defect organelles to reprocess the used materials and win energy by decomposing the substances (Rockenfeller et al., 2015). The ROS-mediated HO-1 expression induces autophagy, which leads to apoptosis by using higher *Cannabidiol* concentration (Böckmann & Hinz, 2020). This aspect of the drug could be used to treat tumour-resistances against chemotherapeutics or even radiation. This mechanism has been shown for an oxaliplatin resistance of human colorectal cancer cells. Adding *Cannabidiol* to the treatment with oxaliplatin leads to autophagic cell death (Jeong et al., 2019). Furthermore, positive effects for breast cancer growth and metastasis could be shown for *Cannabidiol* inhibiting EGF/EGFR (Elbaz et al., 2015).

Summarizing the facts above, it can be speculated that *Cannabidiol* not only is effective in fighting against irradiation-induced inflammation but also can support anti-tumor effects induced by radiation or chemotherapies. Therefore, it is assumed that it might be beneficial to give *Cannabidiol* during or even before start of a standard therapy.

4.5. Perspective

There are some issues which could be improved in the used radiation method. On the one hand the therapy sessions could be more like the ones performed in daily clinical routine. As shown in the example with the breast cancer patients mentioned above, the radiation should not be performed just at a single time point as in the established mouse model. Fractioning the dose of the radiation can be levelled up to reach radiation doses of 1 - 2 Gy used in the clinical treatment. However, to irradiate mice with fractionated doses requires more anaesthesia which can negatively affect the life expectancy of the mice.
The question remains if changing this method into a sophisticated and more expensive method will change the results: is there a different outcome if the experimental method is modified? I do not believe so.
On the other hand, the radiation doses could be increased. As we know now mice survive radiation with 16 Gy for over 50 weeks, the radiation could be increased according to the fractionated radiation dose of 40 - 60 Gy used in the treatment of breast cancer patients (Wöckel & Stüber, 2019). A new radiation model for mice could be applied with a single radiation dose of 20 Gy. Furthermore, it would be possible to try to fractionate the dose in two radiation sessions to see if fractionated doses lead to different effects. Compared to the clinical treatment the radiotherapy would be extremely similar to the one performed in human breast cancer patients.
According to the results of Quirk (Quirk et al., 2020) appropriate respiratory management could be used to decrease the radiation dose of the heart in radiotherapy of left-sided breast cancers. The retrospective study showed that the deep-inspiration breath-hold (DIBH) lowers the radiation dose of the left anterior descending artery LAD from 3.8 Gy in patients with free-breathing to 3.2 Gy in patients with DIBH (Quirk et al., 2020). Therefore, also the respiration should be used to decrease the radiation dose to normal tissues such as the heart and thus decrease the risk of developing radiation-induced cardiovascular diseases.

More important is to prevent radiation-induced cardiovascular diseases or to stop their progression if they have already started. Prevention programmes and clinical guidelines for radiation-induced diseases should be implemented by cardiac specialists. Using, examination and diagnostic tools like ECG, sonography, and blood samples for determining inflammatory markers is a first and feasible step to monitor the health, especially the cardiovascular conditions of a patient after radiotherapy.

When cholesterol levels of LDL and HDL, which are representatives of the lipid metabolism, are not in the normal range, further treatment options should be considered. Lifestyle interventions and well-known medicaments like *Statins and/or Exetimib* should be applied to prevent atherosclerosis. The advantage of using medicaments which are already in daily clinical practice is that, on the one hand, side effects are known and on the other hand, doctors are familiar with these and know how to avoid them. Furthermore, some generic drugs are already available, which are paid by health insurances. It is obvious that the use of medicaments lowering the cholesterol levels are indispensable. Which range of values should be applied to patients after radiation needs to be assessed in further preclinical and clinical trials. Do patients after radiotherapy belong to the group of high-risk patients and hence, have different normal values than patients at the same age, without cardiovascular risk factors or existing conditions? These questions need to be addressed in future clinical trials.

For chronic inflammation, induced by radiotherapy, drugs like *Glucocorticoids* or *NSAIDs* are not recommendable. A permanent use of these medicaments is associated with severe negative side effects. *Pioglitazone,* a PPAR-γ agonist, given to patients suffering from diabetes mellitus II, should be tested. The off-label use of this medicament promises anti-inflammatory effects as well as anti-arteriosclerotic effects. *Pioglitazone* thus prevents chronic inflammation and the onset of atherosclerosis, both late effects of radiation. Furthermore, fenofibrates, which have a similar effector mechanism, should be tested. This agonist of PPAR-α can reduce the cholesterol and triglyceride levels in the blood of patients. Moreover, pro-inflammatory molecules like fibrinogen are reduced by these drugs. In a next step both medicaments should be tested in mice after inflammation-inducing radiotherapy, and inflammatory markers should be tested.
Cannabidiol is another natural drug candidate with a lot of potential. This multi-target drug is very promising, because of its diverse effects. Therefore, new experiments using the anti-inflammatory and anti-oxidative effect of *Cannabidiol* against acute and chronic radiation-induced diseases should be investigated. Toxicity studies have already shown that high doses of 1,500 mg of *Cannabidiol* per day are well tolerated in humans (Machado Bergamaschi et al., 2011), although they induce side effects in animals (Huestis et al., 2019). Phase I dose escalation studies starting from 5 mg/kg up to 20 mg/kg applied to healthy young men (24 years) did not induce any safety concerns (Perkins et al., 2020). Although there are several drug-drug interactions, *Cannabidiol* shows only few serious adverse effects (Chesney et al., 2020).
Reducing the risk of developing myocardial infarction because of radiation-induced cardiovascular diseases is an aim of the use of *Cannabidiol*. The time span, in which the drug should be

given has to be investigated in future experimental studies. Therefore, *Cannabidiol* could be applied before, during or after radiation in mice. Besides that, the dose and the frequency of the medication needs to be elaborated.

It is obvious that the experiments carried out in this dissertation show severe late time damages after radiation. First suggestions about possible targets of treatment for patients after radiotherapy have been made. To work out clearly structured prevention programmes and guidelines for clinical daily routine are required for the development of future clinical studies.

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