Non-invasive imaging of inflammation-related targets in atherosclerosis

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NON-INVASIVE IMAGING OF INFLAMMATION-RELATED TARGETS IN ATHEROSCLEROSIS

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich)

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ABSTRACT

Atherosclerosis is a complex chronic inflammatory disorder of the arterial system causing ischemic and occlusive vascular complications associated with a high mortality and disability worldwide. Most current diagnostic imaging techniques assess primarily the extent of the luminal narrowing within arterial vessels, however plaque composition and activity are in better correlation with subsequent acute vascular events. In this respect, novel non-invasive imaging agents targeting specifically plaque structures related to lesion vulnerability may improve the early identification of plaques at high-risk to cause a cardiovascular event.

In this thesis, a detailed histological characterization of human carotid plaques with a special focus on clinically relevant plaque features was performed. For this purpose, a biobank containing 200 human carotid samples was established in collaboration with the Department of Cardiovascular Surgery at the University Hospital of Zurich. A classification system developed based on histological details was employed for the categorization of all plaques into stable or vulnerable. In this human tissue biobank, we found a significant association between plaque inflammation and vulnerability. In a broad gene expression analysis, targets with high, medium and low potential for atherosclerosis imaging were identified. Thereby, inflammation-related targets were of primary interest. Our group has previously demonstrated that the expression of the T-lymphocyte activation antigens CD80 and CD86, both found on professional antigen-presenting cells, is higher in vulnerable than stable human carotid plaques. Furthermore, literature reports described an association of the fibroblast activation protein alpha (FAP) and plaque vulnerability. We observed a mildly increased FAP expression in human vulnerable compared to stable plaques on protein but not on mRNA level.

To test promising tracers in vivo, we characterized and applied a shear stress-induced apolipoprotein E knockout (ApoE KO) atherosclerosis mouse model. The feeding of a high fat diet to ApoE KO mice led to a 5-fold higher low-density lipoprotein level, 2.8-fold higher total cholesterol, 1.3-fold higher triglyceride, and 1.2-fold higher high-density lipoprotein level compared to ApoE KO mice on a regular diet. Over time, extensive plaque development in the aorta and additionally in segments close to flow-modifying implants was determined by oil red o lipid staining. The histological assessment with hematoxylin/eosin and CD68 immunohistochemistry revealed the formation of vulnerable atherosclerotic plaques in the carotid and aortic arteries, but no lesion development in coronary arteries. Expression analysis of inflammatory markers in murine plaques was performed by quantitative polymerase chain reaction and fluorescence activated cell sorting. The mRNA expressions of the macrophage marker CD68, CD80 and the macrophage mannose receptor MRC1 were significantly increased in diverse plaque-affected vascular segments of the mouse model compared to wild-type animals. Furthermore, CD80 protein
expression in leucocytes of the aorta and carotids of ApoE KO mice on high fat diet was significantly increased compared to ApoE KO mice on normal diet. All investigated plaques in this mouse model showed morphological and immunological features of vulnerability, including samples from locations where stable plaques were expected.

In an attempt to advance non-invasive atherosclerosis imaging towards the imaging of plaque vulnerability, we evaluated inflammation-related targets and developed and evaluated respective radiotracers for positron emission tomography (PET) and single photon emission computed tomography (SPECT). The CD80/CD86-specific indium-111 radiolabeled fusion protein belatacept (Nulojix®) as well as two low molecular weight oxodihydropyrazolocinnoline-based tracers $^{[11]}$C AM7 and $^{[18]}$F AC74 targeting CD80 were characterized in vitro and in vivo.

Belatacept is a homodimer composed of the extracellular portion of human CTLA-4 that is fused to a modified Fc fragment of human IgG1. $^{[111]}$In-DOTA-belatacept specifically accumulated in vivo in CD80/CD86-positive Raji xenografts, lymph nodes and salivary glands determined by SPECT/CT scans and biodistribution experiments. Ex vivo SPECT/CT studies showed an accumulation of the radiolabeled fusion protein in atherosclerotic plaques of the mouse model and the baseline uptake was 1.5-fold higher than the blockade uptake in the aorta and carotids. The binding of the radiotracer to human plaques determined by in vitro autoradiography correlated with the number of immune cells and the size of the lipid/necrotic core. With this study, we demonstrated that the in vivo targeting of CD80/CD86-positive atherosclerotic plaques is feasible with $^{[111]}$In-DOTA-belatacept and that this tracer is not only specifically binding to murine but also human plaques.

Both small molecular weight radiotracers, $^{[11]}$C AM7 and $^{[18]}$F AC74, share a high chemical stability, an extensive plasma protein binding with approximately 1% free tracer in human and murine plasma, a low blood cell binding and a blood half-life < 3 min in vivo in mice. In contrast to $^{[11]}$C AM7, $^{[18]}$F AC74 displayed a higher lipophilicity and a 20-fold higher IC$_{50}$ value. In vivo PET/CT experiments showed after intraperitoneal application of $^{[18]}$F AC74 or $^{[11]}$C AM7 an accumulation in CD80-positive mediastinal lymph nodes. This effect was not observed with fluorine-18 tracers with specificity for other targets. In line with the results of the radiolabeled belatacept, we observed a specific binding of $^{[18]}$F AC74 to human carotid plaques which was significantly increased in plaques compared to normal arteries and correlated with the immune cell number and the size of the lipid/necrotic core. This study demonstrated that the CD80-specific small molecule $^{[18]}$F AC74 accumulated in target-expressing tissues in vitro and in vivo. However, the pharmacokinetic compound characteristics, in particular the rapid hepatobiliary excretion, were not improved by the structural and physicochemical alteration of $^{[11]}$C AM7 to $^{[18]}$F AC74.

The mouse model with vulnerable plaques was used for the evaluation of the CD80 radiotracers $^{[11]}$C AM7 and $^{[18]}$F AC74. These tracers were compared to the metabolic tracers $^{[18]}$F fluorodeoxyglucose ($^{[18]}$F FDG) and its isomer $^{[18]}$F fluorodeoxymannose ($^{[18]}$F FDM). $^{[18]}$F FDG is a clinically used radiotracer in oncology. Both metabolic tracers accumulated in murine atherosclerotic plaques of the mouse model determined by ex vivo PET imaging. The in vivo radiotracer uptake in atherosclerotic segments of the mouse model was quantified by PET/CT scans. Thereby, a higher accumulation in plaques than the background was observed for $^{[11]}$C AM7,
[\textsuperscript{18}F]FDG and [\textsuperscript{18}F]FDM, but not [\textsuperscript{18}F]AC74. The standardized uptake value of [\textsuperscript{11}C]AM7 in plaques was approximately 7-fold lower than for the metabolic tracers. This markedly lower tracer uptake makes a clinical translation of [\textsuperscript{11}C]AM7 without synthetic optimization unattainable.

The published boronic acid based FAP inhibitor MIP-1232 was labeled with iodine-125 and used for \textit{in vitro} autoradiography studies with human carotid plaques. The radiotracer bound \textit{in vitro} to human plaques but additionally to normal artery segments and its use for atherosclerosis imaging is therefore limited.

Besides, the imaging techniques magnetic resonance imaging (MRI) and differential phase contrast X-ray tomography were used to explore plaque morphology of a human stable and a vulnerable plaque non-invasively. Both techniques visualized detailed plaque structures such as the fibrous cap or the necrotic core in good correlation with histopathology. Phase contrast X-ray tomography thereby provided a higher resolution and a better soft tissue contrast compared to MRI. Non-invasive techniques such as MRI or PET have a good prospect to identify high-risk plaques. Specific imaging protocols, correlation analyses between different imaging techniques, and preclinical imaging studies as obtained in this thesis are important steps towards a clinical application.

In conclusion, the imaging of inflammation-related plaque components, in particular CD80, by non-invasive PET has potential to identify highly inflamed vulnerable plaques. The shear stress-induced mouse model of atherosclerosis is suitable to characterize the respective tracers \textit{in vivo}. Future studies have to approach the optimization of CD80-targeting PET tracers towards an improved pharmacokinetic profile to allow their further validation in models of atherosclerosis and finally in humans.
KURZFASSUNG


Kurzfassung


Abschliessend lässt sich sagen, dass die Darstellung von inflammations-bezogenen Plaquekomponenten und insbesondere CD80 Potential hat stark entzündete vulnerable Plaques durch nichtinvasives PET zu identifizieren. Das durch Scherspannung-erzeugte Atherosklerose-Mausmodell ist geeignet Radiotracer in vivo zu charakterisieren. Zukünftige Studien müssen die CD80-spezifischen PET Tracer optimieren hinsichtlich einem verbesserten pharmacokinetischen
Kurzfassung

Profil um weitere Validierungsstudien in Atherosklerosemodellen und schliesslich in Menschen zu ermöglichen.
ABBREVIATIONS

°C        degrees Celsius
β+        positron
µ         micro
$^{11}$C  carbon-11 isotope
$^{18}$F  fluorine-18 isotope
$^{3}$H   hydrogen-3 isotope, tritium
$^{125}$I  iodine-125 isotope
$^{111}$In indium-111 isotope
5-HT      5-hydroxytryptamine, serotonin

A         adenine
APC       antigen-presenting cell
ApoE      apolipoprotein E
AUC       area under the curve

BSA       bovine serum albumine

C         cytosin
CD        cluster of differentiation
CD11c     Integrin alpha-X
CD68      Macrosialin
CD80/CD86 T-lymphocyte activation antigens
CEA       carotid endarterectomy
CT        computed tomography
CTLA-4    cytotoxic T-lymphocyte protein 4
CVD       cardiovascular disease

d         days
DAPI      4',6-diamidino-2-phenylindole
DC        dendritic cell
DOTA      1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DPC       differential phase contrast
DPP       dipeptidyl peptidase
DS        downstream
DW        diffusion-weighted
Abbreviations

$E_{\beta+}$: positron energy
$E_{\gamma}$: gamma energy
EC: electron capture
e.g.: for example
et al.: and others
EtOH: ethanol
ex vivo: out of the living

$f_u$: fraction unbound in plasma
FACS: fluorescence activated cell sorting
FAP: fibroblast activation protein alpha
FDG: fluorodeoxyglucose
FDM: fluorodeoxymannose
FITC: fluorescein isothiocyanate
FOV: field of view
FPLC: fast protein liquid chromatography

g: gram
G: guanine
GBq: gigabequerel
GLUT: glucose transporter
Gy: Gray

h: hours; human
HDL: high-density lipoprotein
HE: hematoxylin and eosin
HFD: high fat diet
HK: hexokinase
HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzym A
HPLC: high-performance liquid chromatography

IC$_{50}$: half-maximum inhibitory concentration
ID: injected dose
Ig: immunoglobulin
IHC: immunohistochemistry
in vitro: in glass
in vivo: within the living
i.p.: intraperitoneal
i.v.: intravenous

$K_d$: dissociation constant
KO: knockout
Abbreviations

L  liters
LC-MS  liquid chromatography-mass spectrometry
LDL  low-density lipoprotein
LDLr  LDL receptor
LN  lymph node
log  logarithm
logD_{7.4}  logarithmic distribution coefficient between octanol and water at pH 7.4
m  milli; murine
M  molar (mol/liter)
max  maximum
MeCN  acetonitrile
mGluR5  metabotropic glutamate receptor 5
MHC  major histocompatibility complex
MI  myocardial infarction
min  minimum; minutes
MMP  matrix metalloproteinase
MMR  macrophage mannose receptor (protein)
MRC  macrophage mannose receptor (gene)
MRI  magnetic resonance imaging
mRNA  messenger ribonucleic acid
MSc  Master of Science
MTC  magnetization transfer contrast
MW  molecular weight
n  nano; number (amount)
n.d.  non determined
ND  normal diet
Ns  non significant
oxLDL  oxidized low-density lipoprotein
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PD  proton density
PET  positron emission tomography
pH  negative logarithm of the hydrogen ion concentration
p.i.  post injection
POP  prolyl oligopeptidase
PTAH  phosphotungstic acid hematoxylin
qPCR  quantitative polymerase chain reaction
Abbreviations

r  recombinant
RCY  radiochemical yield
RNA  ribonucleic acid
rt  room temperature

s.c.  subcutaneous
SD  standard deviation
SEC  size-exclusion chromatography
SEM  standard error of the mean
SMA  smooth muscle actin
SMC  smooth muscle cell
SPECT  single photon emission computed tomography
SPR  surface plasmon resonance
SR-BI  scavenger receptor class B type I
SUV  standardized uptake value
Sv  Sievert

T  thymidine
T1/2  half-life
T1  spin-lattice relaxation time (MRI)
T2  spin-spin relaxation time (MRI)
TAC  time-activity curve
TCFA  thin-cap fibroatheroma
TCHO  total cholesterol
TCR  T cell receptor
TE  echo time (MRI)
TFA  trifluoroacetic acid
TG  triglycerides
Th  T helper cells
TLC  thin-layer chromatography
TOMCAT  tomographic microscopy and coherent radiology experiments
TR  repetition time (MRI)
TSPO  translocator protein

U  uracil
UPLC  ultra performance liquid chromatography
US  upstream

VOI  volume of interest

y  years
1 GENERAL INTRODUCTION

1.1 ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease affecting the arterial system involving lipid metabolism, the adaptive and innate immune systems as well as complex molecular and cellular mechanisms associated with vascular remodeling resulting in the formation of atherosclerotic plaques. Consequences of atherosclerosis are ischemic and occlusive vascular events referred to as cardiovascular disease (CVD) which is the main cause of death globally [1]. Today, the majority of deaths related to CVD occur in developing countries and projections indicate that CVD-related deaths will continue to dramatically increase in the near future primarily driven by the rising prevalences of obesity and insulin resistance worldwide [2]. Atherosclerosis constitutes a significant public health problem having not only a clinical but also a considerable economic impact due to high treatment costs and productivity losses [3].

Risk factors for atherosclerosis are either modifiable or non-modifiable. Modifiable risk factors include an unhealthy diet, physical inactivity, and tobacco smoking giving rise to elevated blood glucose and lipid levels, increased blood pressure and obesity. These factors substantially influence disease development since over 90% of myocardial infarctions (MI) are attributable to modifiable risk factors [4]. Gender, age, hereditary and socio-economic factors on the contrary determine the non-modifiable risk for atherosclerosis and CVD.

Several risk stratification tools have been proposed for the assessment of future vascular events. Among them, risk scores based on the Framingham heart study are most frequently used. The Framingham score estimates the 10-year risk for CVD based on major cardiovascular risk factors such as age, blood lipid levels, hypertension, diabetes and smoking [5].

1.1.1 The Pathophysiology of Atherosclerosis

The underlying mechanisms of atherosclerosis are multifaceted, combining altered endothelial shear stress, endothelial dysfunction, lipoprotein retention, infiltration of immune cells, foam cell formation, apoptosis, necrosis, calcification, angiogenesis, hemorrhage and arterial remodeling. Plaque evolution is a slow process with initial vascular changes such as intimal thickening and reversible fatty streak formation already present in children [6]. Predilection sites for atherogenesis are located in curved arteries and close to branching points where low and oscillatory endothelial shear stress patterns predominate. Under these conditions, endothelial gene expression is altered towards an upregulation of proatherogenic genes [7]. Endothelial dysfunction along with the retention of lipoprotein particles in the intimal vascular layer are initial mechanisms in atherosclerotic plaque formation and high blood low-density lipoprotein (LDL) levels accelerate
this process [8]. Arterial endothelial cells and macrophages produce upon stimulation adhesion molecules, chemokines and growth factors recruiting diverse immune cells [9]. Notably, the innate and adaptive immune systems are both involved in atherosclerosis development from disease onset on. Monocytes and to a lesser extent T cells are attracted to evolving plaques by monocyte chemotactic protein-1 (MCP-1) which is expressed by cellular plaque constituents including macrophages accordingly potentiating immune cell infiltration [10]. Crucial components in atherogenesis are mediators facilitating the differentiation of monocytes into macrophages such as the macrophage colony-stimulating factor (M-CSF), the granulocyte-macrophage colony-stimulating factor (GM-CSF) or chemokine ligand 4 (CXCL4) [11, 12]. In addition, monocytes serve as precursors for dendritic cells (DCs) [13]. LDL lipoproteins trapped within the intima undergo oxidative modifications and these heterogeneous oxidized LDL (oxLDL) species are phagocytized by macrophages and DCs [14]. The resulting lipid loaded cells, referred to as foam cells, are a distinctive characteristic of atherosclerosis.

Overall, numerous immune cell types are present in an atherosclerotic plaque including monocyte-derived macrophages, DCs, and to a lesser extent T cells thereof in particular T helper (Th) cells. Regulatory T cells (Treg), B1a cells and IL-10 secreting regulatory B cells (Breg), all of them atheroprotective, are minor cell populations in atherosclerotic lesions [8, 15]. The plaque microenvironment influences macrophage polarization significantly leading to an enormous macrophage heterogeneity not limited to pro-inflammatory M1 and anti-inflammatory M2 macrophages, but additionally various intermediate macrophage types occur [12]. During antigen presentation, macrophages, DCs and B cells collectively designated professional antigen-presenting cells (APCs) present processed antigens to T cells which initiates a Th1 or Th2 immune response. Activated Th1 cells produce pro-inflammatory mediators and promote plaque growth [8].

Atherosclerosis is characterized by a defective inflammation resolution. Macrophages and foam cells produce proatherogenic mediators and contribute to an insufficient clearance of apoptotic cells. Until today, the exact mechanisms of apoptosis and efferocytosis are not fully understood. However, evidence suggests an involvement of endoplasmic reticulum stress in macrophage apoptosis in advanced atherosclerotic plaques [16]. Under a progressive inflammation, the production outweighs the removal of apoptotic cells which results in secondary necrosis and consequently in the formation of a necrotic core [17]. Intraplaque hemorrhage due to leaky neovessels originating from the adventitial vasa vasorum lead to a sustained plaque inflammation and an enlargement of the necrotic core [9]. Angiogenesis is induced by vascular endothelial growth factor A (VEGF-A) secretion and is linked to plaque hypoxia and inflammation [18]. A common atherosclerotic plaque feature is the occurrence of calcifications which correlates with plaque burden, age and future cardiovascular events [19, 20].

Another important characteristic of atherosclerotic plaques is the fibrous cap formed by smooth muscle cells (SMCs) together with their secreted products collagen, elastin and proteoglycans. Over time, the number of lesional SMCs increases based on an influx of medial SMCs and a local SMC proliferation within the fibrous cap [9]. In the plaque microenvironment, a phenotypic switching
occurs by transformation of medial into synthetic SMCs [21]. Synthetic SMCs are characterized by an increased production of extracellular matrix components and a higher expression of receptors involved in lipid uptake; the latter promoting a foam-like SMC cell type [20, 21].

These changes on the molecular or cellular level are accompanied by extensive vascular remodeling processes. To maintain the vascular lumen area, expansive remodeling prevails in early atherosclerotic lesions, however constrictive vascular remodeling occurs in progressive plaques [9].

1.1.2 The Concept of Plaque Vulnerability

The formation of an atherosclerotic plaque is a dynamic process that subclinically progresses over many decades and involves different overlapping and indefinite stages of the disease eventually resulting in a highly inflamed, thrombosis-prone lesion. However, it is recognized that only 2 – 3% of plaques result in subsequent acute cardiovascular syndromes [17].

Plaque classification according to vulnerability is based on the risk for thrombosis and acute cardiovascular events. Vulnerable plaques, also designated high-risk, unstable or thrombosis-prone plaques, are the causal link to thrombus formation via rupture or erosion [22]. The underlying trigger for a MI is in 76% plaque rupture, while the remainder is attributable to plaque erosion or other unknown factors [10]. Rupture-prone plaques are thin-cap fibroatheromas (TCFAs) containing a soft and large lipid/necrotic core and a thin fibrous cap infiltrated by inflammatory cells [22, 23]. Additional morphological characteristics are expansive remodeling, large lesion size, microvessel formation, hemorrhage and calcifications. A common feature of erosion-prone plaques is a heterogeneous plaque composition rich in proteoglycans, whereas other characteristics are unspecific such as negligible calcifications, infrequent remodeling and low degree of inflammation [22, 23].

The knowledge about vulnerable plaque morphology is mainly derived retrospectively from autopsy studies. In human atherosclerotic plaques, a significant correlation of plaque instability with intraplaque hemorrhage, lipid content, plaque size and an extensive necrotic core was described [24, 25]. All these individual features lead to a sustained plaque inflammation indicating a key role of lesion inflammation in vulnerability. Shoulder segments of plaques with an overlying thin cap in close proximity to a necrotic core and highly infiltrated by macrophages and foam cells are most susceptible to rupture [9, 17]. Controversially discussed is the impact of calcifications on plaque instability. Growing evidence suggests a correlation of coronary artery calcifications with vascular events, however only a weak association with plaque instability. Vulnerable plaques contain speckled or fragmented calcifications in the fibrous cap whereas stable plaques show more extensive calcifications [19].
1.1.3 Atherosclerosis-Associated Vascular Complications

Plaques with extensive inward remodeling limit blood supply to downstream tissues impairing organ functions. Hence, clinical symptoms include renal ischemia, cerebral ischemia, intermittent claudication and angina pectoris depending on the location of the artery affected. In an early stage, patients are asymptomatic at rest, however during exercise or a medical stress test, symptoms of inadequate blood flow to vital organs become evident. In later stages of the disease with a severe occlusion, blood supply is even insufficient under resting conditions.

A complete occlusion of an artery due to a thrombus is an acute medical emergency. A prompt treatment is essential to prevent permanent damage to distal tissues and in severe cases an irreversible loss of organ function. MI is the consequence after occlusion of a coronary artery, whereas a stroke is caused in 80% by an occlusion in the cerebral circulation, while the remainder is attributable to cerebral hemorrhages [26].

1.1.4 Pharmacological and Surgical Interventions in Atherosclerosis

A cornerstone in the prevention of atherosclerosis and the treatment of cardiovascular events is the change of modifiable risk factors. Patients are advised to adhere to a healthy diet, be physically active, maintain a healthy weight, reduce visceral obesity, quit smoking, and reduce stress.

Pharmacologic interventions aim primarily at altering the blood lipid profile and reducing blood pressure. Hypolipidemic drugs such as statins, fibrates and nicotinic acid reduce LDL while increasing high-density lipoprotein (HDL) levels. The most commonly used drug class are the statins which selectively inhibit HMG-CoA reductase. This results in an impaired biosynthesis not only of cholesterol but also of farnesylated and geranylated proteins causing pleiotropic effects that influence among others cell proliferation, angiogenesis and oxidative stress [27]. The antihypertensive treatment is based on diuretics, angiotensin-converting enzyme inhibitors, β-blockers, calcium-channel blockers, and angiotensin II receptor antagonists. For secondary prevention of cardiovascular events, low-dose acetylsalicylic acid is commonly prescribed to patients to reduce the risk of blood clot formation. Other pharmacologic interventions aim at treating comorbidities such as diabetes.

The goal of surgical treatments is to remove atherosclerotic plaques, to reopen an occluded arterial lumen and/or to maintain blood supply to a downstream tissue or organ. The gold standard for carotid steno-occlusive pathology is carotid endarterectomy (CEA) which is the best stroke prevention with low risk for complications [28]. Another surgical technique is balloon angioplasty with or without a subsequent stent placement in the reopened arterial segment. Owing to a reduced restenosis risk, drug-eluting stents are preferred over bare metal stents [29]. In coronary artery bypass surgery, an artery is diverted to reestablish normal blood supply to the myocardium [27].

Despite these effective and successful therapeutic options, many patients do not profit from them because of an inadequate diagnosis and prediction of future vascular events.
1.1.5 Diagnostic Challenges in Atherosclerosis

Patients affected by atherosclerosis are usually asymptomatic until an acute vascular event occurs or a late-stage chronic occlusive disease is established. These medical conditions require immediate and life-long treatment aiming at preserving but often not ameliorating organ functions. The diagnosis of subclinical atherosclerosis represents an unmet clinical need that would allow the identification of patients at high risk for vascular complications in an early phase of the disease. Consequently, a more effective treatment tailored to the medical condition of the patient and the reduction of atherosclerosis-associated disability and mortality would be feasible. Today, differential diagnosis of atherosclerosis and CVD is predominantly performed in symptomatic patients.

In atherosclerosis diagnostics, various techniques are applied depending on the symptoms of the patient, the location of a potential constriction and risk factors. Symptomatic patients undergo a physical examination, blood tests and a stress test if a chronic ischemic vascular disorder is suspected. Further examinations include electrocardiography, electroencephalography and imaging.

Imaging techniques can be subdivided into invasive and non-invasive methodologies. Invasive techniques comprise among others angiography, coronary intravascular ultrasound (IVUS), optical coherence tomography (OCT) and near infrared spectroscopy (NIRS). Non-invasive are the nuclear imaging modalities positron emission tomography (PET) and single photon emission computed tomography (SPECT), besides computed tomography (CT), and magnetic resonance imaging (MRI). For atherosclerosis diagnostics non-invasive imaging approaches are preferred due to a lower risk for complications and fewer contraindications.

Until lately, imaging has focused on the assessment of the degree of luminal stenosis to identify potential culprit lesions. Therefore a wide-range of techniques were used with invasive angiography as the gold standard. However, culprit plaques do most often not cause a severe vascular stenosis since outward remodeling compensates for lesions occupying up to 40% of the internal elastic lamina area, thus preserving a normal vessel lumen [30]. Nearly 80% of lesions causing a thrombotic occlusion occupy less than 74% of cross-sectional luminal area [31, 32]. Hence for the prediction of acute vascular events, imaging modalities should rather focus on the composition and vulnerability of plaques than on the extent of a flow-limiting stenosis [33]. Notably, a remarkable number of plaque ruptures occur without any symptoms and are considerably more frequent in patients with diabetes or hypertension [33].

To investigate plaque vulnerability, functional data about plaque activity and composition are of high importance. Molecular imaging allows the visualization of biological processes at the molecular or cellular level in vivo with target-specific imaging probes. In general, molecular precede anatomical changes and consequently an earlier identification of a disease is feasible by molecular imaging and facilitates a more effective treatment [27, 34]. Besides the nuclear imaging techniques PET and SPECT, MRI and optical imaging methods provide functional information.
1.1.6 Non-Invasive Imaging Techniques in Atherosclerosis

For atherosclerosis imaging, a non-invasive method providing information about plaque vulnerability and allowing the identification of high-risk patients would be exceedingly desirable. Additional requirements are the possibility for a longitudinal monitoring of disease progression and the visualization of systemic processes not limited to certain specific locations as by invasive imaging. An overview of currently available non-invasive imaging techniques in preclinical and/or clinical practice is shown in Table 1.1.

### Table 1.1 Characteristics of non-invasive imaging techniques, adapted from [35-38].

<table>
<thead>
<tr>
<th>Imaging technique</th>
<th>Detected energy</th>
<th>Spatial resolution [mm]</th>
<th>Maximal sensitivity [M]</th>
<th>Tissue penetration depth [mm]</th>
<th>Information, objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>Annihilation photons</td>
<td>Animal: 1 – 4, clinical: 4 – 5</td>
<td>$10^{-11}$ – $10^{-12}$</td>
<td>&gt; 300</td>
<td>Functional, metabolic, quantification, molecular</td>
</tr>
<tr>
<td>SPECT</td>
<td>Gamma rays</td>
<td>Animal: 0.5 – 5, clinical: 7 – 15</td>
<td>$10^{-10}$ – $10^{-11}$</td>
<td>&gt; 300</td>
<td>Functional, metabolic, molecular</td>
</tr>
<tr>
<td>MRI</td>
<td>Radiofrequency waves</td>
<td>Animal: 0.03 – 0.1, clinical: 0.2 – 1</td>
<td>$10^{-3}$ – $10^{-5}$</td>
<td>&gt; 300</td>
<td>Anatomical, functional, molecular</td>
</tr>
<tr>
<td>CT</td>
<td>X-rays</td>
<td>Animal: 0.03 – 0.4, clinical: 0.3 – 1</td>
<td>$10^{-3}$ – $10^{-5}$</td>
<td>&gt; 300</td>
<td>Anatomical, calcium quantification</td>
</tr>
<tr>
<td>DPC</td>
<td>X-rays</td>
<td>Ex vivo: down to 1 μm</td>
<td>-</td>
<td>&gt; 300</td>
<td>Anatomical</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>High-frequency sound waves</td>
<td>Animal: 0.05 – 0.5, clinical: 0.1 – 1</td>
<td>-</td>
<td>1 – 200</td>
<td>Anatomical</td>
</tr>
<tr>
<td>Fluorescence imaging</td>
<td>Visible to infrared light</td>
<td>Animal: 2 – 10</td>
<td>$10^{-9}$ – $10^{-11}$</td>
<td>1 – 20</td>
<td>Functional, molecular</td>
</tr>
<tr>
<td>Bioluminescence imaging</td>
<td>Visible to infrared light</td>
<td>Animal: 3 – 10</td>
<td>$10^{-13}$ – $10^{-16}$</td>
<td>1 – 10</td>
<td>Functional, molecular</td>
</tr>
</tbody>
</table>

PET radiotracers are labeled with positron-emitting nuclides such as $^{18}$F, $^{11}$C, $^{13}$N and $^{64}$Cu. During annihilation, the masses of the emitted positron ($\beta^+$) and an electron combine and are converted to two 511 keV photons that are emitted in an angle of 180°. Photon pairs are detected by the PET imaging system and individual photons are processed for temporal, spatial and energy information to exclude random, single or scatter coincidences from the data analysis [39]. The positron range within tissues depends on the positron energy $E_{\beta^+}$ and has a great influence on the spatial resolution together with technical aspects of the imaging system. PET radiotracers are versatile tools to target and visualize specific disease-associated processes in a quantitative manner.

SPECT imaging is based on radiotracers containing γ-emitting radionuclides such as $^{99m}$Tc, $^{111}$In and $^{67}$Ga. The gamma rays are detected by a rotating detector equipped with a collimator which
influences the sensitivity and resolution of the imaging system. Three dimensional projections of
the radiotracer distribution in vivo are obtained. Technical progress in SPECT regarding the
absolute quantification of radioactivity concentrations has been achieved lately, but it remains
challenging due to confounding factors such as photon scatter, photon attenuation, and motion
artefacts [40].

MRI visualizes the distribution of tissue protons, in particular of abundant water molecules,
after application of a radiofrequency pulse. For contrast-enhancement mostly gadolinium-based
contrast agents are administered. Depending on the repetition time and the echo time employed in
a MR protocol, different anatomical features are visualized. Information about plaque anatomy and
composition can be registered by non-invasive MRI at adequate resolution for large vessels but
lower sensitivity and a longer acquisition time per frame compared to PET [35, 41].

CT tomographic images are obtained based on a density-specific attenuation of X-ray beams
while penetrating tissues and CT contrast can be enhanced by the administration of contrast agents.
Atherosclerotic plaque burden can be estimated by the coronary artery calcification score
determined in CT scans and furthermore CT is applied during invasive coronary angiography [42].
Most importantly, CT provides detailed anatomical information. Effective doses deposited to the
patient are depending on the scanned field of view in the range of 1 – 11 mSv [42].

Differential phase contrast (DPC) X-ray tomographic imaging is a preclinical technique
restricted to ex vivo applications. It not only measures X-ray attenuation as conventional CT, but
additionally the phase shift due to different tissue densities. Thereby high-resolution images about
tissue anatomy with good soft tissue contrast are generated.

Ultrasound is a widely available technique and images are generated by the detection of high-
frequency sound waves reflected by the tissue. In atherosclerosis, the intima-media thickness, a
predictor for future vascular events, can be measured by non-invasive ultrasound, however only in
superficial arteries e.g. the carotids [43].

In optical imaging, light emitted by biomolecules is used to generate images that show the
distribution of the imaging probe in vivo. The application of this techniques so far predominates in
preclinical settings due to the low tissue penetration depth.

Among all non-invasive imaging modalities, PET and MRI have the greatest potentials for clinical
atherosclerosis imaging due to the possibility to systemically investigate plaque activity and
composition. PET imaging is characterized by the highest molecular sensitivity making it possible
to apply radiotracer amounts below effective pharmacological doses in the picomolar range.
Furthermore, the quantification of radiotracer distribution combined with a good temporal
resolution allows the monitoring of dynamic processes in vivo. In atherosclerosis imaging by PET,
the partial volume effect can affect radioactivity quantification due to the small volume of a single
plaque that might lead to a signal displacement [44]. The optimal [18F]fluorodeoxyglucose
([18F]FDG) dose for the identification of culprit plaques ranges between 185 – 300 MBq
corresponding to a deposited dose to the patient of around 3 – 6 mSv which is comparable to a
chest CT [45-47].
Dual imaging approaches such as PET/CT combine functional and anatomical information and are the standard equipment in preclinical and clinical settings. The more recently introduced hybrid PET/MRI systems have the capability to provide more detailed anatomical information with a higher soft-tissue contrast which facilitates the assessment of atherosclerotic plaque composition. Furthermore, the radiation dose to the patient can be substantially reduced since around 70% of the total radiation exposure in a whole-body $[^{18}\text{F}]$FDG PET/CT scan (300 – 370 MBq) is attributable to the CT [47].
1.2 NUCLEAR IMAGING OF ATHEROSCLEROSIS

For non-invasive PET and SPECT imaging in atherosclerosis, various processes and targets allow a specific tracer accumulation in an evolving plaque (Figure 1.1). The selection of the target determines the stage of atherosclerosis imaged and identified. Lately, numerous imaging targets and respective tracers were suggested and evaluated in humans. Many of the tracers that accumulate in atherosclerotic plaques were identified by retrospective data analysis of PET scans acquired for the clinical diagnosis of other diseases. The first tracer discovered that still predominates clinical atherosclerosis imaging is $^{18}$F-FDG. $^{18}$F-FDG is a metabolic tracer taken up into cells by glucose transporters (GLUT) and subsequently trapped within the cytoplasm after phosphorylation by hexokinase [48-50]. $^{18}$F-FDG-6-phosphate is neither transformed along the glycolysis pathway by glucose-6-phosphatase, nor cleared from the cell via back reaction. $^{18}$F-FDG accumulates in metabolically active cells such as macrophages present in plaques but also in tumor cells and cells within the brain. High uptake in cardiomyocytes hampers the identification of coronary atherosclerosis. Since $^{18}$F-FDG uptake is in competition with glucose metabolism, patients are advised to fast prior to a PET scan [52]. In symptomatic patients, $^{18}$F-FDG carotid plaque uptake was higher in symptomatic than asymptomatic lesions, correlated with plaque inflammation and was independent of the degree of luminal stenosis [45, 53, 54]. Furthermore, arterial $^{18}$F-FDG accumulation is a better predictor for CVD than the Framingham risk score and is suitable to monitor effects of antiatherosclerotic drugs and even lifestyle changes [46, 55]. However owing to the lack of specificity of $^{18}$F-FDG, other targets and tracers were explored for the specific and simultaneous detection of coronary and carotid plaques.

In the following paragraphs, an overview of the currently available clinically tested radiotracers is presented (Figure 1.1).

LDL labelled with technetium-99m was evaluated in patients almost three decades ago. $^{99m}$Tc-LDL uptake depended on plaque composition, but an accumulation in large and coronary arteries was observed in only 25% of patients [56]. In another pilot clinical study, technetium-99m labeled oXLDL accumulated in carotid plaques in seven individuals [57].

In particular immune cells being integral components of human atherosclerotic plaques are a widely used imaging target since macrophage and DC numbers correlate with the vulnerability of a plaque and its inflammatory potential [58, 59]. Preliminary studies with $^{68}$GaDOTATATE, binding to the somatostatin receptor 2 which is upregulated on activated macrophages, showed that radiotracer uptake was increased in calcified plaques in several blood vessels including the coronary arteries. Moreover, the accumulation correlated with preceding vascular events, but did not co-localize with $^{18}$F-FDG accumulation [60, 61]. Another target expressed by activated macrophages is the translocator protein (TSPO). A TSPO-specific radiotracer, $^{11}$C]PK11195, accumulated predominantly in symptomatic human carotid plaques, however displayed high non-specific binding [46, 62]. $^{18}$F]Galacto-RGD binds to macrophages and angiogenic endothelial cells expressing integrin $\alpha_\beta$. In patients prior to CEA surgery, $^{18}$F]Galacto-RGD showed a higher
accumulation in stenotic than non-stenotic carotid areas besides a weak association with macrophage number and microvessel density by immunohistochemistry [63]. $[1^{1}C]$Choline and $[1^{18}F]$Fluoromethylcholine, which target choline transport and are incorporated in cell membrane lipids, were recently evaluated in retrospective PET/CT studies. Thereby, radiotracer accumulation was localized within aortic, iliac and carotid arteries in the majority of patients, but no co-localization with calcifications was determined [64, 65].

The imaging of apoptotic cells was investigated by the application of $[99mTc]$Annexin 5 to four patients suffering from a transient ischemic attack prior to CEA [67]. In two patients, tracer uptake in carotid plaques histopathologically classified as unstable lesions was observed.

Another target relevant in atherosclerosis are calcifications with $[1^{18}F]$NaF as potential tracer. In large arteries of patients, uptake of $[1^{18}F]$NaF was observed in plaques co-localizing with calcifications determined by CT, was associated with CVD risk factors, and provided complementary information to $[1^{18}F]$FDG scans [68-70]. In coronary arteries, $[1^{18}F]$NaF accumulation correlated with the coronary calcium score, previous cardiovascular events, and angina pectoris [71].
All these clinically obtained data about targeting strategies in atherosclerosis are essential for future developments. However, the comparison of the individual tracers remains difficult because of the mainly retrospective PET data evaluation and major differences in study design and analysis.

In addition to these clinical imaging studies, numerous targets and corresponding radiotracers were evaluated in vitro and in animal models of atherosclerosis. Plaque components evaluated for atherosclerosis imaging include the vascular endothelial growth factor 1 (VCAM1) [72, 73], matrix metalloproteinases (MMP) [74, 75], hypoxia [76], lectinlike oxidized LDL receptor 1 (LOX-1) [77], macrophage-specific CD68 [78], folate receptor beta [79, 80], and activated platelets [81].

1.2.1 The T-lymphocyte Activation Antigen CD80 as Target for Atherosclerosis Imaging

The costimulatory molecules CD80 and CD86 are expressed by professional APCs and regulate T cell activity by ligation with the T cell receptors CD28 and cytotoxic T-lymphocyte protein 4 (CTLA-4) (Figure 1.2). CD80 expression is tightly regulated and induced after APC activation. Following CD80 upregulation, surface expression levels are controlled by endocytosis and trans-endocytosis mediated by CTLA-4 [82]. Atherosclerotic plaques contain a population of active APCs that colocalize with T cells which implies that antigen presentation occurs within lesions and is not restricted to draining lymph nodes [59, 83-85].

![Figure 1.2](image)

**Figure 1.2** Antigen presentation with two concomitant signals required for a successful T cell activation. First, the interaction of the major histocompatibility complex (MHC) molecule presenting a processed antigen to the T cell receptor (TCR). And second, the interaction of costimulatory molecules (CD80 or CD86) expressed by APCs with CD28 or CTLA-4 defining T cell response towards a Th1 or Th2 profile along with cytokines released by APCs.

By gene expression analysis and immunohistochemistry, we identified CD80 and CD86 as potential targets associated with plaque vulnerability which was in agreement with a study by Erbel et al. [83, 84]. The first CD80-specific radiolabeled imaging agent developed by us, was a carbon-11 labeled oxodihydropyrazolocinnoline derivative evaluated in vitro and in PET scans with mice [83]. This radiotracer, designated [11C]AM7, accumulated in CD80-positive tissue in vitro and bound to human carotid plaques with a significantly higher relative binding to vulnerable than stable plaques and normal arteries [83]. However, the in vivo uptake in CD80-positive xenografts was
negligible due to a rapid hepatobiliary excretion [83]. Therefore, we developed a second oxodihydropyrazolocinnoline derivative labeled with fluorine-18, designated \(^{18}\text{F} \text{AC74}\), with different physicochemical characteristics (A. Chiotellis et al., manuscript in preparation).

1.2.2 The Fibroblast Activation Protein Alpha as Target for Atherosclerosis Imaging

The fibroblast activation protein alpha (FAP, seprase) is a membrane-bound homodimeric serine protease belonging to the prolyl oligopeptidase family (POP). FAP is an interesting target in oncology due to its restricted expression in adults. Expression is only found at sites of wound healing and a multitude of epithelial carcinomas [86, 87]. An association of FAP expression with plaque vulnerability was recently described by Brokopp et al. [88]. In their study, FAP expression correlated with macrophage burden in human carotid plaques and was increased in thin-cap (< 65 µm) versus thick-cap (≥ 65 µm) human coronary lesions.

1.2.3 Evaluated Radiotracer Candidates for Atherosclerosis Imaging

For atherosclerosis imaging, different radiotracer candidates labeled with PET or SPECT isotopes were evaluated in this thesis (Figure 1.3, Table 1.2). CD80 imaging was performed with the two low molecular weight ligands, \(^{11}\text{C} \text{AM7}\) and \(^{18}\text{F} \text{AC74}\), and the macromolecule belatacept which was conjugated with DOTA and labeled with indium-111. The FAP inhibitor MIP-1232 was labeled with iodine-125.

![Figure 1.3](image)

Figure 1.3 Evaluated radiotracer candidates for atherosclerosis imaging: CD80-specific oxodi-hydropyrazolocinnoline derivates \(^{11}\text{C} \text{AM7}\) and \(^{18}\text{F} \text{AC74}\), CD80/CD86-specific macromolecule \(^{111}\text{In}\) DOTA-belatacept and FAP-targeting \(^{125}\text{I}\) MIP-1232.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Application</th>
<th>Half-life</th>
<th>Decay mode</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon-11</td>
<td>PET</td>
<td>20.3 min</td>
<td>(\beta^+ (99%))</td>
<td>(E_{\beta^+} 0.97) MeV</td>
</tr>
<tr>
<td>Fluorine-18</td>
<td>PET</td>
<td>109.8 min</td>
<td>(\beta^+ (97%), \text{ EC (3%)})</td>
<td>(E_{\beta^+} 0.64) MeV</td>
</tr>
<tr>
<td>Indium-111</td>
<td>SPECT</td>
<td>2.8 d</td>
<td>EC</td>
<td>(E_{\gamma} 173, 247) keV</td>
</tr>
<tr>
<td>Iodine-125</td>
<td>SPECT</td>
<td>60.0 d</td>
<td>EC, Auger electrons</td>
<td>(E_{\gamma} 35) keV</td>
</tr>
</tbody>
</table>
1.3 MOUSE MODELS OF ATHEROSCLEROSIS

Mice are the most widely used animals in atherosclerosis research due to the good knowledge of their genetic background, several genetically modified mouse strains available, simple breeding, and low cost of maintenance. An advantage for the imaging with animal PET systems is the decent body size of mice that allows whole-body scans. A physiological lipid profile characterized by high levels of HDL, the lack of cholesterol-ester transfer protein and a low intestinal absorption of dietary cholesterol renders wild-type mice unsusceptible to atherosclerosis [91, 92]. Genetic modifications to achieve an altered lipoprotein metabolism were obtained via modulation or knockout (KO) of the genes coding for the apolipoprotein E (ApoE), LDL receptor (LDLr) or the scavenger receptor class B type I (SR-BI). Commonly used transgenic mouse strains include ApoE KO and LDLr KO mice, both of them generated on a C57BL/6 background since this mouse strain is most susceptible to atherosclerosis. To investigate murine atherosclerosis a high fat and cholesterol diet (HFD) inducing a persistent state of hypercholesterolemia (> 300 mg/dL) is essential [92]. The exact dietary components vary between different atherogenic diets, but the cholesterol content influences atherosclerosis development more significantly than the amount of saturated fat [93]. Under a standard rodent chow diet containing < 0.02% cholesterol and 4 – 6% fat, hypercholesterolemia is observed in ApoE KO animals, whereas LDLr KO mice show only mildly elevated cholesterol levels. By feeding these mice an atherogenic diet, both strains show severe hypercholesterolemia and the formation of atherosclerotic plaques in the macrovasculature [93]. ApoE KO mice form plaques at predilection sites under normal chow diet, but the plaque burden is markedly smaller than under HFD. In the brachiocephalic artery of old ApoE KO mice, occasional spontaneous plaque ruptures and thrombosis were described, morphologically present as intraplaque hemorrhage, loss of the fibrous cap and coagulation [94, 95]. Brachiocephalic lesions have compared to aortic plaques a lower average cap thickness (9.5 µm versus 12.4 µm) and are exposed to a higher maximum cap stress (568.8 kPa versus 205.8 kPa) which is above the threshold for rupture in human plaques of 300 kPa [96]. A study by Campbell et al. stated that the rupture of murine aortic plaques is improbable [97]. In mice, the highest mechanical stress is directed towards the plaque-free media, whereas in human coronary plaques, regions exposed to the highest stress are the fibrous cap and the plaque shoulder [97].

Further approaches to better reflect specific human CVD-associated conditions or comorbidities are among others the application of vasoconstrictors such as angiotensin II. Despite these efforts to generate a suitable animal model for atherosclerosis research, the formation of vulnerable plaques and subsequent plaque ruptures remain unpredictable and are not causing acute vascular events in mice. To overcome these limitations, the simultaneous modification of two genes by DNA technology were explored. SR-BI/ApoE dKO mice developed severe atherosclerosis on normal chow diet with occlusive coronary diseases, MI and cardiac dysfunction [98]. A diet-inducible model of murine CVD, the SR-BI KO/ApoERG61h/h mouse, was more recently presented [99]. Under a normal diet, the hypomorphic and therefore reduced expression of ApoE4-like murine ApoE is sufficient to impair atherosclerosis development. However under a HFD, an extensive
atherosclerotic plaque burden was observed as well as MI and cardiac dysfunction. Owing to the rapid disease progression in these two mouse strains, animals died within 6 – 8 weeks making an in-house breeding facility indispensable [98, 99].

Overall, no consensus about the most suitable mouse model is defined today to investigate experimental atherosclerosis in vivo. Limitations of the currently used models are that hemodynamic factors, plaque morphology and disease characteristics differ significantly from human end-stage atherosclerosis [97]. Under extreme hypercholesterolemic conditions, atherosclerosis development is promoted in mice within weeks or month, whereas in humans atherosclerosis-associated events usually occur after the fifth decade.

1.3.1 The ApoE KO-cuff Mouse Model

The ApoE KO-cuff mouse model is a shear stress-induced model of experimental murine atherosclerosis based on three atherosclerosis-promoting factors: the use of genetically modified ApoE KO mice, the feeding of a modified Western type diet characterized by a high content of dietary fat and cholesterol, and the surgically altered vascular blood flow and shear stress in the common carotid arteries. These animals develop in addition to the natural predilection sites of atherosclerosis in the aorta, surgically-induced plaques in the carotid vessels by low and/or oscillatory shear stress patterns. The implantation of a flow-constrictive cuff leads to an approximately 70% luminal stenosis which facilitates atherosclerotic plaque formation in the adjacent vascular segments [100]. In our experimental setup, we implanted a constrictive cuff around the right common carotid artery and on the contralateral artery a non-constrictive control (Figure 1.4).

**Figure 1.4** Common carotid arteries with implanted constrictive cuff and non-constrictive control. Atherosclerotic plaques are generated up- and downstream of the cuff. The plaque phenotype is described by histology as stable-like in the downstream segment and vulnerable-like in the upstream segment.

The constriction-induced hemodynamic changes cause local shear stress regions. Vascular segments upstream (US) of the cuff are exposed to a lowered shear stress, whereas within the cuff an increased and downstream (DS) an oscillatory shear stress is observed [100]. Thereby, plaque development US and DS is promoted giving rise to plaques of a vulnerable phenotype US and lesions of a stable phenotype DS as previously described [100-103]. This morphological classification of
the plaques was based on lesion size, lipid and collagen content, SMC number, remodeling processes and intraplaque hemorrhage.
1.4 AIM OF THIS THESIS

Atherosclerosis-associated disability and mortality is a major worldwide public health issue. The first clinical presentation of coronary artery disease in more than 50% of individuals is a MI or sudden cardiac death [104]. The prolonged asymptomatic stage of the disease provides a window of opportunity for early diagnosis and monitoring of disease progression. This would allow a more effective treatment of high-risk patients and thereby prevent acute vascular events. Non-invasive PET is currently the most sensitive translational imaging technique and has potential to become a clinically relevant tool for the risk stratification of atherosclerotic lesions. Recent research efforts in the development of nuclear imaging agents focused on inflammation-related targets in atherosclerosis, but none of the evaluated tracers reached clinical routine utilization besides [18F]FDG.

The principal objective of this thesis was to investigate potential targets and to evaluate respective tracers for atherosclerosis imaging in vitro and in vivo in a mouse model of atherosclerosis. Specific thesis goals were as follows:

i. The investigation of morphological characteristics of a stable and vulnerable human carotid plaque by DPC X-ray tomographic imaging, MRI and histopathology.

ii. The identification and validation of targets abundant in vulnerable atherosclerotic plaques by expression analysis on mRNA and protein level in human carotid plaques.

iii. The radiolabeling of PET radiotracer candidates and their in vitro evaluation in binding assays and by in vitro autoradiography with human atherosclerotic plaques as well as control tissue.

iv. The characterization of the ApoE KO-cuff mouse model with a focus on the blood lipid profile, expression levels of inflammatory markers, plaque development and characteristics.

v. In vivo evaluation of the most promising PET radiotracer candidates for plaque imaging in the mouse model of atherosclerosis.

The investigation of targets relevant for atherosclerosis and the preclinical evaluation of radiotracers aims at improving non-invasive atherosclerosis imaging. In addition, this thesis will contribute to the understanding of disease characteristics in humans and in transgenic animal models.
2 HUMAN ATHEROSCLEROTIC CAROTID PLAQUE CHARACTERIZATION AND IDENTIFICATION OF MARKERS OF PLAQUE VULNERABILITY

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AUTHOR CONTRIBUTIONS

R. Meletta established the classification system and assessed the histological correlations, performed RNA isolations and qPCR experiments, analyzed the data and wrote the chapter. N. Borel performed the histological plaque characterization. L. Steier performed RNA isolations and qPCR experiments and was involved in data analysis. S. D. Krämer supervised the study and revised the chapter. A. Müller Herde supervised the study, performed RNA isolations and qPCR experiments and was involved in data analysis.
2.1 INTRODUCTION

Endarterectomized human atherosclerotic carotid plaques are a valuable basis for expression analysis studies and in vitro evaluation of radiotracer candidates. However, for the interpretation of results obtained with human atherosclerotic plaque collections, a meticulous histological assessment of the samples used in the respective studies is essential.

Different morphological classification schemes for plaques have been proposed and the most frequently used system was developed by the American Heart Association [105, 106]. We refined this scheme based on previous literature reports and created a semi-quantitative classification system for stable and unstable plaques. In this system, stable plaques can be further differentiated into intimal thickening and fibrous cap atheroma, whereas unstable plaques include TCFA and lesions associated with thrombus formation. The system served as the basis for the histological classification of human carotid plaques used in the in vitro experiments. Depending on specific research questions, it was further adapted focusing on individual histological components (Chapter 4 and 5).

To further evaluate our classification scheme and to foster the understanding of atherosclerosis, we studied the correlation between individual classification criteria. The focus of this analysis was on inflammation-related plaque characteristics. Furthermore, the expression of various markers that could serve as imaging targets was evaluated by quantitative polymerase chain reaction (qPCR) in human carotid plaques classified according to our scheme.
2.2 RESULTS

The association of the plaque classification criteria delineated in Table 2.1 was investigated in our human carotid plaque biobank. Nine individual histological features (each with a maximal score of 3) and four major histological criteria were defined: core, vasculature, inflammation and fibrous cap. The major criteria were equally weighted for comparison with a maximal score of nine per category resulting in a maximal total vulnerability score of 36.

Table 2.1 Semi-quantitative classification system for human atherosclerotic carotid plaques, adapted from [107-109]. The score for every criteria is indicated in brackets. A weighting factor was applied for the comparison of the major categories core, vasculature, inflammation and fibrous cap resulting in a maximal score of 9 per category and a maximal total vulnerability score of 36.

<table>
<thead>
<tr>
<th>Histological feature</th>
<th>Stable plaque</th>
<th>Unstable plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 1</td>
<td>Grade 2</td>
</tr>
<tr>
<td></td>
<td>Intimal thickening</td>
<td>Fibrous cap atheroma</td>
</tr>
<tr>
<td>Core</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid/necrotic core</td>
<td>None (0)</td>
<td>Small (1.5)</td>
</tr>
<tr>
<td>Intraplaque hemorrhage</td>
<td>None (0)</td>
<td>Small (1.5)</td>
</tr>
<tr>
<td>Neovascularisation</td>
<td>None (0)</td>
<td>&lt; 10 per section (1.5)</td>
</tr>
<tr>
<td>Foam cells</td>
<td>None (0)</td>
<td>&lt; 50 cells (1.5)</td>
</tr>
<tr>
<td>Immune cells</td>
<td>None (0)</td>
<td>Occasional cells or one group &gt; 50 cells (1)</td>
</tr>
<tr>
<td>Cap infiltration</td>
<td>None (0)</td>
<td>&lt; 10 cells in cap (1)</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>Intact cap (0)</td>
<td>Probably intact (1)</td>
</tr>
<tr>
<td>Minimum cap thickness</td>
<td>-</td>
<td>&gt; 200 µm (0)</td>
</tr>
<tr>
<td>Representative cap thickness</td>
<td>-</td>
<td>&gt; 500 µm (0)</td>
</tr>
<tr>
<td>Overall stability</td>
<td>Definitely stable</td>
<td>Probably stable</td>
</tr>
</tbody>
</table>
The inflammatory activity of a plaque, which refers to the number of foam cells and immune cells present as well as the infiltration of the fibrous cap by these cell subtypes, is summarized in the plaque inflammation score (Table 2.1). A positive correlation with a Pearson’s $r^2$ of 0.7565 was determined between the inflammation score and the total vulnerability score which includes all criteria summarized in Table 2.1 (Figure 2.1, left). Furthermore, a significantly higher inflammatory activity of vulnerable than stable plaques and plaques than normal arteries was observed (Figure 2.1, right).

**Figure 2.1** Correlation of the inflammation score with the total vulnerability score of the plaque samples (left) and with the classification categories normal artery, stable and unstable plaque (right) according to Table 2.1. Maximal score: 36 for total vulnerability score, 9 for inflammation score. Values represent mean ± SEM (bar plot). n: number of samples. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

The presence of inflammatory cells, the size of the lipid/necrotic core, the development of microvascular structures and the thinning of the fibrous cap are central determinants for plaque vulnerability (Table 2.1). In our plaque biobank, a significantly larger lipid/necrotic core was found in plaques of high than of low inflammatory activity (Figure 2.2, left). Moreover, a correlation of plaque inflammation with the presence of intraplaque hemorrhage and neovascularization was observed which was significantly increased in highly inflamed plaques (Figure 2.2, middle). Plaque inflammatory activity was associated with a thinner and inflamed fibrous cap represented by the cap score (Figure 2.2, right). A significantly increased cap score was determined in highly inflamed plaques compared to a low or intermediate plaque inflammation.
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**Figure 2.2** Correlation of the inflammation score with the score of the lipid/necrotic core, the vasculature and the fibrous cap according to Table 2.1. Inflammation score: 0 < low < 3; 3 ≤ medium < 6; 6 ≤ high ≤ 9. Maximal score of 9 for the core, vasculature and fibrous cap. Values represent mean ± SEM. n: number of samples. *p < 0.05, **p < 0.01.

The inflammation-related histological plaque features foam cells, immune cells and cap infiltration were analyzed in detail. Significantly higher foam cell infiltration was determined in plaques containing many total immune cells than plaques with no or only few immune cells (Figure 2.3, left). Furthermore, a significantly increased cap infiltration was observed in plaques containing many immune cells compared to plaques devoid of such cells (Figure 2.3, middle). The cap infiltration did moreover correlate with the number of foam cells present in a plaque (Figure 2.3, right).

**Figure 2.3** Correlation of the individual inflammation criteria of Table 2.1. Maximal score of 3 for foam cells and cap infiltration. The extent of immune cell and foam cell infiltration was described on the x-axis with the grading system of Table 2.1. Values represent mean ± SEM. Statistical analysis for n ≥ 3. n: number of samples. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

In this human biobank, we evaluated several targets by quantitative qPCR in plaque specimens classified as either normal arteries, stable or unstable plaques according to Table 2.1 [75, 80, 83]. The results of the gene expression analysis are listed in Table 2.2. The fold change in expression
and the significance was determined between plaques and normal arteries as well as between stable and unstable plaques.

Table 2.2: Differential gene expression of normal arteries, stable and unstable plaques quantified by qPCR [75, 80, 83].

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Abbreviation</th>
<th>Normal Arteries vs. Plaques*</th>
<th>Stable vs. Unstable Plaques</th>
<th>P</th>
<th>P</th>
</tr>
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<tr>
<td>Matrix metalloproteinase-9</td>
<td>MMP9</td>
<td>260.62</td>
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<td>&lt;0.001</td>
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<tr>
<td>T-lymphocyte activation antigen CD86</td>
<td>CD86</td>
<td>2.96</td>
<td>2.65</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Integrin alpha V</td>
<td>ITGA5</td>
<td>2.66</td>
<td>2.02</td>
<td>0.001</td>
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<td>Apolipoprotein H</td>
<td>APOH</td>
<td>4.58</td>
<td>1.59</td>
<td>0.002</td>
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</tr>
<tr>
<td>Indolamine 2,3-dioxygenase 1</td>
<td>ID01</td>
<td>5.30</td>
<td>2.14</td>
<td>0.004</td>
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<tr>
<td>Matrix metalloproteinase-12</td>
<td>MMP12</td>
<td>27.49</td>
<td>1.42</td>
<td>0.004</td>
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<tr>
<td>Metalloproteinase inhibitor 3</td>
<td>TIMP3</td>
<td>8.93†</td>
<td>0.25</td>
<td>0.004</td>
<td></td>
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<tr>
<td>Matrix metalloproteinase-14</td>
<td>MMP14</td>
<td>7.43</td>
<td>1.50</td>
<td>0.008</td>
<td></td>
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<tr>
<td>DNA damage-inducible transcript 3 protein</td>
<td>DDIT3</td>
<td>7.48</td>
<td>1.32</td>
<td>0.010</td>
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<td>MMP1</td>
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<td>1.53</td>
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<td>T-lymphocyte activation antigen CD80</td>
<td>CD80</td>
<td>4.92</td>
<td>3.26</td>
<td>0.032</td>
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<td>Arachidonate 15-lipoxigenase B</td>
<td>ALOX15b</td>
<td>4.09</td>
<td>1.29</td>
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<td>Tyrosine-protein kinase Mer</td>
<td>MERTK</td>
<td>2.73</td>
<td>1.48</td>
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<td>Matrix metalloproteinase-8</td>
<td>MMP8</td>
<td>82.72</td>
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<td>Matrix metalloproteinase-10</td>
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<td>Vascular cell adhesion molecule 1</td>
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<td>11.60</td>
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<td>0.748</td>
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<td>Matrix metalloproteinase-2</td>
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<td>6.00</td>
<td>0.72</td>
<td>0.127</td>
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<td>Macrotalin</td>
<td>CD68</td>
<td>3.62</td>
<td>1.11</td>
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<td>Aryl hydrocarbon receptor</td>
<td>AHR</td>
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<td>Translocator protein</td>
<td>TSPO</td>
<td>1.83</td>
<td>1.04</td>
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<td>Cannabinoid receptor 2</td>
<td>CNR2</td>
<td>2.01</td>
<td>1.16</td>
<td>0.299</td>
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<td>High affinity immunoglobulin gamma Fc receptor I (CD64)</td>
<td>FCGRA1</td>
<td>10.10</td>
<td>1.25</td>
<td>0.442</td>
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<tr>
<td>Type-1 angiotensin II receptor</td>
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<td>Endothelin-1 receptor</td>
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<td>1.53</td>
<td>0.84</td>
<td>0.269</td>
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<tr>
<td>C-C chemokine receptor type 1</td>
<td>CCR1</td>
<td>6.54</td>
<td>1.32</td>
<td>0.431</td>
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<td>T-cell-specific surface glycoprotein CD28</td>
<td>CD28</td>
<td>5.14</td>
<td>2.35</td>
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<tr>
<td>Folate receptor beta</td>
<td>FOLR2</td>
<td>7.63</td>
<td>1.64</td>
<td>0.201</td>
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<tr>
<td>Renin receptor</td>
<td>ATP6AP2</td>
<td>1.30</td>
<td>1.16</td>
<td>0.343</td>
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<tr>
<td>Cytoxic T-lymphocyte protein 4</td>
<td>CTLA4</td>
<td>3.78</td>
<td>2.61</td>
<td>0.126</td>
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<td>Endothelin B receptor</td>
<td>EDNRB</td>
<td>1.51</td>
<td>1.28</td>
<td>0.357</td>
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<td>4.22</td>
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<td>0.494</td>
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<td>Integrin beta-3</td>
<td>ITGB3</td>
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<td>1.05</td>
<td>0.879</td>
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<tr>
<td>Toll-like receptor 4</td>
<td>TLR4</td>
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<td>0.714</td>
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<tr>
<td>Toll-like receptor 6</td>
<td>TLR6</td>
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<td>1.01</td>
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<td>5'-nucleotidase (CD73)</td>
<td>NT5E</td>
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<td>0.72</td>
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<td>0.79</td>
<td>0.259</td>
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<tr>
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<td>0.943</td>
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<td>TLR1</td>
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<td>0.361</td>
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<tr>
<td>Folate receptor alpha</td>
<td>FOLR1</td>
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<td>1.86</td>
<td>0.109</td>
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<tr>
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<td>1.10</td>
<td>0.84</td>
<td>0.609</td>
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<tr>
<td>Nuclear factor erythroid 2-related factor 2</td>
<td>NFE2L2</td>
<td>1.08</td>
<td>1.44</td>
<td>0.320</td>
<td></td>
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</table>

* Atherosclerotic plaques combine values of stable and unstable plaques.
† Fold change refers to comparison between normal arteries and stable plaques only.
2.3 DISCUSSION

Plaque inflammation is a central element in lesion development. In atherosclerosis, immune cells are initially involved in the inflammation resolution but in advanced stages they sustain lesion growth and vulnerability. Advanced plaques contain a large population of immune cells and foam cells that infiltrate the fibrous cap and thereby destabilize the lesion [9]. The overall inflammatory activity of a plaque is a good predictor for plaque vulnerability as determined in this study and by previous reports [31, 59, 83]. Moreover, plaque inflammation positively correlates with other fundamental classification criteria for plaque vulnerability such as the size of the lipid/necrotic core and the lesion vasculature.

For atherosclerotic plaque imaging, a potential target should ideally display an expression restricted to atherosclerotic plaques or even better to vulnerable plaques. Targets of the latter category which showed a significantly increased mRNA expression in unstable compared to stable plaques include the matrix metalloproteinases MMP-1/9/12/14, the T-lymphocyte activation antigens CD80 and CD86 and the integrin alpha V. Target expression analysis studies on mRNA level provide a first evidence for a potential suitability as an imaging target. However, even more important is the protein expression level since radiotracers bind to proteins and not mRNA. Based on protein expression data of the costimulatory molecule CD80 which showed a correlation with plaque vulnerability in human atherosclerotic lesions [83], we focused in this thesis on the target CD80.

In conclusion, lesion inflammation presents in our human carotid plaque biobank an excellent measure for plaque vulnerability. In this respect, inflammation-related targets featuring an increased expression in unstable compared to stable human carotid plaques are promising targets for atherosclerotic plaque imaging.
2.4 EXPERIMENTAL SECTION

2.4.1 Human Atherosclerotic Carotid Plaques

Human carotid plaque specimens were removed during CEA surgery at the University Hospital Zurich. Written informed consent was obtained from all patients. A total of nine patients (2/9 female) with an average age of 72.1 ± 6.1 years were included in this study. Dissected plaques were stored in RNAlater® solution (Sigma-Aldrich, St. Louis, USA) at -80 °C and were fixated in 4% paraformaldehyde (in 0.9% NaCl) before paraffin-embedding. Histological sections of 2.5 µm were prepared and stained with hematoxylin and eosin. Histological plaque samples were classified according to Table 2.1 by a pathologist (N. B.). The criteria core, vasculature, inflammation and fibrous cap were equally weighted yielding in a maximal total score of 36.

2.4.2 RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from human carotid plaque specimens with Isol-RNA Lysis reagent (5-PRIME, USA) and transcribed into cDNA (QuantiTect Reverse Transcription Kit, Qiagen, Germany). PCR was performed with the GoTaq qPCR Master Mix (Promega, Switzerland) on an AB7900 HT Fast Real-Time PCR system (Applied Biosystems, USA) equipped with SDS software. Relative gene expression was determined using the internal control β-actin. Data quantification was performed according to the \(2^{-\Delta\Delta Ct} \) method [110].

2.4.3 Statistics

Statistical data analysis was performed with GraphPad Prism (GraphPad, La Jolla, CA, USA). Intergroup significance was determined for three groups by a one-way ANOVA with a Tukey’s multicomparison test and for two groups by a two-tailed unpaired student’s t-test. Correlation was analyzed by linear regression analysis. A \( p \)-value < 0.05 was considered significant (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), **** \( p < 0.0001 \)).
3 *EX VIVO* DIFFERENTIAL PHASE CONTRAST AND MAGNETIC RESONANCE IMAGING FOR CHARACTERIZATION OF HUMAN CAROTID ATHEROSCLEROTIC PLAQUES

This chapter is adapted from the original manuscript accepted by the International Journal of Cardiovascular Imaging on 27/06/2015.

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**AUTHOR CONTRIBUTIONS**

R. Meletta was involved in the planning and coordination of the study, performed sample preparation and data analysis, contributed to data interpretation, wrote the manuscript. N. Borel coordinated, supervised and analyzed the histology and immunohistochemistry, revised the manuscript. P. Stolzmann was involved in data interpretation and revised the manuscript. A. Astolfo performed the DPC experiments and revised the manuscript. J. Klohs performed MRI experiments and revised the manuscript. M. Stampanoni and M. Rudin provided access to their research infrastructure. R. Schibli was involved in data interpretation and revised the manuscript. S. D. Krämer was involved in the planning of the study and data interpretation, revised the manuscript. A. Müller Herde initiated the project, planned and coordinated the study, performed data analysis and 3D rendering, contributed to data interpretation and contributed to manuscript writing.
3.1 ABSTRACT

AIMS
Non-invasive detection of specific atherosclerotic plaque components related to vulnerability is of high clinical relevance to prevent cerebrovascular events. The feasibility of magnetic resonance imaging (MRI) for characterization of plaque components was already demonstrated. We aimed to evaluate the potential of ex vivo differential phase contrast X-ray tomography (DPC) to accurately characterize human carotid plaque components in comparison to high field multicontrast MRI and histopathology.

METHODS AND RESULTS
Two human plaque segments, obtained from carotid endarterectomy, classified according to criteria of the American Heart Association as stable and unstable plaque, were examined by ex vivo DPC tomography and multicontrast MRI (T1-, T2-, and proton density-weighted imaging, magnetization transfer contrast, diffusion-weighted imaging). To identify specific plaque components, the plaques were subsequently sectioned and stained for fibrous and cellular components, smooth muscle cells, hemosiderin, and fibrin. Histological data were then matched with DPC and MR images to define signal criteria for atherosclerotic plaque components. Characteristic structures, such as the lipid and necrotic core covered by a fibrous cap, calcification and hemosiderin deposits were delineated by histology and found with excellent sensitivity, resolution and accuracy in both imaging modalities. DPC tomography was superior to MRI regarding resolution and soft tissue contrast.

CONCLUSION
Ex vivo DPC tomography allowed accurate identification of structures and components of atherosclerotic plaques at different lesion stages, in good correlation with histopathological findings.
3.2 INTRODUCTION

The rupture of atherosclerotic plaques in carotid arteries is the main cause for stroke. Every year, 15 million people suffer a stroke worldwide. One-third of the affected die, one-third are left permanently disabled and one-third will recover completely from their attack [111]. Projections show that by the year 2030, an additional 3.4 million people aged ≥ 18 years will have had a stroke corresponding to 20.5% increase in prevalence from 2012 [112]. To prevent stroke, the most frequently performed surgical intervention is carotid endarterectomy.

Most ruptures occur in so called unstable plaques containing a highly inflamed lipid-rich and/or necrotic core covered by a thin fibrous cap [113]. Early identification and characterization of plaques are crucial for risk prediction and prevention of adverse events. Invasive techniques, such as intravascular ultrasound, optical coherent tomography, and carotid angiography can reveal luminal stenosis, wall thickness and plaque volume. However, plaque rupture and stroke are not dependent on the extent of stenosis. Characterizing plaque morphology and, in particular, plaque stabilizing and destabilizing components may provide more information on plaque instability and consequently on the risk of rupture. Besides molecular imaging such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), the two imaging techniques computed tomography (CT) and magnetic resonance imaging (MRI) that are widely accepted by the medical community, have the potential to assess plaque vulnerability non-invasively. However, to detect density differentials within soft tissues, such as in atherosclerotic plaques, a good sensitivity coupled with high-resolution are required, which does not apply to conventional CT and MRI. A recently introduced technique, synchrotron-based differential phase contrast X-ray (DPC) tomography [38, 114, 115], does not only measure the attenuation of X-rays passing through tissue, as in standard X-ray tomography, but in addition it measures the phase shift caused by refraction, which the coherent X-ray beam undergoes when passing through tissue with different refractive indices. This results in a high sensitivity to electron density differences and thereby improving contrast for soft tissue. The objective of our study was to evaluate the feasibility of ex vivo DPC tomography to morphologically characterize two human carotid atherosclerotic plaque specimens. Multicontrast-weighted MRI is capable of imaging vessel wall structures and carotid plaque compositions in vivo [116] and ex vivo [117]. MRI differentiates plaque components on the basis of water content, physical state, and molecular motion or diffusion while providing information-rich images at high spatial resolution. The most common strategy is to analyze multicontrast images (T1-, T2-, proton density (PD)-weighted imaging) that reveal components with different contrast [118]. The content and distribution of lipids is an important aspect of atherosclerotic plaques. To improve the contrast between lipids and fibrous tissue, the low mobility of protons bound to lipids has been exploited by using diffusion-weighted (DW) imaging [119]. For better identification of protein-rich regions magnetization transfer contrast (MTC) was recommended by Qiao et al. [120].

To our knowledge no direct comparison of DPC images, multicontrast MR images and histopathology of human endarterectomized carotid plaques has been carried out. The purpose of
this ex vivo study was (i) to explore the potential of a grating interferometer at a synchrotron X-ray source to measure the DPC between different components in human stable and unstable carotid plaques, (ii) to compare the images with multicontrast high-resolution MRI and (iii) to correlate the generated images from both modalities with histopathology.
3.3 RESULTS

3.3.1 Morphological Characterization of a Stable and Unstable Plaque by Histology

According to histopathology, the specimen originating from the *Arteria carotis externa* was classified as a stable plaque (Figure 3.1, A) and the specimen obtained from *Arteria carotis communis/interna* as an unstable plaque (Figure 3.1, B).

![Figure 3.1](image)

Figure 3.1 Whole mount human endarterectomized carotid plaques. (A) Stable plaque excised from *Arteria carotis externa*. (B) Unstable plaque excised from *Arteria carotis communis/interna*. Scale bars: 0.5 cm.

Histological workup was performed at the end of the ex vivo imaging procedures. Figure 3.2 shows the histological identification of the stable and unstable lesions. With HE, the investigated stable plaque presented a thick fibrous cap with fibrillar eosinophilic material. A high collagen content (blue) and local accumulation of erythrocytes (red) between the fibrous cap and the intima was proven by Masson’s trichrome staining. A staining for iron-containing hemosiderin (Prussian blue) was negative. Elastic fibers, stained black with VG-Elastica, were present in the lamina elastica interna and media. Immunohistochemical analyses using an anti-SMA antibody demonstrated the presence of smooth muscle cells (SMC) in the lamina elastica interna and media.

The unstable plaque presented towards one plaque shoulder a large necrotic core overlaid by a thicker fibrous cap and towards the other plaque shoulder a smaller lipid core overlaid by a thinner fibrous cap. Invasion of many macrophages and immune cells into the lipid core and partially necrotic core was strongly indicated by HE. The necrotic core contained cell debris, necrotic material and cholesterol crystals. A locally extensive calcification (arrow) was clearly detected with HE. Erythrocytes in the lipid core were of intensive red and SMCs in the media were stained light red by Masson’s trichrome. Intracellular hemosiderin pigments, a sign of erythrophagocytosis indicating preceding plaque hemorrhage, were found in macrophages in the lipid core (Figure 3.2, a high-power magnification, Prussian blue). Positive Prussian blue staining in the media (Figure 3.2, b, high-power magnification) is linked to the ability of SMCs to phagocytize heme-derived iron [121, 122]. The staining of SMCs correlated with the presence of elastic fibers in the media and lamina elastica interna. Few SMCs were also present in regions of the fibrous cap. PTAH staining
identified intraplaque fibrin in the lipid core and necrotic core of the unstable plaque. Fibrin deposition in lipid core/necrotic core regions are known to delineate late stage plaques [123]. Furthermore PTAH staining revealed a ruptured fibrous cap as visualized by a fibrin-positive ulceration site (U) towards the lumen of the blood vessel.

**Figure 3.2** Histopathological sections of the stable and unstable plaque stained with hematoxylin and eosin (HE); Masson’s trichrome coloring connective tissue in blue, erythrocytes and lipid/necrotic core in intense red, smooth muscle cells in light red; Prussian blue showing iron-containing hemosiderin (blue) indicating preceding intraplaque hemorrhage; Van Gieson-Elastica (VG-Elastica) coloring lamina elastica interna and other elastic fibers in black; anti-smooth muscle cell actin (anti-SMA) antibody labeling smooth muscle cells in media and fibrous cap. Phosphotungstic acid hematoxylin staining (PTAH) showing fibrin in purple located in lipid and necrotic core as well as at the ulceration site. Arrow in HE-stained unstable plaque section pointing at an extensive calcification. Boxed higher magnification images of the Prussian blue stained unstable plaque show hemosiderin-loaded macrophages present in the lipid core (a) and hemosiderin-loaded smooth muscle cells present in the media (b). L, lumen of the blood vessel; M, media; Int, intima; FC, fibrous cap; LC, lipid core; NC, necrotic core; U, ulceration site; n.a., not applicable.
### 3.3.2 Appearances of Atherosclerotic Plaque Components in DPC Tomography and Multicontrast MRI

In the following section we demonstrate the similarities and differences in appearance of plaque components among the different imaging modalities and in correlation with histopathology. Figures 3.3, 3.4 and 3.5 show the matched DPC images, MR images and histopathology sections of the stable and unstable carotid plaque. Visual appearances of each plaque component in DPC and MR tomography are summarized in Table 3.1. The high-resolution and sensitivity of DPC tomography enabled high-power magnification images which are shown with the matching HE stained sections in Figure 3.3, B-K.

**Figure 3.3** (A) Representative set of matched images of hematoxylin and eosin staining (HE), differential phase contrast (DPC) tomography and multicontrast MR (T1-, T2-, and proton density (PD)-weighted images, magnetization transfer contrast (MTC), diffusion-weighted (DW) images) of the stable and unstable plaque. For orientation, lumen of the blood vessel (L), media (M), intima (Int), intimal thickening (IT), fibrous cap (FC), lipid core (LC), necrotic core (NC), ulceration site (U) are labelled. Asterisk in stable plaque indicates erythrocyte accumulation; arrowhead in unstable plaque indicates low-density calcifications; arrow indicates high-density calcification. (B) Overview section of the stable (top) and unstable (bottom) plaque with boxed regions of interest for high-power magnification images in C-K. (C-K) Histopathology (HE-stained) and corresponding DPC tomograms of different plaque components of the stable (C-E) and unstable (F-K) plaque.
Table 3.1 Morphologic characteristics of plaque components compared with appearance in DPC and MRI.

<table>
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<tr>
<th>Components</th>
<th>DPC</th>
<th>T1</th>
<th>T2</th>
<th>PD</th>
<th>MTC</th>
<th>DW</th>
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<tbody>
<tr>
<td>Media (stable)</td>
<td>Isodense</td>
<td>Hyperintense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Hyperintense</td>
</tr>
<tr>
<td>Media with hemosiderin (unstable)*</td>
<td>Isodense</td>
<td>Hypointense</td>
<td>Hypointense</td>
<td>Hypointense</td>
<td>Hyperintense</td>
<td>Hypointense</td>
</tr>
<tr>
<td>Intima</td>
<td>Isodense</td>
<td>Hyper-to-Isointense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Hypointense</td>
<td>Hyperintense</td>
</tr>
<tr>
<td>Intimal thickening</td>
<td>Hyperdense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Hypointense</td>
<td>Isointense</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>Isodense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Hypointense</td>
<td>Isointense</td>
</tr>
<tr>
<td>Lipid core</td>
<td>Iso-to-hypodense</td>
<td>Iso-to-hypointense</td>
<td>Hypointense</td>
<td>Iso-to-hypointense</td>
<td>Hyperintense</td>
<td>Hypointense</td>
</tr>
<tr>
<td>Necrotic core</td>
<td>Hyper-to-hypodense</td>
<td>Isointense</td>
<td>Hyper-to-hypointense</td>
<td>Iso-to-hypointense</td>
<td>Isointense</td>
<td>Hyper-to-hypointense</td>
</tr>
<tr>
<td>Calcifications</td>
<td>Low-dense</td>
<td>Isodense</td>
<td>Hyperintense</td>
<td>Isointense</td>
<td>Isointense</td>
<td>Isointense</td>
</tr>
<tr>
<td></td>
<td>High-dense</td>
<td>Hyperdense</td>
<td>Hypointense</td>
<td>Hypointense</td>
<td>Hyperintense</td>
<td>Hypointense</td>
</tr>
</tbody>
</table>

* Media of the unstable plaque contained iron-containing hemosiderin engulfed by SMCs attributable for intense signals in MRI.

TUNICA MEDIA

Histopathology revealed SMCs and elastic fibers in the media of the stable and unstable plaque. The stable plaque displayed a media of homogeneous texture in DPC and MR images (Figure 3.3, A, C). Hyperintense (bright signal) T1-weighted and DW images distinguished the media from the intima. In contrast, media of the unstable plaque displayed hypointense (dark signal) T1-, T2-, DW-, and PD-weighted images, hyperintense MTC images, and an isodense signal in DPC images. Staining for hemosiderin disclosed blue precipitates as iron-containing particles engulfed by SMCs in the media of the unstable plaque which account for prominent signal intensities.

TUNICA INTIMA

The stable plaque consisted of an intact intima, with a homogeneous texture and intermediate intensity in most image contrasts, except for MTC and DW images where they appeared hypointense and hyperintense, respectively (Figure 3.3, A, C).
**INTIMAL THICKENING**

The intimal thickening in the stable plaque appeared homogenous and hyperdense in DPC delimiting it from the intima. MTC images revealed a hypointense intimal thickening (Figure 3.3, A, D).

**ERYTHROCYTE ACCUMULATION**

The gap between intima and intimal thickening/fibrous cap of the stable plaque was clearly defined in DPC and T1 contrast, but less in the other MR settings (Figure 3.3, A). The region was appreciated as low homogenous signal with a cloudy appearance in the zoomed DPC image (Figure 3.3, E). The presence of erythrocytes was only proven by histological staining.

**FIBROUS CAP**

The fibrous cap of both the stable and unstable plaque showed homogeneous and intermediate intensities in DPC images and MR images, except in MTC were it appeared hypointense (Figure 3.3, A, F).

**LIPID CORE**

A lipid core was only visible in the unstable plaque composed of hemosiderin-containing macrophages and erythrocytes as well as fibrin. The lipid core appeared hypointense in T1-, T2-, DW-, and PD-weighted images, and hyperintense in MTC (Figure 3.3, A). The lipid core could be delineated from intima and fibrous cap in all setups. The lipid core had an inhomogeneous texture from isodense to hypodense in DPC (Figure 3.3, G).

**NECROTIC CORE**

The necrotic core was clearly visible in all MR images with varying signal intensities, however, with more homogenous appearance than in DPC (Figure 3.3, A). In hypodense areas of the DPC images we identified cholesterol crystals or dissolved crystals and in hyperdense regions cell debris were identified (Figure 3.3, H and I, respectively).

**CALCIFICATIONS**

The highest and distinct signal intensity of all plaque components was found in regions of calcification in the unstable plaque (Figure 3.3, A, J). High-dense calcification (high amount of calcium, arrow) appeared hyperdense/-intense in DPC and MTC. In T1-, T2-, DW- and PD-weighted images it appeared hypointense. This calcification was sharply delineated from the surrounding soft tissue. In contrast, low-dense calcification (arrowhead) was distinguished only on T1-weighted images as hyperintense.
ULCERATION

The focal ulceration site in the unstable plaque was fibrin-positive and appreciated as a ruptured cap in HE and DPC (Figure 3.3, K) and was not clearly discernable in MR images.

![Figure 3.4 Serial, matched images of hematoxylin and eosin staining (HE), differential phase contrast (DPC) tomography and multicontrast MRI (T1-, T2-, proton density (PD)-weighted images, magnetization transfer contrast (MTC), diffusion-weighted (DW) images) of the stable plaque.](image)
Figure 3.5 Serial, matched images of hematoxylin and eosin staining (HE), differential phase contrast (DPC) tomography and multicontrast MRI (T1-, T2-, proton density (PD)-weighted images, magnetization transfer contrast (MTC), diffusion-weighted (DW) images) of the unstable plaque.
3.3.3 Three-Dimensional Visualization of Endarterectomized Atherosclerotic Plaques in DPC

The three-dimensional rendering of DPC images of the carotid plaque specimens are shown in Figure 3.6. The needle-shaped crystal in the unstable plaque appeared, in the given field of view, with an approximate length of 1.5 mm (Figure 3.6, E). Due to the limited field of view in DPC tomography only a part of the calcified crystal was imaged, however, allowing estimating that a relatively large calcification was present in this plaque specimen.

**Figure 3.6** Three-dimensional DPC images of the endarterectomized plaques. Black schematic plaque with arrow indicates viewing direction. (A) Carotid segment with the stable plaque at different orientations, white arrowheads pointing to the plaque; scale bars: 3 mm. (B) Cropped region of the stable plaque showing intact and smooth surface of the fibrous cap (FC), intima (Int) and the cleft (asterisk) containing erythrocytes; scale bar: 1 mm. (C) Carotid segment with the unstable plaque at different orientations; scale bars: 1 mm. (D) Cropped region of the unstable plaque showing thin fibrous cap (FC) covering the necrotic core (NC); scale bars: 0.5 mm. (E) Left image showing the whole unstable plaque in a maximal intensity projection. Right image showing cropped region with calcified crystal; scale bars: 1 mm.
3.4 DISCUSSION

In this *ex vivo* feasibility study we were able to demonstrate that detailed information on morphological characteristics and composition of a human stable and unstable endarterectomized plaque can be obtained with DPC tomography and multicontrast MRI. Our observations are in good correlation with histopathology.

Assessment of anatomy and composition of atherosclerotic plaques by MRI have been extensively studied *ex vivo* [117, 124-127] and *in vivo* [116, 128-130] using animal models of disease and in patients. However, a comprehensive evaluation of different MRI sequences has not been determined. With our combination of high-resolution MRI sequences, we were able to accurately identify fibrocellular tissue, lipid-rich and calcified regions as well as iron-containing deposits in the atherosclerotic plaques. Our intention was to apply the findings of MR images and histopathology to understand appearances of plaque characteristics in DPC images. In general, the DPC images presented a lower contrast than MR images, however, homo- and heterogeneous textures within the plaques are more pronounced in DPC images. All fibrocellular tissue, e.g. media, fibrous cap and intimal thickening, was recognized as a homogeneous pattern and delimited to the plaque. Regions of active remodeling within the unstable plaque (lipid/necrotic core) were clearly recognized as areas with heterogeneous appearance and different signal intensities. High-dense, but not low-dense, calcification gave a distinct hyperdense signal that clearly allowed for differentiation from background structures. Contrary to MRI, hemosiderin deposits presented no significant signal in DPC. There are a few studies [131-135] reporting on the application of phase contrast imaging to atherosclerotic vessels and findings are in good agreement with our results.

With our study we provided a foundation for directing and validating the interpretation of MR and DPC images and attempted to develop and optimize imaging parameters. The relatively new technique, DPC tomography was conducted at a synchrotron radiation facility, which, so far, cannot be used in clinical practice and limits it to a benchmarking feasibility work. Recording one tomogram with the experimental parameters used in this study would result in a deposition of approximately 30'000 Gy [114]. For humans, a whole-body irradiation of up to 1 Gy is unlikely to cause long range symptoms, whereas a dose of > 30 Gy is always fatal [136]. MRI has distinct advantages over DPC including no ionizing radiation and it can be applied *in vivo* [41] and hence to e.g. monitor plaque progression in longitudinal studies.

There are a number of limitations to this proof-of-concept study. First, the low sample number of only one stable and one unstable carotid plaque prevent us to raise quantitative data about the sensitivity, specificity and accuracy of DPC tomography and MRI. In this present study we primarily focused on finding optimal imaging parameters receiving best image quality and contrast. With this we gained information on how to understand and interpret the signals and contrasts of the different plaque components in DPC and multicontrast MRI. Second, assessment of sensitivities for different lesion components in DPC and MRI was done retrospectively. For further studies, the morphological appearances of lesion components in DPC or MRI, as listed in Table 3.1, need to be tested for suitability in the detection and differentiation of plaque characteristics in a prospective
and blinded way, including a significant number of plaques to allow statistical analysis. Third, the natural postmortem degradation processes of the endarterectomized plaques have to be taken into account including shrinkage during histological processes. Forth, in spite of technological progress, DPC tomography currently remains an experimental method. Further improvements are necessary to achieve an X-ray source with acceptable radiation doses and acquisition time before applying to humans.

In conclusion, our study demonstrates that DPC tomography can produce remarkable high-resolution images and can discriminate between clinically relevant components of the atherosclerotic vessel wall. Once adequately validated and optimized, DPC tomography might potentially help to define high-risk atherosclerotic plaques. Finally, we want to emphasize the importance of multicontrast imaging since only one contrast cannot differentiate all components. However, using multicontrast MRI will also not allow discriminating each single component, e.g. calcification and iron deposits displayed the same MR signal. More sophisticated diagnostic tools (e.g. new MR protocols) and validation studies will help understanding different contrasts. In this present feasibility study DPC and MRI imaging protocols for human carotid plaques were optimized and validated with a small sample size. This work will help to design and conduct further studies with higher numbers of plaques to obtain quantitative information on plaque morphology at statistical significance.
3.5 EXPERIMENTAL SECTION

3.5.1 Study Design and Experimental Overview

The study was carried out in concordance with the Helsinki declaration. Written informed consent was obtained from a 75-year-old male patient scheduled for carotid endarterectomy. Two carotid specimens, one originating from the *Arteria carotis externa* and the other from the *Arteria carotis communis/interna*, were immediately transferred into Rnalater® (Sigma, St. Louis, USA) solution and stored at 4 °C overnight according to our standardized procedure for plaque harvesting and bio-banking [83]. The next day, tissues were fixed in 4% formalin for 24 h at 4 °C and finally stored in PBS at 4 °C.

First, the two specimens were scanned by DPC tomography at the TOMCAT (Tomographic Microscopy and Coherent Radiology Experiments) beamline of the Swiss Light Source, followed by imaging on a small animal MR system using different MR contrasts. In a second step, both plaques were characterized by histological workup and, finally, the DPC and MR images were matched with the corresponding histological sections.

3.5.2 Differential Phase Contrast Imaging and Three-Dimensional Rendering

The presented data sets were obtained by synchrotron-based X-ray tomography using the differential phase contrast technique [114, 115] at a photon energy of 25 keV. Carotid artery segments were placed in a falcon tube filled with PBS. To measure the phase shift, a grating interferometer was used and series of projection images were taken while the carotid plaque specimens rotated. A 300 μm thick LAG:Ce scintillator converted the X-rays to visible light, which were captured by a CMOS camera (PCO.Edge, PCO AG, Kelheim, Germany). The total scan time for a segment with a field of view (FOV) of 12 mm x 3.5 mm was about 90 min. More details about components and parameters are provided in Table 3.2.

To obtain 3-dimensional images of the DPC data sets, individual Tiff files were converted to a multilayer Tiff file and processed by Imaris software (Bitplane AG, Zurich, Switzerland).
Table 3.2 Components and parameters of the grating interferometer-based DPC experiment

<table>
<thead>
<tr>
<th>Component or Parameter</th>
<th>Specification or Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source</td>
<td>Synchrotron</td>
</tr>
<tr>
<td>Ring current</td>
<td>400 mA</td>
</tr>
<tr>
<td>Beam energy</td>
<td>25 keV</td>
</tr>
<tr>
<td>Monostripe</td>
<td>W/Si</td>
</tr>
<tr>
<td>Field of View</td>
<td>12 mm x 3.5 mm</td>
</tr>
<tr>
<td>Detector</td>
<td>Complementary Metal Oxide Silicon (CMOS)</td>
</tr>
<tr>
<td>Sample Magnification</td>
<td>1.0</td>
</tr>
<tr>
<td>Scintillator</td>
<td>LAG:Ce 300 μm</td>
</tr>
<tr>
<td>Pixel</td>
<td>2048 x 2048</td>
</tr>
<tr>
<td>Pixel size</td>
<td>6.5 x 6.5 μm²</td>
</tr>
<tr>
<td>Grating interferometer</td>
<td>7 phase steps</td>
</tr>
<tr>
<td>Scan settings</td>
<td></td>
</tr>
<tr>
<td>Projections</td>
<td>1441 over 180°</td>
</tr>
<tr>
<td>Number of darks</td>
<td>32</td>
</tr>
<tr>
<td>Number of flats</td>
<td>100</td>
</tr>
<tr>
<td>Angular step</td>
<td>0.125°</td>
</tr>
</tbody>
</table>

3.5.3 Magnetic Resonance Imaging

MRI was performed with a Bruker Pharmascan 7/16 small animal MR system equipped with a gradient system capable of a maximum gradient strength of 760 mT/m, with a 80 μs rise time and a quadrature birdcage resonator. The specimens were placed in a Falcon tube, filled with PBS, inside the volume resonator and kept at 24 °C. Reference data was acquired in coronal and sagittal orientations for accurate positioning of the plaque specimen. Before imaging a fieldmap-based local shimming was performed on the specimen using the automated MAPshim routine to reduce field inhomogeneities. The imaging protocol consisted of different spin echo (SE) sequences using different dimensions of the FOVs, matrix sizes, echo times (TE) and repetition times (TR) as shown in Table 3.3. For all sequences 30 averages were performed.
Table 3.3 MRI sequence parameters

<table>
<thead>
<tr>
<th>Sequence</th>
<th>TE [ms]</th>
<th>TR [ms]</th>
<th>Acquisition time</th>
<th>Geometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>6.5</td>
<td>810</td>
<td>1h 17 min</td>
<td>For T1, T2, PD, MT:</td>
</tr>
<tr>
<td>T2</td>
<td>30</td>
<td>2000</td>
<td>3h 12 min</td>
<td>Slice thickness: 0.5 mm</td>
</tr>
<tr>
<td>Proton density (PD)</td>
<td>13</td>
<td>2000</td>
<td>3h 12 min</td>
<td>No slice gap</td>
</tr>
<tr>
<td>Magnetization transfer (MT&lt;sub&gt;on&lt;/sub&gt;, MT&lt;sub&gt;off&lt;/sub&gt;)</td>
<td>5</td>
<td>750</td>
<td>1h 36 min (each)</td>
<td>FOV: 15 x 15 mm</td>
</tr>
<tr>
<td>Diffusion-weighted (DW)</td>
<td>17.5</td>
<td>2500</td>
<td>4 h</td>
<td>Matrix: 256 x 256</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>In-plane resolution: 59 x 59 μm</td>
</tr>
</tbody>
</table>

To calculate the MTC, images with (MT<sub>on</sub>) and without (MT<sub>off</sub>) the application of a saturation pulse were acquired [120]. For MT offset the frequency and amplitude were optimized and a Gaussian saturation pulse with an offset frequency of 3.5 kHz and amplitude of 30 μT was found optimal. MT subtraction maps were calculated using the equation:

\[ \text{MTC} = \text{MT}_{\text{on}} - \text{MT}_{\text{off}}. \]

For DW imaging, diffusion-encoding was applied (gradient pulse duration = 2.5 ms, gradient pulse separation = 8.1 ms) with a b-value of 650 s/mm².

3.5.4 Histological Processing and Histopathology

After DPC and MRI examinations, the two carotid plaques were paraffin-embedded and serial sections of 2.5 μm were prepared for further histological and immunohistochemical investigations. Sections were routinely stained with hematoxylin and eosin (HE), Masson's trichrome, Elastica van Gieson (VG-Elastica), phosphotungstic acid hematoxylin (PTAH) showing fibrin deposits in lesions, and Prussian blue staining to identify iron-containing hemosiderin from previous hemorrhage. For immunohistochemistry, the monoclonal antibody anti-human alpha smooth muscle cell actin (anti-SMA, 1:400, mouse, M0851, Dako, Baar, Switzerland) was used. The detection system included the Dako RealKit (Dako) on the immunostainer (Dako). All sections were digitized by a slide scanner with a pixel size of 0.221 x 0.221 μm² (Pannoramic 250, 3D Histech, Sysmex, Horgen, Switzerland). Pathological classification of the two plaque types was done in a first instance macroscopically by the surgeon (Z.R.) according to their surface morphology and further investigated on the basis of the modified American Heart Association (AHA)-classifications [105] by a board-certified pathologist (N.B.) using histology and specific staining methods. The experienced pathologist (N.B.) identified vessel layers and plaque components, such as media (M), intima (Int), intimal thickening (IT), fibrous cap (FC), lipid core (LC), necrotic core (NC), inflammatory cell infiltration, hemosiderin deposits, neovascularization, and calcifications.
3.5.5 Interpretation of DPC and MRI Data

The DPC (stable or unstable plaque: 580 slices), MR (stable plaque 25 slices; unstable plaque 40 slices) and histological images (stable plaque 36 slices; unstable plaque 180 slices) were manually matched (TIFF files, Adobe Photoshop CS6 extended, Version 13.0.1) using the known location and distance between DPC images, MR images and histological cross sections. Furthermore, gross morphological features, such as plaque shape, vessel wall thickness and shape as well as calcium deposits were used to optimize the matches. We did not account for shrinkage of the specimen caused by histological processing, as it can vary across the specimen and would require multiple landmarks for accurate matching [137]. The above mentioned plaque components were identified in both imaging modalities using the histological sections as reference. Three independent investigators (R.M., P.S., A.M.H.) analyzed DPC and MR images for plaque signal characteristics in comparison with surrounding structures.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Bernd R. Pinzer and Sabina Wunderlin for technical support. The Scientific Center for Optical and Electron Microscopy (ScopeM) of the ETH Zurich is acknowledged for support. We thank the surgeon Zoran Rancic (Z.R.) from the Clinic for Cardiovascular Surgery, University Hospital Zurich, for the initial macroscopic classification of the plaques. The team of Prof. Philipp A. Kaufmann from the Department of Nuclear Medicine, Cardiac Imaging, University Hospital Zurich, is acknowledged for coordinating the plaque collection.

FUNDING

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4 IMAGING OF THE COSTIMULATORY MOLECULES CD80 AND CD86 WITH INDIUM-111 LABELED BELATACEPT IN ATHEROSCLEROSIS

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AUTHOR CONTRIBUTIONS

R. Meletta planned and coordinated the study, established and performed the chelator conjugation, radiolabeling, stability evaluation, cell assays, immunocytochemistry, autoradiography and mouse experiments, analyzed the data and wrote the manuscript. A. Müller Herde supervised the study, performed autoradiography and biodistribution experiments, was involved in data interpretation and revised the manuscript. P. Dennler established the chelator conjugation and radiolabeling, performed the LC-MS measurements, was involved in the discussion of the results and revised the manuscript. E. Fischer was involved in the study design, data evaluation and discussion of the results and revised the manuscript. R. Schibli contributed to the study design and discussion of the results and revised the manuscript. S. D. Krämer supervised the project and was involved in data interpretation and revision of the manuscript.
4.1 ABSTRACT

PURPOSE

The inflammatory nature of atherosclerosis provides a broad range of potential molecular targets for atherosclerosis imaging. Growing interest is focused on targets related to plaque vulnerability such as the costimulatory molecules CD80 and CD86 expressed by professional antigen-presenting cells (APCs). We investigated in this proof-of-concept study the applicability of the CD80/CD86-binding fusion protein belatacept as a probe for atherosclerosis imaging.

METHODS

Belatacept was labeled with indium-111 and the binding affinity was determined in cell-based assays with CD80/CD86-positive Raji cells. The in vivo distribution and binding to CD80/CD86-positive tissue was determined in Raji xenograft-bearing CD1 nu/nu mice in SPECT/CT scans, biodistribution and ex vivo autoradiography studies. Ex vivo SPECT/CT experiments were performed with aortas and carotids of ApoE KO mice. Accumulation in human carotid atherosclerotic plaques was investigated by in vitro autoradiography.

RESULTS

$^{111}$In-DOTA-belatacept was obtained in a fast and robust radiolabeling procedure in > 70% yield and > 99% radiochemical purity. The specific activity was ~40 GBq/µmol and the stability was > 80% after 72 h in human and murine plasma at 37 °C. The labeled belatacept bound with high affinity to Raji cells. In vivo, $^{111}$In-DOTA-belatacept accumulated specifically in CD80/CD86-positive Raji xenografts, lymph nodes and salivary glands. Ex vivo SPECT experiments revealed displaceable accumulation in atherosclerotic plaques of ApoE KO mice fed a diet enriched with cholesterol and fat. In human atherosclerotic plaques, binding correlated with the infiltration by immune cells and the presence of a large lipid and necrotic core.

CONCLUSION

$^{111}$In-DOTA-belatacept accumulates in CD80/CD86-positive tissues rendering it a research tool with great potential for the assessment of inflammatory activity in atherosclerosis and possibly other conditions with involvement of APCs. Owing to its attractive binding profile, the tracer is suitable for preclinical imaging of costimulatory molecules of both human and murine origin. Radiolabeled belatacept could serve as a benchmark for future CD80/CD86-specific imaging agents.
4.2 INTRODUCTION

The innate and adaptive immune systems interact in a well-orchestrated manner during antigen presentation. In the context of atherosclerosis, professional APCs encounter antigens such as oxidized low-density lipoprotein particles and heat shock proteins [138]. Among APCs, DCs are the primary cell type involved in antigen presentation. After antigen exposure, APCs lose phagocytic action simultaneously gaining antigen-presenting capability by the upregulation of major histocompatibility complex (MHC) receptors and the costimulatory molecules CD80 (B7.1) and CD86 (B7.2) [138]. So far, the best-characterized costimulatory pathway involves CD80 and CD86 expressed by APCs and their corresponding T cell receptors cytotoxic T-lymphocyte protein 4 (CTLA-4, CD152) and CD28. This pathway is a complex interplay of inflammatory and inhibitory molecules controlled by expression patterns restricted to certain cell types and inflammatory stages and additionally differences in affinity [139]. Costimulation is known to be an essential mechanism in a variety of diseases to define T cell response towards activation or tolerance [140, 141].

An association between atherosclerosis and the T cell costimulatory molecules CD80 and CD86 was initially postulated by Buono et al. [142]. In their study, LDLr KO mice lacking CD80 and CD86 displayed a delayed atherosclerosis progression compared to control LDLr KO mice. An involvement of CD80 and CD86 in vascular remodeling processes was presented by Ewing et al. in a femoral artery cuff mouse model [143]. We recently described that the relative mRNA levels of CD80 and CD86 were significantly increased in human vulnerable compared to stable carotid plaques supported by immunohistochemistry data [83]. Our results were in line with a study on human carotid and coronary plaques by Erbel et al. [84]. They observed a higher CD86 mRNA expression in human carotid plaques of symptomatic than asymptomatic patients. Furthermore, immunohistochemical staining revealed that virtually all mature DCs were CD86-positive and in close proximity to activated T cells. Further evidence supporting an involvement of mature APCs in atherosclerotic lesions was presented by an expression analysis in human carotid endarterectomized plaques [59]. A significantly increased mRNA expression of CD11c, CD80, CD83 and CD86 in vulnerable than stable plaques was observed. The detected increase in vulnerable plaques was more pronounced for the mature subpopulation than the total number including immature DCs [59]. Altogether, these studies suggest that advanced atheroma contain a population of fully maturated DCs and macrophages expressing costimulatory molecules and that these APCs could be efficient regulators of T cell activity. In atherosclerosis, data indicate that antigen presentation is not restricted to lymph nodes but it additionally occurs in atherosclerotic plaques as supported by co-localization data of DCs and T cells [84, 85].

The intensively studied CD80/CD86-CD28/CTLA-4 costimulatory axis is of interest in several other diseases with involvement of the immune system, such as cancer, rheumatoid arthritis and transplant rejection. The idea to target CD80 and CD86 by means of a modified form of CTLA-4 in therapy has led to the design of abatacept (CTLA4-Ig, Orencia®, Bristol Myers Squibb) and belatacept (LEA29Y, Nulojix®, Bristol Myers Squibb). These proteins are composed of the
extracellular domain of human CTLA-4 fused to a modified Fc fragment of a human IgG1. The second generation fusion protein belatacept contains two amino acid substitutions in the CTLA-4 domain that resulted in a 10-fold increased inhibition of T cell costimulation in vitro [144]. Surface plasmon resonance measurements revealed high binding affinities of belatacept to human and murine CD80 as well as CD86 [144, 145]. European and American authorities approved belatacept for its use to prevent organ rejection after kidney transplantation in 2011.

Since activated APCs expressing CD80 and CD86 are a primary component of atherosclerotic lesions and the amount of APCs correlates with plaque vulnerability, CD80- and CD86-positive DCs and macrophages present a promising imaging target in atherosclerosis [58, 59, 85]. For non-invasive positron emission tomography (PET) imaging of atherosclerosis, we recently evaluated a small molecule with high-affinity binding to CD80, designated [11C]AM7. The compound bound in a displaceable manner to endarterectomized human carotid plaques and bound to CD80-positive Raji xenografts in vitro [83]. However, the low tissue distribution was a limiting factor in pilot PET experiments with mice [83].

In the present proof-of-concept study, we evaluated the potential of CD80/CD86-specific belatacept for imaging in oncology and atherosclerosis. We labeled belatacept with the long-lived gamma-emitting nuclide indium-111 and characterized its binding affinity to CD80/CD86 and its stability in plasma in vitro. The 111In-labeled probe was further evaluated by in vivo and ex vivo single photon emission computed tomography (SPECT) regarding its accumulation in CD80/CD86-positive tissues in xenograft-bearing mice. Moreover, ex vivo SPECT scans were acquired in a mouse model of atherosclerosis. Finally, the in vitro binding of [111In]DOTA-belatacept to human carotid plaques was evaluated.
4.3 RESULTS

4.3.1 Conjugation, Radiolabeling and Quality Control of $[^{111}\text{In}]$DOTA-belatacept

The bifunctional chelating agent $p$-SCN-Bn-DOTA was successfully conjugated to belatacept under aqueous conditions by reaction with lysine amino groups of the protein according to a published procedure [146]. For all experiments, belatacept with an average of 1.9 DOTA per protein was used as determined by LC-MS. The protein conjugate was then labeled with indium-111 and purified by semi-preparative FPLC. After purification, $[^{111}\text{In}]$DOTA-belatacept was obtained in a radiochemical yield of 73 – 78%, a radiochemical purity of > 99% and a specific activity of 36 – 41 GBq/µmol ($n = 3$).

4.3.2 Stability of $[^{111}\text{In}]$DOTA-belatacept

The stability of $[^{111}\text{In}]$DOTA-belatacept at 37 °C in PBS (pH 7.4), human and murine plasma, respectively, was investigated by FPLC up to 72 h (Figure 4.1). More than 75% of radiolabeled product was stable after 72 h incubation. After 48 h, corresponding to the time of in vivo experimentation, 96% intact product was present in murine plasma. In general, $[^{111}\text{In}]$DOTA-belatacept displayed better stability in plasma than in PBS.

![Figure 4.1](image)

**Figure 4.1** Stability of $[^{111}\text{In}]$DOTA-belatacept in PBS, human and murine plasma analyzed by FPLC up to 72 h. The percentage of intact $[^{111}\text{In}]$DOTA-belatacept at different analysis time points is indicated and was calculated based on AUC values of the intact product ($t_{R1} \sim 9$ min) and degraded protein fragments ($t_{R2} \sim 12$ min).

4.3.3 Binding of $[^{111}\text{In}]$DOTA-belatacept to CD80/CD86-Positive Raji Cells

Immunocytochemistry experiments confirmed the presence of CD80, CD86 and CTLA-4 on the surface and/or in the cytoplasm of Raji cells (Figure 4.2, A), in agreement with previous studies [147, 148]. A dissociation constant ($K_d$) of 17.6 nM for $[^{111}\text{In}]$DOTA-belatacept was determined in a saturation binding assay with CD80/CD86-positive Raji cells (Figure 4.2, B). The binding potential of $[^{111}\text{In}]$DOTA-belatacept to Raji and control NCI-H69 cells was in addition evaluated in Lindmo assays. In Raji cells, the immunoreactivity of $[^{111}\text{In}]$DOTA-belatacept was 28.3% and binding was considered specific as incubation with 0.1 mM unlabeled belatacept resulted in a reduced
radiotracer binding (Figure 4.2, C). Binding to NCI-H69 cells was below 5% and non-specific (data not shown).

Figure 4.2 (A) Representative immunofluorescence microscopy images of Raji cells stained with DAPI (blue) and anti-CD80, anti-CD86 or anti-CTLA-4 antibody (all in green). The control samples were incubated with secondary antibody only. Scale bar 20 µm. (B) Saturation binding assay of [111In]DOTA-belatacept with Raji cells. The calculated Kd value of the specific binding was 17.6 nM. (C) Lindmo binding assay with an increasing Raji cell number and a constant [111In]DOTA-belatacept concentration of 0.5 nM.

4.3.4 Accumulation of [111In]DOTA-belatacept in CD80/CD86-Positive Raji Xenografts in Vivo

The in vivo distribution of [111In]DOTA-belatacept and its accumulation in CD80/CD86-positive tissues was evaluated in Raji xenograft-bearing CD1 nu/nu mice. SPECT/CT scans were acquired 48 h after tracer injection to achieve an optimal signal-to-background ratio. In SPECT/CT scans under baseline conditions, [111In]DOTA-belatacept accumulated in CD80/CD86-positive Raji xenografts located on both shoulders of CD1 nu/nu mice, in axial and inguinal lymph nodes, the salivary glands, the liver and the urinary bladder (Figure 4.3, A). By co-injection of the radiotracer and an excess of unlabeled belatacept, radiotracer accumulation was reduced to background level in the Raji xenografts and the lymph nodes (Figure 4.3, B). The radioactivity was concentrated in the liver and the salivary glands, however for the latter a reduced signal compared to under baseline conditions was noticed.

Ex vivo autoradiography experiments of CD80/CD86-positive Raji and control NCI-H69 xenografts [83] were in agreement with the SPECT/CT data. In autoradiograms, we observed an overall higher radioactivity signal in Raji xenografts under baseline than under blockade conditions with a focal distribution pattern (Figure 4.3, C). The control NCI-H69 xenograft displayed a low
radiotracer accumulation under baseline and blockade conditions indicating a non-specific accumulation of $[^{111}\text{In}]$DOTA-belatacept that was comparable to the signals observed in Raji xenografts under blockade conditions (Figure 4.3, C).

Quantitative radiotracer distribution in xenograft-bearing CD1 nu/nu animals was evaluated in an *ex vivo* biodistribution experiment with three animals each, 48 h after injection of $[^{111}\text{In}]$DOTA-belatacept (baseline) or $[^{111}\text{In}]$DOTA-belatacept together with unlabeled belatacept (blockade) (Figure 4.3, D; Table 4.1). Under baseline conditions, the highest percentage of injected dose per g tissue (% ID/g) was found in the spleen, the axial and inguinal lymph nodes, the liver and the salivary glands, followed by 8.2 ± 1.3% ID/g in Raji xenografts and 7.6 ± 2.3% ID/g in blood (Table 4.1). A significantly reduced radiotracer accumulation under blockade conditions was observed for the Raji xenografts ($p = 0.00572$) and the salivary glands ($p = 0.00189$). Furthermore, a significantly higher baseline radiotracer uptake was determined in Raji than NCI-H69 xenografts.

**Figure 4.3** (A, B) *In vivo* SPECT/CT images of CD1 nu/nu mice bearing Raji xenografts (human) located on both shoulders 48 h p.i. of ~10 MBq $[^{111}\text{In}]$DOTA-belatacept (25 µg) (A) and additionally 500 µg unlabeled belatacept (B) via the tail vein. a. Raji xenografts, b. lymph nodes, c. salivary glands, d. liver, e. urinary bladder. Yellow, maximal, and blue, minimal SPECT signal (0.0001 – 0.0032 kBq/voxel); grey, computed tomography (CT). (C) *Ex vivo* autoradiograms of Raji and NCI-H69 xenografts dissected 48 h p.i. of ~10 MBq $[^{111}\text{In}]$DOTA-belatacept. (D) Biodistribution of $[^{111}\text{In}]$DOTA-
belatacept in CD1 nu/nu mice with subcutaneous Raji (right shoulder) and NCI-H69 (left shoulder) xenografts. Values are mean percentages injected dose per gram tissue (% ID/g). The biodistribution data is in addition shown in Table 4.1. ** p < 0.01.

Table 4.1 Ex vivo biodistribution data [% ID/g tissue] of baseline (n = 3) and blockade (n = 3) CD1 nu/nu mice with subcutaneous Raji and NCI-H69 xenografts. The data is in addition shown graphically in Figure 4.3, D.

<table>
<thead>
<tr>
<th>Organ/tissue</th>
<th>Baseline [% ID/ g]</th>
<th>Blockade [% ID/ g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>37.4 ± 20.0</td>
<td>7.6 ± 2.1</td>
</tr>
<tr>
<td>Lymph nodes (axial, inguinal)</td>
<td>19.7 ± 2.8</td>
<td>12.2 ± 4.4</td>
</tr>
<tr>
<td>Liver</td>
<td>14.9 ± 2.6</td>
<td>15.1 ± 1.5</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>11.3 ± 2.1</td>
<td>2.5 ± 0.2**</td>
</tr>
<tr>
<td>Blood</td>
<td>7.6 ± 2.3</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>Raji xenograft</td>
<td>8.2 ± 1.3</td>
<td>4.1 ± 0.3**</td>
</tr>
<tr>
<td>Kidneys</td>
<td>4.5 ± 0.5</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>Heart</td>
<td>3.5 ± 1.3</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>NCI-H69 xenograft</td>
<td>3.2 ± 0.6</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

** p < 0.01

4.3.5 Accumulation of \[^{111}\text{In}\]DOTA-belatacept in Murine Atherosclerotic Plaques

Radiotracer accumulation in atherosclerotic plaques of mice was evaluated by ex vivo SPECT/CT imaging. Tissues were dissected 48 h after injection of \[^{111}\text{In}\]DOTA-belatacept. In addition to the spontaneously occurring predilection sites of atherosclerosis located in the aortic arch and the descending aorta in ApoE KO mice fed a high fat diet, shear stress-induced atherosclerotic plaques were induced by constriction of the common carotid arteries of two mice, referred to as ApoE KO-cuff mice. In these animals, \[^{111}\text{In}\]DOTA-belatacept accumulated in defined regions of the carotids besides the aortic arch and the descending aorta (Figure 4.4, A). The radiotracer signal co-localized with the lipid staining for atherosclerotic plaques. Radiotracer accumulation was reduced under blockade conditions with an excess of unlabeled belatacept (Figure 4.4, B). Plaque burden was comparable in the baseline and the blockade tissue. Quantification of the SPECT signal revealed a higher radiotracer accumulation in the baseline than the blockade aorta and carotids (Table 4.2).

In the two ApoE KO animals without a cuff, fed a high fat diet, radiotracer accumulation was detected in the aortic arch and the descending aorta (Figure 4.4, C). The SPECT signal co-localized with the lipid staining. Under a standard rodent diet, no \[^{111}\text{In}\]DOTA-belatacept accumulation was observed in ApoE KO (Figure 4.4, D) and C57BL/6 mice (Figure 4.4, E). Oil red o staining revealed for the ApoE KO animals under normal diet a small lipid accumulation limited to the aortic arch, whereas in C57BL/6 animals the lipid staining was negative.
Figure 4.4 Ex vivo SPECT/CT images (upper row) and the corresponding oil red o staining (bottom row) of the aorta and the carotids of ApoE KO-cuff mice (A, B), ApoE KO animals fed a high fat diet (C), ApoE KO animals fed a normal diet (D) and C57BL/6 mice fed a normal diet (E). Animals were injected intravenously with ~10 MBq $[^{111}\text{In}]$DOTA-belatacept (baseline, A, C, D, E) or additionally with an excess of unlabeled belatacept (blockade, B). Color scales for minimal to maximal accumulation. Scale bars 1 mm. ApoE KO-cuff mice have flow-altering devices implanted around their carotids visible as transparent or reddish material fixed with a black suture.

Table 4.2 Ratios of the maximum counts of the aorta and carotids for the baseline and the blockade ApoE KO-cuff mouse (Figure 4.4, A and B).

<table>
<thead>
<tr>
<th>Ratios maximum counts</th>
<th>Aorta and carotids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline-to-background</td>
<td>3.47</td>
</tr>
<tr>
<td>Blockade-to-background</td>
<td>2.29</td>
</tr>
<tr>
<td>Baseline-to-blockade</td>
<td>1.52</td>
</tr>
</tbody>
</table>

4.3.6 Accumulation of $[^{111}\text{In}]$DOTA-belatacept in Human Carotid Plaques

After the encouraging in vivo results with mice, we investigated whether $[^{111}\text{In}]$DOTA-belatacept accumulates in human atherosclerotic plaques and whether the accumulation correlates with characteristics of plaque vulnerability. Radiotracer binding to human carotid plaques was evaluated by in vitro autoradiography. Plaques were classified based on the following equally weighted criteria: Size of the lipid/necrotic core, number of immune cells, cap thickness and cap rupture (Table 4.3). The scale of total scores ranged from 0 to 8. A sample with score 0 was classified as normal artery whereas all samples with a score ≥ 1 were classified as plaques.
Table 4.3 Scoring system of histologically analyzed plaque features and the number of samples per classification category. The classification of the immune cells was performed according to the number of cells present relative to the overall plaque size.

<table>
<thead>
<tr>
<th>Sample features</th>
<th>Score</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid and necrotic core</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>2</td>
</tr>
<tr>
<td>Immune cells</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Few</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Many</td>
<td>2</td>
</tr>
<tr>
<td>Minimum cap thickness</td>
<td>&gt; 200 µm</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt; 200 µm</td>
<td>1</td>
</tr>
<tr>
<td>Representative cap</td>
<td>&gt; 500 µm</td>
<td>0</td>
</tr>
<tr>
<td>thickness</td>
<td>&lt; 500 µm</td>
<td>1</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>Intact</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Possibly ruptured</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Definitely ruptured</td>
<td>2</td>
</tr>
</tbody>
</table>

In total 37 plaque samples were included in this study with a heterogeneous distribution on the scoring spectrum and the majority of samples displaying a score between 0 and 2 (Table 4.4).

Table 4.4 Overview of all samples and their respective score according to Table 4.3. In some cases, no scoring of the sample was possible due to fragmented plaque tissues e.g. no cap discernible hampering the morphological assessment of the plaques (indicated as n.d., non determined).

<table>
<thead>
<tr>
<th>Overall score</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>n.d.</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 4.5 shows the hematoxylin/eosin (HE) staining of four plaque specimens classified with a score of 0, 2, 4 and 7, respectively. The sample with a vulnerability score of 0 displayed an intact and structured endothelium with no enclosed lipids or immune cells (Figure 4.5, I). A small focal accumulation of lipids and immune cells was observed in the sample classified with a score of 2 (Figure 4.5, II, III). The sample classified with a vulnerability score of 4 featured a large lipid and necrotic core covered by a thick fibrous cap (Figure 4.5, IV, V). The plaque was focally infiltrated by
numerous immune cells and clusters of foam cells (Figure 4.5, VI). The sample with the highest score consisted of a large lipid and necrotic core, displayed a heterogeneous plaque composition (Figure 4.5, VII) and the presence of many immune cells (Figure 4.5, IX). The representative and the minimum cap thicknesses were < 500 µm and < 200 µm, respectively (Figure 4.5, VII, VIII).

**Figure 4.5** Hematoxylin/eosin staining of the plaque sections corresponding to the autoradiograms of Figure 4.6. Higher-magnification images show the intact endothelium (I), endothelial thickening (II, III), a homogenous and thick fibrous cap (IV, V), foam cells (VI), a heterogeneous fibrous cap (VII), a thin fibrous cap (VIII) and immune cells (IX). Scale bar lower-magnification 2 mm, higher-magnification 200 µm and VI/IX 50 µm. Lu: lumen.

In the *in vitro* autoradiography experiment, $[^{111}\text{In}]$DOTA-belatacept bound to human carotid plaques and the radiotracer binding was reduced under blockade conditions with excess of unlabeled belatacept (Figure 4.6, A corresponding plaque samples to Figure 4.5). Average radiotracer specific binding to plaques was higher than to normal arteries, though not with statistical significance (Figure 4.6, B). However, the specific binding of $[^{111}\text{In}]$DOTA-belatacept correlated significantly with the score for immune cell infiltration (Figure 4.6, C). Moreover, a significantly higher specific radiotracer binding was observed to plaques featuring a large lipid and necrotic core than plaques with a small core (Figure 4.6, D). Radioactivity accumulation and cap thickness did not correlate. The correlation between the relative specific radioactivity and the total score was weak with a Pearson's $r^2$ of 0.275 (data not shown). In these experiments, unspecific binding to Fc receptors was blocked by addition of human gamma globulin to the autoradiography buffer. The respective signal reduction was 10.9 ± 2.6% ($n = 3$).
**Figure 4.6** (A) *In vitro* autoradiograms of human carotid plaques with different vulnerability scores under baseline ([¹¹¹In]DOTA-belatacept) and blockade condition ([¹¹¹In]DOTA-belatacept with excess unlabeled belatacept) corresponding to Figure 4.5. Scale bar 3 mm. (B-D) Relative specific binding of [¹¹¹In]DOTA-belatacept determined by *in vitro* autoradiography. Samples were classified into normal arteries (n = 9) or plaques (n = 28); no (n = 11), few (n = 18) or many (n = 8) immune cells; no (n = 11), small (n = 17) or large (n = 9) lipid/necrotic core. Ns: non significant, *p < 0.05.*
4.4 DISCUSSION

In this proof-of-concept study, we successfully established a radiolabeling procedure for the CD80/CD86-targeting fusion protein belatacept with indium-111 using $\rho$-SCN-Bn-DOTA as bifunctional chelating agent. After purification, the product was obtained in high radiochemical yield and purity in a fast and robust labeling reaction. Under the applied conditions, approximately two DOTA chelators were coordinated to belatacept which is the intended range to minimize undesired chelator-mediated effects on pharmacokinetics [149]. A high stability of the radiolabeled product was determined in PBS and plasma of murine and human origin, respectively, up to 72 h allowing \textit{in vivo} investigations.

The radiolabeled product bound to Raji cells with a calculated nanomolar $K_d$ value. Efforts to assess the binding affinity to mouse recombinant CD80 by differential scanning fluorimetry were not successful due to similar melting temperatures $T_m$ of the recombinant protein and belatacept. However, the results of this study indicate that the radiolabeled belatacept binds to murine CD80/CD86 with a sufficient affinity for \textit{in vivo} imaging. The contribution of Fc receptor mediated-binding to the total binding of belatacept was negligible as concluded from \textit{in vitro} autoradiography with human tissue and as expected from published data [145].

\textit{In vivo} SPECT/CT scans and \textit{ex vivo} autoradiography experiments indicated that $[^{111}\text{In}]$DOTA-belatacept specifically accumulated in CD80/CD86-positive tissues. Besides Raji xenografts, strong and specific uptake was observed in lymph nodes. Lymph nodes exhibit a resident population of APCs capable of expressing CD80 and CD86 in particular during the process of antigen presentation. Moreover, biodistribution studies revealed the highest radiotracer accumulation in spleen, a peripheral lymphoid organ, and a high specific accumulation in salivary glands of xenograft-bearing mice. This is consistent with the fact that APCs are constituents of normal human and murine salivary glands being involved in immune surveillance [150, 151]. The high non-specific hepatic radiotracer accumulation might be related to the IgG1 Fc part of the fusion protein [152].

In a next step, we evaluated $[^{111}\text{In}]$DOTA-belatacept in an animal model of atherosclerosis. Owing to the proximity of atherosclerosis-prone locations in the vasculature to other tissues of radiotracer accumulation e.g. the liver, the salivary glands and the thymus, SPECT/CT scans of aorta and carotids were performed \textit{ex vivo}. Furthermore, perfusion of the excised tissues allowed to exclude any blood-related tracer radioactivity. $[^{111}\text{In}]$DOTA-belatacept accumulated in atherosclerotic plaques in agreement with lipid staining and tracer accumulation was higher under baseline than blockade condition. Moreover, $[^{111}\text{In}]$DOTA-belatacept accumulation in the aortas of ApoE KO animals depended on an atherosclerosis-promoting diet. In human carotid tissue samples, $[^{111}\text{In}]$DOTA-belatacept binding was associated with the infiltration of immune cells and the presence of a large lipid/necrotic core. It can be assumed that the majority of inflammatory cells within a plaque are APCs with macrophages representing the predominant cell type [8]. The increasing number of macrophages and mature DCs in advanced lesions might promote the
formation of a necrotic core due to a loss of efferocytosis activity, an intensification of the inflammatory response and degradation of matrix components [17, 138, 153].

Limitations of our study are the low number of animals used for in vivo and ex vivo imaging and the lack of control animals to study lymph node accumulation of [111In]DOTA-belatacept in the absence of CD80/CD86-positive xenografts or atherosclerosis. In addition, belatacept binds with similar affinity to CD80 and CD86, which may reduce selectivity for a particular type of cell activation as compared to a CD80- or CD86-selective tracer. Finally, a fusion protein would not fulfill all requirements for clinical application as a PET imaging agent primary due to its prolonged biological half-life of 8 - 9 days in the case of belatacept and concerns with respect to immunogenicity of proteins [154].

Our results suggest that [111In]DOTA-belatacept accumulates specifically in atherosclerotic plaques, depending on their degree of inflammation and vulnerability. The myocardial tracer uptake was low and unspecific in CD80/CD86-positive and -negative xenograft-bearing mice. Together with motion correction this could allow the detection of coronary atherosclerotic plaques by non-invasive imaging. Future studies have to focus on CD80 or CD86-selective small molecules, truncated versions of belatacept as well as abatacept, which has a higher affinity and selectivity for CD80 in mouse than belatacept [145]. In this respect, [111In]DOTA-belatacept could be used as a benchmarking tracer in the preclinical development of novel CD80/CD86 imaging agents.
4.5 CONCLUSIONS

CD80 and CD86 expressed by activated APCs are promising imaging targets in atherosclerosis. In this context, [¹¹¹In]DOTA-belatacept could serve as a research tool to study the approach of targeting APCs not limited to atherosclerosis but also other inflammation-related disorders such as cancer, autoimmune disease and organ rejection after transplantation. A highly expedient characteristic of belatacept is its immunoreactivity with both murine and human CD80/CD86 allowing easy translation between species.
4.6 EXPERIMENTAL SECTION

4.6.1 Conjugation, Radiolabeling and Quality Control

For radiolabeling, all chemicals and solvents were purchased in trace analysis grade from Fluka (Buchs, Switzerland) or Sigma-Aldrich (Germany) and were used without further purification. Commercially available belatacept (Nulojix®, Bristol Myers Squibb) was conjugated to p-SCN-Bn-DOTA (S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetra acetic acid, B205, Macrocyclics) according to a published procedure [146]. Briefly, conjugation of 92.3 µM belatacept was performed under metal-free conditions using a 20-fold molar excess of chelating agent. The reaction mixture was incubated overnight at 4 °C yielding after purification in 1.4 mL protein conjugate solution with a concentration of 5.56 ± 0.02 mg/mL determined by a NanoDrop® ND-1000 spectrophotometer (Witec AG, Littau, Switzerland). Aliquots were stored at -20 °C.

For radiolabeling, each 10 µg protein conjugate was reacted with 4 MBq $^{111}$In (Mallinckrodt, Dublin, Ireland) as determined by incubation of a fixed amount of belatacept-DOTA with increasing amounts of $^{111}$In. The reaction mixture for the radiolabeling contained 300 – 350 µg DOTA-belatacept (175 µL), 0.1 M ammonium acetate buffer (pH 6, 60 µL) and $^{111}$In ([$^{111}$In]Cl in 0.02 N HCl, 213 – 228 MBq, 215 µL, Mallinckrodt). After 1 h incubation at room temperature, the reaction was quenched by the addition of 50 mM EDTA solution (10% (v/v)), mixed and allowed to incubate for 5 min. Semi-preparative purification of the product (500 µL) was performed by size-exclusion FPLC on a Shimadzu SCL-10A VP system with a superose 12 column (GE Healthcare). Isocratic conditions with PBS (pH 7.4) as mobile phase and a flow rate of 0.5 mL/min were applied. Quality control of the purified product was performed under isocratic conditions (0.3 M NaCl, 0.05 M NaH$_2$PO$_4$ x H$_2$O, pH 6.2) with a TSKgel G30000SWxl column (7.8 mm x 30 cm, 5 µm; Tosoh Bioscience LLC). FPLC data were analyzed with the software RadioStar.

4.6.2 LC-MS Analysis

LC-MS analysis was performed on a Waters LCT Premier mass spectrometer. Prior to analysis, conjugated belatacept was deglycosylated using a Protein Deglycosylation Mix (P6039, New England BioLabs Inc.). 2.5 µL 10x G7 reaction buffer and 2.5 µL deglycosylation enzyme cocktail were added to 20 µL of 1.1 mg/mL belatacept in H$_2$O and the mixture was incubated at 37 °C overnight. Twenty-microliters of the deglycosylation mixture were mixed with 20 µL PBS (pH 7.4) and 2 µL 1 M DTT at 37 °C for 30 min to reduce deglycosylated belatacept. Samples were chromatographed on an Aeris WIDEPORE XB-C18 column (3.6 µm, 100 mm x 2.1 mm; Phenomenex) heated to 80 °C using the following gradient: 0 min to 3 min: 5% A, 90% B, 5% C; 3 min to 15 min: 5 to 75% A, 90 to 20% B, 5% C; 15 min to 20 min: 75 to 90% A, 20 to 5% B, 5% C (solvent A: acetonitrile + 0.1% formic acid, solvent B: water + 0.1% formic acid, solvent C: isopropanol) at a flow rate of 0.5 mL/min. The eluent was ionized using an electrospray source (ESI+). Data were collected with MassLynxV4.1 and deconvolution was performed using MaxEnt1. Payload ratios were calculated based on mass peak intensity.
4.6.3 Stability of [\(^{111}\text{In}\)]\text{DOTA}\)-belatacept

The in vitro stability of [\(^{111}\text{In}\)]\text{DOTA}\)-belatacept was evaluated in PBS (pH 7.4), human and murine (male NMRI nu/nu mice) blood plasma, respectively. Approximately 1.4 MBq radiotracer (20 µL, 76.5 nM) was added to 500 µL PBS or plasma and the samples were incubated at 37 °C. Fifty-microliter samples were analyzed after 0, 24, 48 and 72 h by size-exclusion FPLC on a SCL-10A VP system (Shimadzu) equipped with a UV/Vis and a radiodetector. Chromatographic analysis corresponded with the conditions applied for quality control of the radiolabeled product.

4.6.4 Cell Culture

Raji (human Burkitt’s lymphoma cell line, ATCC CCL-86) and NCI-H69 cells (human lung small cell carcinoma cell line, ATCC HTB-119) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Raji cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin. NCI-H69 cells were cultured in RPMI 1640 medium with GlutaMAX and 25 mM HEPES (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, penicillin-streptomycin and 1 mM sodium pyruvate. Cells were cultured in suspension at 37 °C in a humidified atmosphere containing 5% CO\(_2\). Routine culture treatment was performed twice a week.

4.6.5 Immunocytochemistry

For immunocytochemistry, the following primary antibodies were used: anti-CD80 1:100 (2A2, ab86473, Abcam, Cambridge, UK), anti-CD86 1:200 (EP1158Y, ab53004, Abcam, Cambridge, UK) and anti-CTLA-4 1:100 (MO6, clone 2F1, H00001493-M06, Abnova, Taipei, Taiwan). Additionally the following secondary antibodies were used: goat anti-rabbit IgG H&L 1:1000 (FITC, ab6717, Abcam, Cambridge, UK) and goat anti-mouse IgG H&L 1:1000 (FITC, ab6785, Abcam, Cambridge, UK). All incubation steps were performed at room temperature unless otherwise stated. Raji cells were seeded on 6-well tissue culture plates on the day of experiment and each well contained a disinfected coverslip. The cell culture media was carefully removed and cells were fixated by incubation with 4% paraformaldehyde in PBS (pH 7.4) for 30 min. Thereafter, cells were permeabilized by incubation with methanol (10 min) and blocked with 2% bovine serum albumin (BSA) in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.4) for 30 min. The primary antibody was diluted to the appropriate concentration in 2% BSA/TBS and cells were incubated at 4 °C overnight. The next day, cells were washed in TBS (3x, 10 min) and the secondary antibody was added in 2% BSA/TBS. The samples were incubated for 1 h and kept from that point on in dark. After washing in TBS (3x, 10 min), 4',6-diamidino-2-phenylindole (DAPI) (1:5000) was added for 4 min to stain the nuclei of the cells. After three more washing steps with TBS (10 min each), the coverslips were mounted onto slides using ProLong® Gold antifade reagent (Life Technologies, Carlsbad, CA, USA) with cell-side facing down. For negative control, only secondary antibody was added to the cells. Slides were examined using an Axioskop 2 microscope equipped with 20/0.50 Plan-Neofluar,
40/1.3 oil Plan-Neofluar, and 63/1.25 oil Plan-Neofluar objectives and an AxioCam digital camera (Carl Zeiss AG, Feldbach, Switzerland). Images were acquired using AxioVision software (Carl Zeiss AG).

4.6.6 Binding Studies

Saturation binding and Lindmo assays with $[^{111}\text{In}]$DOTA-belatacept were performed with Raji and NCI-H69 cells. For the saturation binding assay, the cells (approx. $8.5 \times 10^6$ cells per tube) were washed twice with PBS and incubated for 90 min on ice with increasing concentrations of radiotracer (0.03 – 330 nM) in triplicates. The total volume per tube was adjusted with RPMI medium to 200 µL. Non-specific binding was determined by adding 100 µM unlabeled belatacept as displacer. After 2 h incubation, the cells were washed three times with 2 mL ice-cold PBS (pH 7.4) and centrifuged at 1260 x g for 5 min at 4 °C (Sorvall RC-5C centrifuge, Thermo Fisher). Samples were analyzed in a $\gamma$-counter (Packard Cobra II Auto Gamma, PerkinElmer). The dissociation constant ($K_d$) was calculated by fitting the specific radiotracer binding with non-linear regression analysis assuming a 1:1 binding (GraphPad Prism Software). In the Lindmo assay a fixed amount of $[^{111}\text{In}]$DOTA-belatacept (0.5 nM) was incubated with a standard dilution (dilution factor 2) of Raji and NCI-H69 cells, respectively, in RPMI medium. The highest cell concentration for the Raji cells was approximately $2.9 \times 10^7$ cells/tube and for the NCI-H69 approximately $1.6 \times 10^7$ cells/tube. The total volume per tube was adjusted with RPMI medium to 200 µL. To assess the non-specific binding, 100 µM unlabeled belatacept was used as displacer. The samples were incubated for 2 h at 4 °C, thereafter washed three times with 2 mL ice-cold PBS and centrifuged at 1260 x g for 5 min at 4 °C. Samples were analyzed in a $\gamma$-counter (Packard Cobra II Auto Gamma, PerkinElmer). Specific binding was calculated by subtracting the non-specific binding ($[^{111}\text{In}]$DOTA-belatacept and excess unlabeled belatacept) from the total binding ($[^{111}\text{In}]$DOTA-belatacept only).

4.6.7 In Vivo Studies with Xenograft-Bearing Mice

All animal experiments were approved by the Veterinary Office of the Canton Zurich (Switzerland) and were in accordance with the Swiss law of animal protection. Five-week old female CD1 nu/nu mice (Crl: CD1-Foxn1nu) were purchased from Charles River Laboratories (Sulzfeld, Germany) and were fed a normal chow diet ad libitum. At the age of six weeks, immune-deficient CD1 nu/nu mice were subcutaneously inoculated in their shoulders with $1 \times 10^7$ Raji cells in 100 µL Matrigel (BD Biosciences, Oxford, UK) (4 animals both sides, 6 animals right side). $1 \times 10^7$ NCI-H69 cells in 100 µL Matrigel were subcutaneously inoculated in the shoulders of CD1 nu/nu mice two weeks afterwards (2 animals both sides, 6 animals with Raji xenografts, see above, opposite side). In vivo SPECT/CT scans, ex vivo autoradiography and biodistribution experiments were performed two weeks after the second inoculation with in total twelve mice. Animals were injected intravenously with 10 MBq $[^{111}\text{In}]$DOTA-belatacept (25 µg, baseline) or 10 MBq $[^{111}\text{In}]$DOTA-belatacept and 500 µg unlabeled belatacept (blockade). In vivo and ex vivo experiments were performed 48 h after tracer injection.
4.6.8 In Vivo Studies with ApoE KO and C57BL/6 Mice

Eleven-week old male C57BL/6 mice and four-week old male ApoE KO mice (B6.129P2-Apoetm1Unc/J) were ordered from Charles River Laboratories (Sulzfeld, Germany). Two C57BL/6 mice and two ApoE KO mice were fed a normal chow diet ad libitum. All other ApoE KO mice (n = 4) received a modified Western type diet containing 21% fat, 0.25% cholesterol and 19.5% casein (Kliba Nafag, Kaiseraugst, Switzerland) ad libitum. In two ApoE KO animals fed a modified Western type diet, a flow constrictive device was placed around the right common carotid artery and a non-constrictive control around the contralateral side according to previous publications [100, 102] (Chapter 6). Mice were used for ex vivo SPECT/CT experiments 19 weeks after this surgical intervention. 10 MBq [111In]DOTA-belatacept (25 µg, baseline) or 10 MBq [111In]DOTA-belatacept and 500 µg unlabeled belatacept (blockade) was injected into the tail vein of the animals. Mice were euthanized 48 h after tracer injection for ex vivo SPECT/CT imaging and oil red o staining of the excised aorta and carotids.

4.6.9 SPECT/CT Imaging

In vivo and ex vivo SPECT/CT scans were conducted with a four-head multiplexing multipinhole camera (NanoSPECT/CT, Bioscan Inc. Washington, DC, USA). Images were acquired with the Nucline Software (Bioscan Inc., Poway, CA, USA). The acquisition time per view depended on the amount of radioactivity at scan start in the field of view and the resulting scan times ranged from 20 min to 1 h (in vivo scans) and 14.5 h to 15.5 h (ex vivo scans). In vivo scans were performed 48 h after tracer injection. For ex vivo studies, animals were euthanized 48 h after tracer injection and SPECT/CT images were acquired after sample preparation. For in vivo SPECT/CT scans, animals were anesthetized with a mixture of isoflurane (1.5 – 4%) and oxygen. SPECT data were reconstructed with HiSPECT software (Scivis GmbH, Göttingen, Germany). The fused SPECT and CT data were analyzed using VivoQuant image post-processing software (inviCRO Imaging Services and Softwares, Boston, USA) and PMOD biomedical image quantification software (PMOD Technologies Ltd., Zurich, Switzerland). Volumes of interest (VOIs, ~1 cm³) were drawn manually to include aorta and carotids. A VOI of equal size was drawn outside the tissues to define background radioactivity. Tissue-to-background ratios were calculated from the averaged 5 voxels with highest radioactivity per VOI.

4.6.10 Biodistribution Experiments

Post-mortem biodistribution studies with inoculated CD1 nu/nu mice (Raji xenograft right and NCI-H69 xenograft left shoulder) were performed with three baseline animals and three blockade animals. Animals were euthanized with CO₂ followed by cervical dislocation. Tissues, organs and a defined volume of original injectate were weighed and counted for radioactivity in a γ-counter (Packard Cobra II Auto Gamma, PerkinElmer). Accumulated tissue radioactivity was expressed as % ID/g.
4.6.11 *Ex Vivo* Autoradiography

For *ex vivo* autoradiographic studies, CD1 nu/nu mice bearing either Raji or NCI-H69 xenografts on both shoulders were injected with ~10 MBq tracer and euthanized after 48 h as described above. Tumors were dissected, embedded in Tissue-Tek O.C.T. medium, frozen at -80 °C and sections of 5 µm were prepared using a cryostat. After drying, frozen sections were exposed to super resolution imager plates (PerkinElmer, Waltham, MA, USA) for 5 min and analyzed by a Cyclone® Plus Storage Phosphor System (PerkinElmer). Data evaluation was performed with the software OptiQuant (5.0, PerkinElmer).

4.6.12 Oil Red O Staining

Lipid staining of the aorta and the carotids was accomplished with the dye oil red o at room temperature according to a previously published procedure [155]. In brief, the tissue was fixated in 4% PFA for 10 min, thereafter washed twice with PBS (pH 7.4) and preincubated in 60% 2-propanol for 5 min. Tissues were stained with 0.3% (w/v) oil red o (Sigma-Aldrich) in 60% 2-propanol for 30 min on a shaking platform. Finally, the tissue was washed under running tap water for 2 min. Images of the stained tissues were obtained with a Nikon SMZ1000 microscope equipped with a Nikon 1 J3 digital camera (Nikon, Melville, NY, USA).

4.6.13 Human Atherosclerotic Carotid Plaques

Atherosclerotic carotid plaques were obtained from patients undergoing carotid endarterectomy (CEA) at the University Hospital of Zurich. Written informed consent was obtained from all patients prior to surgery. Plaque material was collected from the common, external and internal carotid artery. Twenty three patients were included in this study with an average age of 75.4 ± 6.8 years and 87% of male gender. Plaque specimens were stored in RNAlater® solution (Sigma-Aldrich, St. Louis, USA) at -80 °C. After thawing, plaques were embedded in Tissue-Tek O.C.T. medium and cryosections (20 µm) were prepared with a cryostat. Frozen sections were stored at -20 °C until further use.

4.6.14 *In Vitro* Autoradiography

Radiotracer binding was evaluated by *in vitro* autoradiography with frozen sections of human carotid plaques (20 µm). Cryosections were thawed at room temperature for 30 min and then preincubated for 10 min in HEPES buffer (50 mM HEPES, 5 mM MgCl₂, 125 mM NaCl, 1 mM CaCl₂, pH 7.4) supplemented with 0.5% milk powder and 40 µM gamma norm (Octapharma AG, Lachen, Switzerland) on ice. Gamma norm contains human immunoglobulins that block unspecific binding of belatacept to ubiquitous Fc receptors. Tissues were incubated with 17 nM [¹¹¹In]DOTA-belatacept (specific activity 36.6 GBq/µM) diluted in the above specified buffer for 1 h on ice. For blockade conditions, additionally 10 µM unlabeled belatacept was added to the tracer solution. To minimize non-specific binding, a washing procedure starting with HEPES buffer containing milk powder and gamma norm (5 min, 4 °C), 3x HEPES buffer (5 min, 4 °C) and finally 2x distilled water
(10 sec, 4 °C) was performed. The sections were dried at room temperature. For quantification and normalization of the radiotracer binding, a standard dilution of the tracer solution was prepared on filter papers. The sections and the standard dilution were exposed to a super resolution imager plate (PerkinElmer) for 30 min and 14 h. The plate was scanned by a Cyclone® Plus Storage Phosphor System (PerkinElmer) and data analysis was performed with the software OptiQuant (5.0, PerkinElmer). Integrated radioactivity signals per area (DLU/mm²) were obtained and specific binding was calculated. The specific binding was normalized to the radiotracer standards. Hematoxylin and eosin (HE) staining of all sections included in autoradiography experiments was performed to investigate tissue morphology and score the plaques according to a scoring system (Table 4.3). In total 37 carotid specimens were used for autoradiography experiments thereof 9 classified as normal arteries and 28 as plaques. The distribution of the plaques per classification category is summarized in Table 4.4.

4.6.15 Statistics

Statistical data analysis was performed with GraphPad Prism (GraphPad, La Jolla, CA, USA) or Microsoft Excel. To assess the intergroup difference of two groups, a two-tailed unpaired student's t-test was performed. For more than two groups, data was analyzed with a one-way ANOVA with a Tukey's multicomparison test. A p-value < 0.05 was considered significant.

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5 THE COSTIMULATORY MOLECULE CD80 IN LYMPH NODES OF ANATHEROSCLEROSIS MOUSE MODEL AND CD80 IMAGING WITH A FLUORINE-18 LABELED RADIOTRACER CANDIDATE

This chapter will be submitted in a modified version to Molecular Pharmaceuticals. Binding affinity and logD data will be published by A. Chiotellis et al. (manuscript in preparation).

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AUTHOR CONTRIBUTIONS

R. Meletta planned and coordinated the study, performed in vitro tracer evaluation and autoradiography experiments, in vivo PET/CT scans, biodistribution, qPCR and FACS analysis, data analysis and interpretation, and wrote the manuscript. A. Müller Herde supervised the study, performed in vitro tracer evaluation, autoradiography, in vivo PET/CT scans, biodistribution, and was involved in data evaluation and interpretation. A. Chiotellis produced [¹⁸F]AC74 and performed chromatographic analyses for stability evaluation. L. Steier performed in vitro autoradiography studies. E. Russo carried out the FACS experiment and data analysis. C. Halin was involved in the planning of the FACS experiment and data interpretation. R. Schibli contributed to result discussion. S. M. Ametamey was involved in the design of [¹⁸F]AC74, supervised the (radio)chemistry and contributed to results discussion. S. D. Krämer supervised the study, was involved in data evaluation and interpretation, and revised the manuscript.
5.1 ABSTRACT

Given the involvement of the costimulatory molecule CD80 in diverse pathological conditions such as atherosclerosis, cancer and autoimmune disorders, a diagnostic PET tracer allowing the monitoring of CD80 levels in vivo would be desirable. In this respect, the novel CD80-targeting radiotracer candidate $^{18}$FAC74, based on an oxodihydropyrazolocinnoline lead structure, was evaluated in vitro and in vivo in mice and was compared to the original tracer $^{11}$CAM7.

Physicochemical and pharmacokinetic compound characteristics were investigated in cellular and other in vitro systems as well as in mice. Radiotracer binding to human atherosclerotic plaque material classified based on the criteria of immune cell infiltration, the size of the lipid/necrotic core, cap thickness and rupture was determined by in vitro autoradiography. Moreover, the in vivo radiotracer accumulation in lymph nodes (LNs) and the systemic distribution pattern in mice was investigated by PET/CT imaging and ex vivo biodistribution. Additionally, CD80 expression levels in LNs were measured by qPCR and FACS analysis.

$^{18}$FAC74 displayed good plasma and metabolic stability and moderate lipophilicity. In vitro, blood cell binding was below 11%, whereas binding to plasma proteins was extensive. $^{18}$FAC74 was rapidly cleared from blood by hepatobiliary excretion with a blood half-life of below 3 min. AC74 and AM7 displayed nanomolar IC$_{50}$ values to human recombinant CD80, whereas the compounds did not bind to murine recombinant CD80. By in vitro autoradiography, a correlation of $^{18}$FAC74 binding with the presence of immune cells as well as the size of the lipid/necrotic core was observed in human carotid plaque samples. In vivo, the accumulation in LNs after paravenous administration was detected only for CD80-specific radiotracers independent of the mouse strain used, the diet or the surgery status. Ex vivo biodistribution showed a significantly reduced tracer accumulation in mediastinal LNs under blockade condition indicating specificity. No difference in CD80 expression was observed on mRNA and protein level in different LN types.

In conclusion, $^{18}$FAC74 bound to human carotid plaques and binding was associated with the presence of immune cells and the size of the lipid/necrotic core. Moreover, $^{18}$FAC74 and $^{11}$CAM7 accumulated in LNs in vivo after intraperitoneal administration potentially due to a passive enrichment in LNs facilitating a low affinity binding to murine CD80. The structural modifications of $^{11}$CAM7 resulting in $^{18}$FAC74 did not improve pharmacokinetics and therefore the lead structure needs to be further optimized towards higher specificity and slower elimination while maintaining high binding affinity.
5.2 INTRODUCTION

T cell costimulation is an essential immunologic process defining the T cell response towards immune activation or tolerance. The best-characterized costimulatory and coinhibitory ligand-receptor complexes involve the costimulatory molecules CD80 and CD86 and their receptors CD28 and cytotoxic T-lymphocyte activation protein 4 (CTLA-4, CD152). CD80 (B7.1) and CD86 (B7.2) are membrane-bound glycoproteins expressed by professional antigen-presenting cells (APCs) such as macrophages, dendritic cells (DCs) and activated B cells. They can additionally be induced in other cell types. These costimulatory molecules interact in the process of antigen presentation after APC activation with their corresponding T cell receptors CD28 and CTLA-4. The expression of these surface molecules and their interaction is strictly regulated by the cellular activation state, the spatiotemporal arrangement and differences in affinity allowing a situation-adapted, organized and dynamic regulation of immune responses [139]. The basal state in APCs is characterized by a low constitutional expression of CD86. After activation, CD86 and with delay additionally CD80 levels are increased. On T cells, CD28 is constitutively expressed by the majority of naïve CD4- and CD8-positive T cells providing a costimulatory signal by ligation to CD80 and CD86. CTLA-4 is induced following T cell activation and delivers a coinhibitory signal [156]. The binding affinity between CD80 and CTLA-4 is approximately 10-fold higher than the interaction between CD80 and CD28, mainly defined by the periodic arrangement of both homodimeric receptors in the immune synapse [157, 158]. After activation, the expression levels of these APC and T cell receptors remain dynamic mediated by endocytosis, trans-endocytosis and intracellular signaling mechanisms [82, 159].

Minor changes in this complex and highly regulated interplay cause eventually amplified effects and can induce an unbalanced immune response. The CD80/CD86-CD28/CTLA-4 pathway is linked to numerous pathologic conditions of the immune system including atherosclerosis, carcinogenesis [160, 161], rheumatoid arthritis [162] and transplant rejection [163]. Thereby, the level of CD80 expression can lead to beneficial or detrimental consequences since the high affinity interaction with CTLA-4 attenuates immune responses whereas ligation of CD28 sustains inflammatory processes. In atherosclerosis, plaques contain a large population of fully maturated APCs capable of interacting with T cells [58]. Moreover, data indicate that antigen presentation occurs in advanced atherosclerotic plaques in addition to lymph nodes as common site of T cell activation [85]. Recent evidence and our own findings suggest an association of CD80 expression with plaque vulnerability [59, 83].

Several promising attempts to block or modulate this costimulatory process have been presented by means of low molecular weight ligands targeting CD80 [164-168]. Key requirements for a small molecule to achieve a disruption of the protein-protein interaction are a high specificity and potency. Huxley et al. presented a small library of compounds binding with low nanomolar affinity to human CD80 determined in surface plasmon resonance measurements [166]. Moreover, these compounds interrupted T cell costimulation in cell-based assays. In a previous study, we radiolabeled one of these compounds exhibiting a $K_d$ value of 3.1 nM with carbon-11, denoted as
\[^{11}\text{C}]\text{AM7}\), for non-invasive PET imaging \([83]\). Subsequently we evaluated this radiotracer with respect to physicochemical and pharmacokinetic characteristics \([83]\). \[^{11}\text{C}]\text{AM7}\) displayed a sufficient chemical and metabolic stability and bound \textit{in vitro} to CD80-expressing tissue. \textit{By in vivo}\ PET/CT imaging of mice bearing CD80-positive Raji xenografts, we determined a low radiotracer accumulation and \[^{11}\text{C}]\text{AM7}\) was rapidly excreted via the hepatobiliary route. Tissue distribution of this tracer was negligible due to the rapid clearance in combination with a high plasma protein binding \([83]\). To optimize these inadequate \textit{in vivo} compound properties, the second-generation tracer \[^{18}\text{F}]\text{AC74}\) was developed (A. Chiotellis \textit{et al.}, manuscript in preparation). Therefore, the lead structure of AM7 was modified aiming at altered physicochemical characteristics and the amenability for fluorine-18 labeling. The latter has the advantage of the longer half-life compared to carbon-11 and a better suitability not only for a preclinical but also for a potential clinical application.

The purpose of this study was (i) the \textit{in vitro} evaluation of \[^{18}\text{F}]\text{AC74}\) with regard to its binding affinity to recombinant human as well as murine CD80, stability, lipophilicity, plasma protein and blood cell binding. Furthermore, the \textit{in vivo} metabolic stability and blood half-life was determined in mice. Since atherosclerotic plaque imaging is a potential application field of a CD80-specific radiotracer candidate, (ii) binding of \[^{18}\text{F}]\text{AC74}\) to human atherosclerotic carotid plaques was investigated \textit{in vitro}. In addition, (iii) LN accumulation of \[^{18}\text{F}]\text{AC74}\) was evaluated in different mouse strains by PET/CT imaging and \textit{ex vivo} biodistribution studies and CD80 expression in tissues of interest was analyzed on mRNA and protein level.
5.3 RESULTS

5.3.1 Physicochemical and Pharmacokinetic Evaluation of \([^{18}\text{F}]\text{AC74}\)

5.3.1.1 In Vitro Characterization of \([^{18}\text{F}]\text{AC74}\)

Stability of \([^{18}\text{F}]\text{AC74}\) in PBS (pH 7.4), human and murine plasma, respectively, was analyzed by radio-UPLC up to 4 h. Within this timeframe, no degradation products of \([^{18}\text{F}]\text{AC74}\) were observed in any of the samples.

The lipophilicity of \([^{18}\text{F}]\text{AC74}\) was determined by the shake flask method at physiological pH in a 1-octanol/buffer system. A \(\log D_{\text{pH7.4}}\) value of 0.903 ± 0.044 was obtained.

The radiotracer fraction of \([^{18}\text{F}]\text{AC74}\) binding to blood cells was analyzed in whole blood of human and murine origin, respectively. The equilibrium in whole blood was rapidly reached resulting in a stable unbound fraction of \([^{18}\text{F}]\text{AC74}\) throughout the analysis period of 2 h with no significant difference between the two blood types used. The fraction of \([^{18}\text{F}]\text{AC74}\) recovered in human plasma was 0.898 ± 0.027 and in murine plasma 0.892 ± 0.022.

The in vitro plasma protein binding of \([^{18}\text{F}]\text{AC74}\) in human and murine plasma, respectively, was determined by equilibrium dialysis. The unbound radiotracer fraction (\(f_u\)) in human plasma was 0.0091 ± 0.0003 and in murine plasma 0.0122 ± 0.0009.

5.3.1.2 In Vitro Competition Binding Assay

The half-maximal inhibitory concentration (IC\(_{50}\)) of AC74 and AM7 to human and murine recombinant CD80, respectively, was investigated in a displacement binding assay using the radiotracer \([^3\text{H}]\text{AM7}\). The IC\(_{50}\) values of AC74 and AM7 determined for human CD80 were in the nanomolar range with AM7 displaying a 20-fold lower IC\(_{50}\) value than AC74 (Table 5.1). No specific binding of \([^3\text{H}]\text{AM7}\) was observed with recombinant murine CD80 (Table 5.1).

The IC\(_{50}\) of \([^{11}\text{C}]\text{AM7}\) determined in Raji (human Burkitt’s lymphoma) or Jurkat (human T lymphocyte) cells was 6.3 – 7.3 µM.

\[\begin{array}{|l|c|c|}
\hline
\text{Compound} & \text{Calculated IC}_{50} \text{ values [nM]} \\
\hline
AC74 & 40.1 ± 20.9 & \text{n.d.} \\
AM7 & 2.0 ± 0.2 & \text{n.d.} \\
\hline
\end{array}\]

*Table 5.1* Calculated IC\(_{50}\) values of AC74 and AM7 to human and murine recombinant CD80 determined in a displacement assay with the radiotracer \([^3\text{H}]\text{AM7}\).
5.3.1.3 *In Vivo* Characterization of $[^{18}\text{F}]\text{AC74}$ in Mice

To evaluate the metabolic stability of $[^{18}\text{F}]\text{AC74}$ in C57BL/6 mice, animals were sacrificed 30 min and 60 min post injection (p.i.), respectively. Blood, urine and liver tissues were collected and after sample preparation analysed by radio-UPLC and TLC. At 30 min p.i., the fraction of parent tracer to total radioactivity in plasma was 73.9%. One hydrophilic metabolite was detected. Two more polar metabolites were determined in urine samples. At 60 min p.i., 10% of the total radioactivity was attributable to metabolites in urine, whereas no radiometabolites were detected in liver.

The half-life of $[^{18}\text{F}]\text{AC74}$ in blood after intravenous injection was determined by dynamic PET/CT scans using exponential fits of blood radioactivity derived from the heart of CD1 nu/nu mice. The blood half-life of $[^{18}\text{F}]\text{AC74}$ was on average 2.77 min.

5.3.2 $[^{18}\text{F}]\text{AC74}$ for Atherosclerosis Imaging: *in Vitro* Autoradiography Study with Human Carotid Plaques

As $[^{18}\text{F}]\text{AC74}$ unambiguously bound to human recombinant CD80, we first tested its accumulation in human CD80-positive atherosclerotic tissue. Radiotracer binding to human carotid plaques was evaluated by *in vitro* autoradiography. All samples used for autoradiography studies were stained with hematoxylin/eosin (HE) and were histologically classified according to the scoring system of Table 5.2. Plaque samples were classified based on the size of the lipid/necrotic core, the number of immune cells, cap thickness and fibrous cap rupture. All these criteria were equally weighted resulting in a score per sample from 0 to 8. Samples with a score $\geq 1$ were classified as plaques whereas normal arteries had a score of zero.

**Table 5.2** Scoring system of histologically analyzed plaque features and the number of samples per classification category. The classification of the immune cells was performed according to the number of cells present relative to the overall plaque size. The lipid/necrotic core of one fragmented sample was not classified.

<table>
<thead>
<tr>
<th>Sample features</th>
<th>Score</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid and necrotic core</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>2</td>
</tr>
<tr>
<td>Immune cells</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Few</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Many</td>
<td>2</td>
</tr>
<tr>
<td>Minimum cap thickness</td>
<td>$&gt; 200 \mu\text{m}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$&lt; 200 \mu\text{m}$</td>
<td>1</td>
</tr>
<tr>
<td>Representative cap</td>
<td>$&gt; 500 \mu\text{m}$</td>
<td>0</td>
</tr>
<tr>
<td>thickness</td>
<td>$&lt; 500 \mu\text{m}$</td>
<td>1</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>Intact</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Possibly ruptured</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Definitely ruptured</td>
<td>2</td>
</tr>
</tbody>
</table>
In total, 39 samples were included in this study with a homogeneous distribution based on the scoring system (Table 5.3).

**Table 5.3** Overview of all samples and their respective score according to Table 5.2. In some cases, no scoring of the sample was possible due to fragmented plaque tissues e.g. no cap present hampering the morphological assessment of the plaques (indicated as n.d., non determined).

<table>
<thead>
<tr>
<th>Overall score</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>n.d.</td>
<td>7</td>
</tr>
</tbody>
</table>

By *in vitro* autoradiography under baseline conditions, an increasing trend in maximal binding of $[^{18}F]AC74$ was observed with increasing vulnerability score (Figure 5.1, A). Regions of high tracer accumulation were located within the core of the plaque. A reduced radiotracer binding was observed under blockade conditions by addition of an excess of unlabeled compound.

Signals in the autoradiograms were quantified for further detailed analyses (Figure 5.1, B, C). The normalized specific binding displayed a weak correlation with the vulnerability score of the samples (Figure 5.1, B) and was significantly higher in plaques than normal artery segments (Figure 5.1, C). Furthermore, an association of the radiotracer binding and the number of immune cells within the plaque and the size of the lipid/necrotic core was observed (Figure 5.1, C). The specific binding was significantly higher in plaques containing few immune cells than in plaques devoid of immune cells. However, no significant difference was determined for plaques with many immune cells compared to lesions with no immune cells. Moreover, a significant difference in specific tracer binding was determined between an absent and a large lipid/necrotic core.
Figure 5.1 (A) Representative autoradiograms of human carotid plaques with different vulnerability scores under baseline or blockade condition. Hematoxylin/eosin (HE) staining displays plaque morphology. Scale bar 2 mm. (B/C) Specific binding of $[^{18}F]$AC74 determined by *in vitro* autoradiography. (B) $[^{18}F]$AC74 binding plotted versus the vulnerability score. (C) Specific binding of $[^{18}F]$AC74 in normal arteries ($n = 7$) or plaques ($n = 32$); in plaques with no ($n = 10$), few ($n = 17$) or many ($n = 12$) immune cells; in plaques with no ($n = 11$), a small ($n = 10$) or a large ($n = 17$) lipid/necrotic core; * $p < 0.05$.

5.3.3 Accumulation of $[^{18}F]$AC74 in Lymph Nodes *in Vivo*

5.3.3.1 Radiotracer Accumulation in an Axillary Lymph Node close to a Raji Xenograft after Paravenous $[^{18}F]$AC74 Administration

In a next step, we evaluated the tracer *in vivo* with a xenograft expressing human CD80. Radiotracer distribution and accumulation in CD80-positive tissue was evaluated by PET imaging in Raji xenograft-bearing CD1 nu/nu mice. In this mouse model, we successfully tested the $^{111}$In-labeled fusion protein belatacept revealing a specific accumulation of this tracer in CD80/CD86-positive xenografts and LNs (Chapter 4).
After administration of [18F]AC74, radioactivity accumulated in the liver and intestines with a generally low background activity. Negligible [18F]AC74 accumulation in hCD80-positive Raji xenografts was observed (Figure 5.2, A, B). However, in one animal injected with [18F]AC74, a high radioactivity accumulation was localized in a LN close to a Raji xenograft on the shoulder of the mouse (Figure 5.2, A, arrow). This effect was only observed after paravenous and not after intravenous radiotracer application. Time activity curves (TACs) of this mouse indicated a high SUV value in the LN up to approximately 20 min, thereafter decreasing exponentially (Figure 5.2, B).

![Figure 5.2](image-url) (A) PET/CT images of [18F]AC74 in a Raji xenograft-bearing mouse. Arrows point at the lymph node. Raji xenograft is encircled. PET images are averaged 0 – 21 min, PET in color, CT in white/gray. (B) Time activity curves for the liver, kidney, lymph node, Raji xenograft and background (muscle) of the same animal as in panel (A).

5.3.3.2 Radiotracer Accumulation in Mediastinal Lymph Nodes of ApoE KO and C57BL/6 Mice after Intraperitoneal [18F]AC74 Administration

The accumulation of [18F]AC74 in LNs was further studied in ApoE KO-cuff and C57BL/6 mice by PET/CT imaging after intraperitoneal radiotracer application. ApoE KO-cuff mice had a shear stress-modifying cuff implanted around their right common carotid artery and a non-constrictive control around the contralateral carotid artery. The constriction in combination with the feeding of a high fat diet (HFD) facilitated the development of atherosclerotic plaques in these carotid segments (Chapter 6).

To investigate whether the LN accumulation was specific for [18F]AC74 or whether this is a common phenomenon for paravenously applied radiotracers, a head-to-head comparison with the following four 18F-radiotracers was performed in an ApoE KO-cuff HFD mouse: the CD80-specific [18F]AC74, the metabolic tracer [18F]FDG, the mGluR5-binding [18F]PSS232 and the 5-HT3 antagonist [18F]Fluoropalonosetron (Figure 5.3). Radiotracer characteristics are summarized in Table 5.4. [18F]PSS232 was selected due to a similarly high plasma protein binding as [18F]AC74. [18F]Fluoropalonosetron has an identical logD_{pH7.4} value and therefore a similar lipophilicity as [18F]AC74. [18F]FDG was selected due to its markedly different characteristics such as the low
lipophilicity and molecular weight. All radiotracers were intraperitoneally administered to the mouse.

![Chemical structures](image)

**Figure 5.3** Evaluated fluorine-18 and carbon-11 labeled radiotracers in ApoE KO and C57BL/6 mice. CD80-binding \[^{18}F\]AC74 and \[^{11}C\]AM7, the metabolic tracer \[^{18}F\]FDG, the mGluR5-specific \[^{18}F\]PSS232 and 5-HT3 antagonist \[^{18}F\]Fluoropalonosetron. Radiotracer characteristics are listed in Table 5.4.

**Table 5.4** Characteristics of the evaluated fluorine-18 and carbon-11 labeled radiotracers \([83, 169, 170]\). Data concerning \[^{18}F\]Fluoropalonosetron were kindly provided by Dr. Linjing Mu. Hs: *Homo sapiens*, Mm: *Mus musculus*, Rn: *Rattus norvegicus*. MW: molecular weight, \(f_u\): unbound fraction in plasma.

<table>
<thead>
<tr>
<th>Target</th>
<th>[^{18}F]AC74</th>
<th>[^{11}C]AM7</th>
<th>[^{18}F]FDG</th>
<th>[^{18}F]PSS232</th>
<th>[^{18}F]Fluoropalonosetron</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdlogP</td>
<td>0.8</td>
<td>1.3</td>
<td>-2.0</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>(\text{logD}_{pH7.4})</td>
<td>0.9</td>
<td>0.1</td>
<td>-</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>MW [g/mol]</td>
<td>449.4</td>
<td>419.5</td>
<td>181.2</td>
<td>315.4</td>
<td>313.4</td>
</tr>
<tr>
<td>(f_u)</td>
<td>Mm 0.0122</td>
<td>Mm 0.022</td>
<td>-</td>
<td>Rn 0.03</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Hs 0.0091</td>
<td>Hs 0.057</td>
<td></td>
<td>Hs 0.03</td>
<td></td>
</tr>
</tbody>
</table>

In this head-to-head comparison, radiotracer accumulation in mediastinal LNs was only observed for the CD80-specific tracer \[^{18}F\]AC74 whereas \[^{18}F\]PSS232 and \[^{18}F\]Fluoropalonosetron showed no significant uptake in the mediastinal region (Figure 5.4, A). The metabolic tracer \[^{18}F\]FDG accumulated in the heart and furthermore in the costal musculature engaged in the process of breathing.

In addition, ApoE KO-cuff HFD mice were scanned with the CD80-specific radiotracer \[^{11}C\]AM7 and this tracer accumulated in the mediastinum in LNs as well (Figure 5.4, B). Furthermore, LN accumulation of \[^{18}F\]AC74 was investigated in wild-type C57BL/6 mice on normal diet (ND). Also in this mouse strain, \[^{18}F\]AC74 accumulated in mediastinal LNs (Figure 5.4, C). No radiotracer accumulation was observed in other LNs within the PET field of view.
Figure 5.4 PET/CT images of (A) a head-to-head comparison of $[^{18}\text{F}]\text{AC74}$, $[^{18}\text{F}]\text{FDG}$, $[^{18}\text{F}]\text{PSS232}$ and $[^{18}\text{F}]\text{Fluoropalonosetron}$ ($[^{18}\text{F}]\text{FPS}$) injected intraperitoneally into an ApoE KO-cuff HFD mouse (10 weeks post-surgery). (B) i.p. administration of $[^{11}\text{C}]\text{AM7}$ to an ApoE KO-cuff HFD mouse and (C) i.p. injection of $[^{18}\text{F}]\text{AC74}$ to a C57BL/6 ND mouse. Arrowheads indicate lymph nodes, asterisks liver and hashtag heart. Averaged PET images from 0 – 60 min. Color bar: standardized uptake value (SUV). PET in color, CT in white/gray.

5.3.3.3 Ex Vivo Biodistribution of $[^{18}\text{F}]\text{AC74}$ in ApoE KO Mice

The specificity of the accumulation of $[^{18}\text{F}]\text{AC74}$ in mediastinal LNs was evaluated by biodistribution experiments with ApoE KO HFD mice (Table 5.5). Different tissues and organs...
including mediastinal and cervical (negative control) LNs were dissected from animals after intraperitoneal injection of tracer or tracer and an excess of unlabeled compound.

The highest concentrations of $^{18}$FAC74 were found in the urine, gallbladder, liver, intestines and kidneys, followed by blood, pancreas, stomach and fat. The highest tracer accumulation in a CD80-expressing tissue was observed in the mediastinal LNs. Under baseline conditions, cervical LNs displayed a significantly lower radiotracer accumulation than mediastinal LNs. Other CD80-positive organs such as the spleen and the thymus showed intermediate radiotracer accumulations. Arterial segments affected by atherosclerosis in particular the aorta and carotids displayed an intermediate to low $^{18}$FAC74 accumulation. The lowest values were observed in the bones and the brain indicating that no radiotracer defluorination and no tracer passage across the blood-brain barrier occurred, respectively. Radiotracer accumulation under blockade conditions was significantly reduced in the mediastinal LNs, but not in other tissues.

Table 5.5 Ex vivo biodistribution of $^{18}$FAC74 in ApoE KO HFD mice. Animals were sacrificed 30 min after injection of 14 MBq $^{18}$FAC74 (0.6 nM, baseline, n = 3) or additionally with 600 nM AC74 (blockade, n = 4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Baseline [norm. % ID/g]</th>
<th>Blockade [norm. % ID/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>1.615 ± 1.471</td>
<td>1.395 ± 0.861</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>1.022 ± 0.541</td>
<td>1.590 ± 0.681</td>
</tr>
<tr>
<td>Liver</td>
<td>0.461 ± 0.083</td>
<td>0.454 ± 0.102</td>
</tr>
<tr>
<td>Intestines</td>
<td>0.355 ± 0.215</td>
<td>0.285 ± 0.206</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.166 ± 0.056</td>
<td>0.198 ± 0.068</td>
</tr>
<tr>
<td>Blood</td>
<td>0.109 ± 0.111</td>
<td>0.110 ± 0.055</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.104 ± 0.064</td>
<td>0.041 ± 0.016</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.066 ± 0.038</td>
<td>0.066 ± 0.010</td>
</tr>
<tr>
<td>Fat</td>
<td>0.059 ± 0.017</td>
<td>0.111 ± 0.075</td>
</tr>
<tr>
<td>Mediastinal lymph nodes</td>
<td>0.055 ± 0.010</td>
<td>0.014 ± 0.013 **</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.051 ± 0.014</td>
<td>0.074 ± 0.059</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.048 ± 0.031</td>
<td>0.037 ± 0.020</td>
</tr>
<tr>
<td>Descending aorta</td>
<td>0.046 ± 0.002</td>
<td>0.047 ± 0.014</td>
</tr>
<tr>
<td>Skin</td>
<td>0.041 ± 0.045</td>
<td>0.011 ± 0.004</td>
</tr>
<tr>
<td>Control carotid artery</td>
<td>0.039 ± 0.007</td>
<td>0.034 ± 0.013</td>
</tr>
<tr>
<td>Cuff carotid artery</td>
<td>0.029 ± 0.010 **</td>
<td>0.030 ± 0.013</td>
</tr>
<tr>
<td>Aortic arch</td>
<td>0.027 ± 0.007</td>
<td>0.029 ± 0.009</td>
</tr>
<tr>
<td>Lung</td>
<td>0.019 ± 0.005</td>
<td>0.021 ± 0.004</td>
</tr>
<tr>
<td>Heart</td>
<td>0.016 ± 0.005</td>
<td>0.015 ± 0.002</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.010 ± 0.003</td>
<td>0.011 ± 0.003</td>
</tr>
<tr>
<td>Cervical lymph nodes</td>
<td>0.006 ± 0.003</td>
<td>0.005 ± 0.002</td>
</tr>
<tr>
<td>Bone</td>
<td>0.005 ± 0.001</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Brain</td>
<td>0.001 ± 0.001</td>
<td>0.001 ± 0.001</td>
</tr>
</tbody>
</table>

** $p < 0.01$
5.3.3.4 Gene Expression Analysis of CD80, CD86 and CD68 in Murine Lymph Nodes

RNA expression of the costimulatory molecules CD80 and CD86 as well as CD68, a marker for activated macrophages, relative to β-actin was investigated in mediastinal and cervical LNs of ApoE KO-cuff mice fed a HFD, ApoE KO-cuff mice fed a ND and C57BL/6-cuff ND mice.

No significant difference in CD80, CD86 and CD68 expression was determined for the different animal groups (Figure 5.5). Furthermore, mediastinal and cervical (control) LNs displayed similar gene expression levels for CD80, CD68 and CD86.

![Graphs showing mRNA expression of CD80, CD86, and CD68 in mediastinal and cervical lymph nodes of different mouse groups.](image)

**Figure 5.5** Relative mRNA expression of CD80, CD86 and CD68 in mediastinal and cervical lymph nodes of ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice. Values represent mean ± SEM of n = 3 – 5 animals or a single value with no error bar (mediastinal LNs of C57BL/6-cuff mice). No significant intergroup differences were observed.

Human and murine CD80 as well as CD86 mRNA expression was analyzed in the axillary LN of a CD1 nu/nu mouse with implanted Raji xenografts since this LN showed a substantial radioactivity signal in PET/CT scans (Figure 5.2). Only murine but not human CD80 and CD86 were detected in the evaluated LN.
5.3.3.5 CD80 Protein Expression in Lymph Nodes of ApoE KO Animals

The CD80 protein expression in single cell suspensions derived from mediastinal, cervical, inguinal and mesenteric LNs of ApoE KO animals on HFD or ND was investigated by FACS. CD11c is an integrin that is expressed by conventional DCs [171]. Following an intraperitoneal administration, the lymphatic fluid passes through the mesenteric and thereafter the mediastinal LNs before it is released into the left subclavian vein. Cervical and inguinal LNs served as controls. For both ApoE KO mouse groups fed either a HFD or a ND, no difference in CD80 expression in CD11c-positive cells was observed between mediastinal, cervical and inguinal LNs (Figure 5.6). Only the mesenteric LNs in ApoE KO HFD animals displayed a significantly higher CD80 expression than inguinal LNs.

The CD80 expression in leucocytes derived from the aorta and carotids of the same animals was analyzed revealing a significantly higher expression in HFD than ND animals (Chapter 6).

![Figure 5.6](image.png)

**Figure 5.6** Difference in median fluorescence intensity (ΔMFI) in CD11c-positive cells of mediastinal, cervical, inguinal and mesenteric lymph nodes of ApoE KO HFD (n = 4) or ApoE KO ND (n = 3) mice. Lines indicate mean values. *p < 0.05.
5.4 DISCUSSION

Based on our recent findings [83] (Chapter 4), we consider the targeting of CD80 a valuable diagnostic strategy for the imaging of diverse disease areas. These include atherosclerosis, oncology and autoimmune disorders. Besides the CD80/CD86-targeting $^{111}$In-labeled polypeptide belatacept, we developed the two CD80-specific low molecular weight radiotracers for PET imaging, $^{11}$CAM7 [83] and $^{18}$FAC74 (A. Chiotellis et al., manuscript in preparation). With the latter, we intended to alter the pharmacokinetic properties of $^{11}$CAM7 by replacing its basic tertiary amine by the neutral triazole moiety, which at the same time made it amenable for labeling with fluorine-18.

We did not observe the desired prolongation in blood half-life despite the increase in logD$_{pH 7.4}$ from 0.1 to 0.9, which would in principle increase tissue distribution [172], and despite the change from a cationic to a neutral species at physiological pH. We expected that this major change in electronic features would affect recognition of the tracer by hepatobiliary efflux transporters. Similar as $^{11}$CAM7, $^{18}$FAC74 was rapidly cleared from the blood into bile, albeit 99% of the tracer in plasma was bound to protein.

The structural alteration resulted in a 20-fold decrease in affinity to recombinant human CD80. For imaging, the higher binding affinity of AM7 is definitely an advantage, although for some pathophysiological conditions with a locally high CD80 expression, the lower binding affinity of AC74 might still be sufficient. We did not observe any specific binding of the tritiated AM7 to recombinant mouse CD80 and, therefore, do not expect significant binding of the new tracer to the recombinant mouse protein. The low or absent affinity of AM7 to the recombinant murine CD80 could result from several conditions. The human and murine CD80 protein have less than 50% sequence identity. In fact, Erbe et al. delineated that two CD80 low molecular weight inhibitors that were structurally related to our tracers, bound to a binding pocket of the N-terminal V-set domain exclusively present on human CD80, but not on murine CD80 or human CD86 [164]. Furthermore, we investigated the binding affinity to recombinant soluble proteins rather than to the proteins in their native microenvironment where they tend to assemble in oligomers in the immunological synapses [173]. Both sequence and conformation differences could affect binding affinity. Differences in glycosylation between native and recombinant protein can be excluded as both the human and murine proteins were produced by mouse myeloma cells (NS0) and are therefore fully glycosylated.

Binding assays with CD80-positive cells may thus appear preferable. However, the IC$_{50}$ values of $^{11}$CAM7 in cell-based assays using the human Raji or Jurkat cells were considerably higher than in the assay with recombinant human CD80, in agreement with previous reports [164, 166]. The apparently reduced affinity in the cell assays most probably results from the fact that Raji cells not only express the APC costimulatory molecules CD80 and CD86, but also the respective T cell receptor CTLA-4 (Chapter 4, Figure 4.2). In the binding assay, the CD80-targeting test molecules have to compete with CTLA-4 for the binding to CD80. Stamper et al. described the crystal structure of CD80 bound to CTLA-4 as a highly periodic assembly of multiple homodimeric CTLA-4 as well as
CD80 molecules allowing an interaction with high avidity [158]. Moreover, the surface density of CD80 receptors could potentially be reduced by trans-endocytosis [82].

We recently determined a significantly higher accumulation of [11C]AM7 in human carotid plaques than normal arteries and additionally in vulnerable than stable plaques by in vitro autoradiography [83]. In the present study, we focused on inflammation-related plaque features that are expected to correlate with CD80 expression, such as the number of immune cells, besides other relevant plaque characteristics such as the size of the lipid/necrotic core. Despite the reduced affinity of the triazole derivative, a significantly higher [18F]AC74 binding to plaques than to normal arteries was observed. Furthermore, radiotracer accumulation correlated best with a large lipid/necrotic core and few immune cells within the plaque core. These findings demonstrate that CD80-targeting radiotracers, including [18F]AC74, are of interest for atherosclerosis imaging. Notably, the tissues used in autoradiography were of human origin.

Despite the lack of binding affinity of [3H]AM7 to recombinant murine CD80, we found an intense tracer accumulation within a LN in a PET/CT scan of an animal paravenously injected with [18F]AC74 into the tail. This effect was absent after intravenous radiotracer application. The visualized LN was in close proximity to the implanted CD80-positive Raji xenograft. Since Raji cells are a human hematopoietic cell line, qPCR analysis was performed to investigate a potential spreading of human CD80 within the mouse. However, no human CD80 or CD86 was detected in murine LNs indicating either unspecific radiotracer enrichment in this LN or specific binding to murine CD80 in vivo, which was dependent on the route of administration.

After intraperitoneal [11C]AM7 and [18F]AC74 injection, respectively, into mice without a xenograft, a signal in mediastinal LNs was observed reproducibly. This accumulation was independent of the mouse strain, diet, blood lipid profile, precedent surgery, and the CD80 radiotracer. The accumulation was not observed for [18F]PSS232, which has a similarly high plasma protein binding, or for other tested radiotracers [169, 170]. This indicates that the accumulation is, at least to some extent, compound-specific and probably also CD80-specific. A high and displaceable accumulation in LNs was also observed with the 111In-labeled belatacept, which binds to human and murine CD80 and CD86 (Chapter 4).

Ex vivo biodistribution experiments with i.p. injection of [18F]AC74 into ApoE KO mice revealed a specific tracer accumulation in mediastinal LNs since a significantly reduced radioactivity was found in mediastinal LNs of blockade animals. Furthermore, accumulation in mediastinal LNs was significantly higher than in cervical LNs. These findings suggest a specific accumulation of [18F]AC74 in CD80-positive LNs as determined by qPCR on mRNA and by FACS on protein level. However, differences in CD80 expression between mouse strains and lymph nodes were not detected.

The accumulation pattern in LNs of these CD80 tracers was dependent on the administration route. After injection into the peritoneal cavity, the fluid drains to the mesenteric and periportal LNs before entering the mediastinal LNs via the thoracic duct [174]. Finally, the lymph is released into the left subclavian vein [175]. In rats, substances can be transported via this lymphatic route...
within hours or even minutes [176]. Based on our experimental findings, we reasoned that both CD80 radiotracers were transported via the lymphatic system after i.p. administration and were enriched in mediastinal LNs thereby allowing binding to CD80-expressing immune cells. The lymphatic transport of unbound radiotracer is most plausible since cell-mediated transport is considerably slower in the range of hours to days [175]. After release of the lymphatics into the blood circulation, the radiotracer candidates were rapidly excreted via the hepatobiliary route resulting in a weak signal in the circulating blood pool. After the paravenous \[^{18}\text{F}]\text{AC74}\) administration into the tail vein of the xenograft mouse, a different lymphatic transport route of the compound is probable leading to a PET signal in axillary LNs.

Since CD80 is expressed on APCs, \textit{in vivo} accumulation of \[^{18}\text{F}]\text{AC74}\) was expected in lymphoid tissues such as spleen, thymus and LNs under physiological conditions. Following intravenous administration to CD1 nu/nu mice, radiotracer accumulation was predominantly localized in the liver, intestines, gallbladder and kidneys as determined by \textit{in vivo} PET/CT imaging. The fast clearance from the blood pool within a few minutes and the high plasma protein binding complicate the imaging with this compound because of the negligible tissue distribution and therefore the negligible target accumulation. The modification of the lead structure of AM7 by introducing a triazole moiety did not improve the pharmacokinetic characteristics in mouse. Both CD80-targeting radiotracers display similar limitations such as a rapid clearance via the hepatobiliary route, a low unbound radiotracer fraction in plasma, and a low affinity to murine recombinant CD80. Therefore, further modifications of the AM7/AC74 lead structure and completely different lead structures should be developed, motivated by the successful imaging of Raji xenografts with the \(^{111}\text{In}\)-labeled CD80/CD86-targeting fusion protein belatacept, the \textit{in vivo} accumulation of both low molecular weight tracers in CD80-positive LNs and the \textit{in vitro} binding of \[^{18}\text{F}]\text{AC74}\) to human carotid plaques.
5.5 CONCLUSIONS

The second generation CD80-specific tracer $^{[18F]}$AC74, further developed from $^{[11C]}$AM7, displayed a similar *in vitro* behavior with an overall good stability, low blood cell binding and high plasma protein binding. However, the determined IC$_{50}$ to human CD80 was lower for AC74 than AM7. The high plasma protein binding in combination with a rapid excretion hampers the use of $^{[11C]}$AM7 and $^{[18F]}$AC74 for preclinical PET imaging. However, targeting APCs with structurally optimized CD80-specific radiotracers has potential. *In vitro*, both radiotracer candidates bound to human carotid plaques and accumulated *in vivo* in CD80-positive LNs after a paravenous administration.
5.6 EXPERIMENTAL SECTION

5.6.1 Radiochemistry

The radiolabeling of $^{18}$FAC74 was performed as previously described (A. Chiotellis et al. manuscript in preparation). $^{18}$FAC74 formulated in 5% EtOH in saline was obtained in on average 98% radiochemical purity and a specific activity of 58 – 95 GBq/μmol. $^{13}$CAM7 and $^{18}$FPSS232 were produced in-house according to previous reports and $^{18}$FFDG was supplied by the University Hospital Zurich [83, 169]. $^{18}$FPalonosetron was produced in-house according to an unpublished procedure.

5.6.2 Determination of Plasma Stability, logD$_{pH7.4}$, Blood Cell Binding and Plasma Protein Binding of $^{18}$FAC74

Plasma stability was investigated by incubation of 700 kBq $^{18}$FAC74 in human and murine blood plasma and in PBS (70 μL each), respectively, at 37 °C up to 4 h. Proteins were precipitated by the addition of ice cold acetonitrile (140 μL) and the PBS samples were diluted by the same amount of acetonitrile after 0.5, 1, 2 and 4 h incubation. The samples were centrifuged (5 min, 5000 x g, 4 °C) and the supernatants were filtered and subsequently analyzed by analytical radio-UPLC.

The partition coefficient (logD$_{pH7.4}$) was determined by the shake flask method as previously described [177]. $^{18}$FAC74 (~300 kBq) was incubated in a mixture of 0.5 mL phosphate buffer (pH 7.4) and 0.5 mL 1-octanol at room temperature for 15 min in an overhead shaker. The two phases were separated by centrifugation (5000 x g, 5 min) and a 50 μL aliquot of each phase was analyzed by a γ-counter (Wizard, PerkinElmer). LogD is expressed as the logarithm of the ratio between radioactivity concentrations in 1-octanol and the buffer phase. Values represent mean ± standard deviation determined in three independent experiments in triplicates.

To test the blood cell binding of the radioligand, 50 kBq $^{18}$FAC74 (0.526 pmol) was added to fresh mouse or human whole blood (100 μL), in triplicates. Samples were incubated at 37 °C up to 120 min and blood cells were separated from plasma at different time points by centrifugation (16'000 x g, 5 min, 4 °C). Blood cell binding was determined as the ratio of radioactivity in plasma and blood cells determined in a γ-counter (Wizard, PerkinElmer).

Plasma protein binding was determined by equilibrium dialysis in dialysis cells (Dianorm, Munich, Germany) using cellulose membranes (10 kDa cut-off, Dianorm). In brief, 80 kBq radiotracer in 4% bovine serum albumin (BSA) in PBS, human or murine plasma (200 μL each) was dialyzed against PBS containing dextran (15 mg/mL, pH 7.4) for 5 h at room temperature, in triplicates. After dialysis, 50 μL of each dialysis cell was analyzed in a γ-counter (Wizard, PerkinElmer) and the unbound radiotracer fraction was calculated as the ratio between plasma resp. BSA/PBS and dextran/PBS.
5.6.3 In Vitro Displacement Assay

The binding of the two CD80 ligands AC74 and AM7 to recombinant human CD80 (140-B1-100, R & D Systems) and mouse CD80 (740-B1-100, R & D Systems), respectively, was determined by immunoprecipitation with Pierce® Protein A/G Agarose beads (Thermo Scientific). 0.17 μg CD80, 1 nM [3H]AM7 (2.8 TBq/mmol, RC TRITEC AG, Switzerland) and different concentrations of each compound ranging between 0.01 – 1000 nM were incubated in TRIS buffer (25 mM TRIS, 150 mM NaCl, pH 7.2) overnight in an overhead shaker at room temperature. Thereafter, 100 μL resin slurry (approx. 50 μL of settled immobilized protein A/G) was added to each tube and the samples were equilibrated at room temperature for 2 h. In all subsequent steps, the liquid was separated from the protein A/G beads by centrifugation (16'000 x g, 1 min) at room temperature. The unbound radiotracer fraction was removed by washing three times with TRIS buffer (500 μL each). In a next step, the CD80 fraction bound to protein A/G was released by incubation in glycine buffer (150 mM, pH 2.5, 60 μL each, 3x) for 5 min. The supernatant was collected and pooled from every cycle (50 μL each). Subsequently, 3 mL of scintillation liquid (Ultima Gold, PerkinElmer) was added to the pooled supernatant and the samples were analyzed in a Beckman LS 6500 Liquid Scintillation Counter (Beckman USA). Nonspecific binding to protein A/G beads and the maximum radiotracer binding to CD80 was determined. All samples were prepared in triplicates. IC50 values were determined by non-linear regression analysis of three independent experiments and are reported as mean ± standard deviation.

In cell-based displacement assays, Raji or Jurkat cells were incubated with 1 nM [11C]AM7 and increasing concentrations of AM7 (10^{-4} – 10^{-11} M) in RPMI medium at 37 °C for 30 min (triplicates). Followed by three washing steps with ice-cold RPMI medium and the cell pellets were finally measured in a γ-counter (PerkinElmer).

5.6.4 Human Atherosclerotic Carotid Plaques

Human atherosclerotic plaques from the A. carotis communis, A. carotis interna and externa were obtained from patients undergoing carotid endarterectomy in the Clinic for Cardiovascular Surgery at the University Hospital Zurich (Switzerland). Before surgery, written informed consent was obtained from all patients. Plaque segments were removed by bifurcation advancement technique [28]. In some cases redundant tissue of normal artery wall (A. thyroidea, A. iliaca) was collected and used as control. In total, 32 patients were included in this study on average 76.1 ± 6.4 years old at surgery and 86.4% of male gender. Excised tissue was stored in RNAlater® solution (Sigma, St. Louis, USA) at -80 °C until further use. After thawing, plaques were embedded in TissueTek O.C.T medium and 20 μm frozen sections were prepared and stored at -20 °C.

5.6.5 In Vitro Autoradiography

Radiotracer binding was investigated on sections of human atherosclerotic plaques. Frozen sections were dried for 15 min at room temperature and subsequently incubated in HEPES buffer (50 mM HEPES, 5 mM MgCl2, 125 mM NaCl, 1 mM CaCl2, pH 7.4) supplemented with 0.1% BSA for
15 min on ice, followed by 45 min incubation with 3 nM $[^{18}\text{F}]\text{AC74}$ solution (in HEPES/0.1% BSA) in an humidified chamber at room temperature. For blockade conditions, the tracer solution contained 100 μM unlabeled AC74. After incubation, the sections were washed on ice for 5 min in HEPES/0.1% BSA, for 3 min in HEPES buffer (3x) and for 1 min in distilled water (2x). Sections were dried and exposed for 45 min to a phosphor imager plate (BAS-MS 2025, Fuji Film, Dielsdorf, Switzerland), and the plate was scanned in a BAS-5000 bio-imaging analyzer (Fuji Film, Dielsdorf, Switzerland). Radiotracer binding was quantified by means of a dilution series of tracer solution pipetted onto filter papers (Whatman, Bottmingen, Switzerland) and exposed to the same plate. Data analysis was performed with the AIDA 4.5 software (Raytest, Sprockhövel, Germany). Integrated radioactivity signal intensities per plaque size were determined. Specific binding was calculated by subtraction of the radioactivity signal under blocking conditions and was normalized to the radiotracer standards. Hematoxylin/eosin (HE) staining was performed of all sections included in autoradiography. Based on HE staining, plaques were classified according to the criteria of Table 2. HE sections were scanned with a digital slide scanner (Pannoramic 250, Sysmex, Horgen, Switzerland).

5.6.6 Animals

Animal care and experiments were in accordance with the Swiss law of animal protection. Experiments were approved by the Veterinary Office of the Canton Zürich (Switzerland). Five-week old male C57BL/6N, five-week old immune-deficient female CD1 nude (Crl: CD1-Foxn1nu) and four-week old male ApoE KO (B6.129P2-Apoe tm1Unc/J) mice were purchased from Charles River Laboratories (Sulzberg, Germany). C57BL/6 and CD1 mice were fed a normal chow diet ad libitum. ApoE KO mice had unlimited access to either ND or HFD (21% fat, 0.25% cholesterol and 19.5% casein, Klika Nafag, Kaiseraugst, Switzerland). The procedure of the cuff implantation surgery is described in Chapter 6.

5.6.7 Ex Vivo Metabolite Studies

$[^{18}\text{F}]\text{AC74}$ (60 – 127 MBq) was injected via lateral tail vein into two C57BL/6N mice. Blood samples were collected 5 and 15 min p.i. from the tail vein. At 30 min or 60 min p.i., the animals were sacrificed and blood, urine and the liver were collected. Plasma was obtained after centrifugation (4800 x g, 5 min, 4 °C). Proteins of plasma and urine were precipitated by the addition of ice-cold acetonitrile and separated by centrifugation. Liver tissue was homogenized in 1 mL PBS (pH 7.4) using a PT 1200 Polytron (Kinematica AG). After the addition of ice-cold acetonitrile, the samples were centrifuged, acetonitrile was added to the supernatant and samples were centrifuged. Supernatants were filtered and analyzed by analytical radio-UPLC and radio-TLC.

5.6.8 In Vivo PET Imaging and Blood Kinetics

PET scans were performed with an eXplore VISTA small animal PET/CT tomograph (Sedecal/GE, Madrid, Spain). Animals were anesthetized with isoflurane in an air/oxygen mixture; body temperature and respiratory frequency were monitored throughout the scan.
In vivo distribution and blood kinetics of $[{\text{18}}^\text{F}]$AC74 was evaluated in immune-deficient CD1 mice bearing Raji or Jurkat xenografts ($n = 3$). Subcutaneous inoculation of Raji or Jurkat cells into the shoulder of CD1 $\text{nu/nu}$ mice was performed as previously described [83]. Six weeks after inoculation, mice were injected as indicated with $5 - 15$ MBq $[{\text{18}}^\text{F}]$AC74 and PET scans were acquired over 90 min. PET was followed by CT acquisition and PET data was reconstructed in user-defined time frames (2D-OSEM). Data was analyzed with the biomedical image quantification software PMOD (PMOD Technologies Ltd., Zurich, Switzerland). The standardized uptake value (SUV) of defined regions was calculated based on tissue radioactivity within the volume of interest.

ApoE KO-cuff HFD and C57BL/6-cuff ND mice were intraperitoneally injected with $\sim 12$ MBq $[{\text{18}}^\text{F}]$AC74, $\sim 17$ MBq $[{\text{18}}^\text{F}]$FDG, $\sim 14$ MBq $[{\text{18}}^\text{F}]$PSS232, $\sim 16$ MBq $[{\text{18}}^\text{F}]$Fluoropalonosetron or $\sim 12$ MBq $[{\text{11}}^\text{C}]$AM7. PET scans were acquired over 60 min.

Blood kinetics was analyzed by an exponential fit of the heart time activity curve in two intravenously injected CD1 mice. Heart radioactivity was considered to represent the blood pool since no myocardial accumulation of $[{\text{18}}^\text{F}]$AC74 was observed.

5.6.9 Biodistribution Studies

Biodistribution studies were performed in male ApoE KO mice on a HFD. Animals were intraperitoneally injected with 13.2 – 14.0 MBq $[{\text{18}}^\text{F}]$AC74 (0.6 nM in 100 µL) in 0.9% NaCl solution. 10 min prior to radiotracer injection, vehicle (100 µL DMSO in 0.9% NaCl, $n = 3$) or blocker (100 µL, 600 nM unlabeled AC74 in DMSO/0.9% NaCl, $n = 4$) was administered intraperitoneally. Animals were sacrificed 30 min after administration of the radiotracer by intraperitoneal injection of pentobarbital in 0.9% NaCl (300 µL). Selected tissues and organs were collected, weighed and the accumulated activity was determined in a $\gamma$-counter (PerkinElmer). Activity was normalized to animal weight and the percentage of injected dose per gram tissue weight (% ID/g) was calculated based on reference counts from a standard dilution of a radiotracer injectate.

5.6.10 Total RNA Isolation, cDNA Generation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from mediastinal, cervical and/or axillary lymph nodes of ApoE KO-cuff HFD, ApoE KO-cuff ND, C57BL/6 ND and/or CD1 $\text{nu/nu}$ mice (17 – 18 weeks after cuff placement) by means of the Isol-RNA Lysis reagent (5 PRIME, USA) and the TissueLyser bead-mill system (Qiagen, Germany). cDNA was generated by the QuantiTect Reverse Transcription Kit (Qiagen, Germany). The following custom-made oligonucleotide primers were ordered from Microsynth (Balgach, Switzerland): murine $\beta$-actin (ACTB, NM_007393.3) (forward 5’-AGACCTCTAT-GCCAACACAGT-3’, reverse 5’-TGCTAGGAGGCAAGCAGTAA-3’), murine CD68 (NM_009853) (forward 5’-CTGTTACCTGTACTGCTCT-3’, reverse 5’-AACATGGCCAAGCTTGCTCC-3’), murine T-lymphocyte activation antigen CD80 (NM_009855.2) (forward 5’-CTGTTACCTGTACTGCTCT-3’, reverse 5’-AACATGGCCAAGCTTGCTCC-3’), murine T-lymphocyte activation antigen CD86 (NM_019388) (forward 5’-AAAGTTGTTCTGTACGACG-3’, reverse 5’-GGGCCAGGT-
ACTGGCAT-3′), human β-actin (ACTB, NM_001101.3) (forward 5′-AGACCTGTACGCAACACAG-3′, reverse 5′-TTCTGACCTCCTGGCAAT-3′), human T-lymphocyte activation antigen CD80 (NM_005191) (forward 5′-GGGCACATACGAGTGTGTTG-3′, reverse 5′-TCAGCTTTGACTGATAACGTAC-3′) and human T-lymphocyte activation antigen CD86 (NM_175862) (forward 5′-TATGGGGCGCAAGTTTTGA-3′, reverse 5′-TGGTGGATGCGAATCATTC-3′). For qPCR, GoTaq qPCR Master Mix (Promega, Dübendorf, Switzerland) was used and the analysis was performed on an AB7900 HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA) with SDS software (Applied Biosystems). Reactions were run in duplicates in two experiments and dissociation curve analysis was performed. Relative gene expression was determined with β-actin as internal control by 2−ΔΔCt quantification [178].

5.6.11 FACS Analysis of Lymph Nodes of ApoE KO Mice

Mediastinal, cervical, inguinal and mesenteric LNs of 29-week old ApoE KO animals fed a HFD (n = 4) and animals fed a ND (n = 3) were dissected. Tissue suspensions were passed through a 40 µm cell stainer (BD Bioscience) with PBS containing 2.5% fetal bovine serum (FBS) and 2 mM EDTA. Thereafter, cell suspensions were centrifuged for 2 min at 1200 rpm (4 °C) and resuspended in 2.5% FBS and 2 mM EDTA. Cell suspension were stained with the following antibodies: rat anti-mouse CD4-APC (clone GK1.5, Biolegend), rat anti-mouse CD45-PerCP (clone 30-F11, Biolegend), rat anti-mouse CD4-PE (clone GK1.5, Biolegend), hamster anti-mouse CD80-PE (clone 16-10A1, Biolegend), hamster IgG isotype control (eBioscience), mouse anti-mouse CD45.2-FITC (clone 104, BD Pharmingen), rat anti-mouse CD16/32 (clone 93, Biolegend), hamster IgG isotype control APC (clone Htk888, Biolegend), hamster anti-mouse CD11c-APC (clone N418, Biolegend), rat IgG2a κ isotype control (BD Pharmingen), rat anti-mouse I-A/I-E-PerCP (clone M5, Biolegend), mouse anti-mouse IgG1 κ isotype control FITC (clone MOPC-21, Biolegend). FACS analysis was performed on a BD FACSCanto with FACSDiva software (BD Bioscience). For data analysis FlowJo software (Treestar) was used. The number of CD45-positive cells and the difference in median fluorescence intensity (ΔMFI) between CD45 and the isotype control was determined.

5.6.12 Statistics

The significance of intergroup differences was evaluated by GraphPad Prism (GraphPad, La Jolla, CA, USA) or Microsoft Excel. According to the number of groups, either a two-tailed unpaired student’s t-test or a one-way ANOVA with a Tukey’s multicomparison test was performed. A p-valued < 0.05 was considered significant.

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6 CHARACTERIZATION OF A MOUSE MODEL OFATHEROSCLEROSIS AND EVALUATION OF PET RADIOTRACER CANDIDATES FOR THE IMAGING OF PLAQUE INFLAMMATION

This chapter will be submitted in a modified version to Atherosclerosis, Thrombosis, and Vascular Biology.

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AUTHOR CONTRIBUTIONS

R. Meletta planned and coordinated the study, established and carried out the blood lipid analysis, sampling for in vitro experiments, RNA isolation, qPCR experiments, oil red o staining, FACS, ex vivo PET/CT scans, performed [¹⁸F]FDM radiolabelings, [¹⁸F]FDM and [¹⁴C]AM7 quality control, data analysis and interpretation, and wrote the manuscript. L. Steier was involved in the RNA isolation, qPCR experiments and oil red o staining, data analysis and interpretation. C. Keller performed the cuff implantation surgery and the PET/CT scans. L. Mu established the [¹⁸F]FDM radiosynthesis and quality control and contributed to manuscript writing. A. Chiotellis produced [¹⁸F]AC74 for animal experiments. E. Russo performed the FACS experiment and evaluated FACS data. C. Halin supervised the FACS experiment. N. Borel supervised and analyzed the histology and immunohistochemistry staining. S. M. Ametamey was involved in the design of [¹⁸F]AC74, supervised the (radio)chemistry and contributed to the discussion and interpretation of the results. R. Schibli was involved in the discussion and interpretation of the results. S. D. Krämer contributed to the supervision and planning of the study, was involved in data analysis and interpretation, and
revised the manuscript. A. Müller Herde supervised the experiments, the planning and data interpretation, was involved in the sampling for *in vitro* experiments, *ex vivo* PET/CT scans and cuff implantation surgery.
6.1 ABSTRACT

Plaque inflammation is a key feature of vulnerable atherosclerotic lesions. Consequently, the targeting of inflammation-related processes is a promising strategy for atherosclerosis imaging. The identification of patients at high risk for acute vascular events is an unmet clinical need and a non-invasive imaging technique such as positron emission tomography (PET) would be ideal for atherosclerosis risk stratification.

Shear stress patterns in the arterial tree significantly influence atherosclerotic plaque formation in mice and humans. A shear stress-induced mouse model of atherosclerosis, referred to as the ApoE KO-cuff mouse model, was recently presented giving rise to stable- and vulnerable-like lesions in the same animal [100]. In the present study, a flow constrictive cuff was implanted around the right common carotid artery of ApoE KO mice fed a high fat diet (HFD). On the contralateral artery, a non-constrictive control was placed around the vessel. The blood lipid profile of ApoE KO and wild-type C57BL/6 mice was evaluated over five month. At different disease stages in ApoE KO-cuff HFD mice, the atherosclerotic plaque burden was characterized with respect to lesion location and morphology by oil red o staining, hematoxylin/eosin staining and CD68 immunohistochemistry. Gene expression of the macrophage marker CD68, the T-lymphocyte activation antigen CD80, and the macrophage mannose receptor 1 (MRC1) was investigated by qPCR in vessel segments of ApoE KO-cuff and control mice. CD80 protein expression was examined by FACS analysis in the aorta and carotids of ApoE KO mice on a HFD or normal chow diet (ND). For preclinical radiotracer evaluation, in vivo PET/CT imaging was performed in ApoE KO-cuff HFD mice with two CD80-specific tracers, [11C]AM7 and [18F]AC74, containing an oxo dihydropyrazolocinnoline as common basic chemical structure. These radiotracer candidates developed in our group were compared to the glucose analogs [18F]fluorodeoxyglucose ([18F]FDG) and [18F]fluorodeoxymannose ([18F]FDM) which were additionally evaluated in ex vivo PET/CT scans.

Blood lipids in ApoE KO-cuff mice displayed a diet-dependent significant increase in total cholesterol, triglycerides and low-density lipoproteins (LDL). Furthermore, ApoE KO-cuff mice showed for high-density lipoprotein (HDL), LDL and total cholesterol significantly higher values than wild-type animals. Plaques developed in this mouse model at the natural predilection sites of atherosclerosis within the arterial tree and additionally in the vessel segments adjacent to the implants. No plaques were observed in the coronary arteries. Histologically, plaques featured clusters of inflammatory cells, numerous foam cell accumulations, extensive calcifications, a heterogeneous morphology and substantial constrictive vascular remodeling. All examined plaques contained CD68-positive macrophages. Lesions were classified as vulnerable plaques based on histology. No distinct differences in plaque morphology between the constrictive and non-constrictive implant as well as between the upstream and downstream segments were observed. The inflammatory markers CD68 and CD80 were elevated in ApoE KO-cuff HFD compared to wild-type mice. No significant change in MRC1 gene expression was noted. Moreover, a significantly
increased CD80 protein expression in leucocytes was observed in the aorta and carotids of ApoE KO mice on HFD compared to mice on ND.

In *ex vivo* PET/CT experiments with ApoE KO-cuff HFD mice, $[^{18}F]$FDG and $[^{18}F]$FDM accumulation was found at sites of atherosclerotic plaque formation within the aorta and carotids co-localizing with oil red o lipid staining. Radiotracer uptake in regions upstream and downstream of the cuff was increased for $[^{11}C]$AM7, $[^{18}F]$FDG and $[^{18}F]$FDM compared to the background. $[^{18}F]$AC74 displayed a similar signal in the cuff regions and the background. The most pronounced tracer accumulation in regions of atherosclerotic plaques was observed for $[^{18}F]$FDG.

In conclusion, modified shear stress conditions in combination with a HFD led to an overall elevated blood lipid profile, extensive atherosclerotic plaque development of a vulnerable phenotype and an increase in mRNA as well as protein expression levels of inflammatory markers in the ApoE KO-cuff mouse model. Furthermore, the CD80-specific radiotracer $[^{11}C]$AM7 and the metabolic tracers $[^{18}F]$FDG and $[^{18}F]$FDM accumulated at sites of atherosclerotic plaques *in vivo.*
6.2 INTRODUCTION

Atherosclerosis is an inflammatory disease affecting preferentially arterial segments exposed to oscillatory or low shear stress conditions as they often occur near bifurcations or in inner curvatures of arteries [179, 180]. High cardiovascular mortality and disability is associated with the rupture of atherosclerotic plaques and subsequent thrombus formation resulting in reversible ischemic episodes or even complete occlusions of arteries thereby impairing the function of downstream tissues and organs. This can cumulate in life-threatening conditions such as a myocardial infarction or stroke. Atherosclerotic lesions evolve over decades with initial fatty streaks already found in children. Genetic, life-style and probably other to date unknown factors sustain the accumulation of lipids and inflammatory cells in atherosclerotic plaques and can eventually lead to the formation of vulnerable plaques that are prone to rupture.

Positron emission tomography (PET) is a non-invasive imaging technique providing quantitative information about functional processes. Together with computed tomography (CT) or magnetic resonance imaging (MRI) supplying anatomical information, PET allows to investigate three-dimensional, dynamic distribution patterns of radiotracers in vivo at excellent sensitivity. Therefore, PET has high potential to become a meaningful tool for the risk assessment of future cardiovascular events. The metabolic PET tracer $[^{18}F]FDG$ is a clinically established radiotracer in oncology and moreover accumulates at sites of active inflammation including atherosclerotic plaques. In atherosclerosis, studies demonstrated a higher $[^{18}F]FDG$ signal in the ascending aorta and left main coronary artery of patients with acute coronary syndrome than stable angina [181]. However, $[^{18}F]FDG$ non-specifically accumulates at systemic sites of inflammation and generally in regions of high metabolic activity such as the myocardium. Therefore, a radiotracer specifically binding to atherosclerotic plaques that allows the identification of both coronary and carotid plaques is required for plaque imaging in clinical routine. Research efforts have focused on the identification of targets involved among others in plaque inflammation [60, 62], angiogenesis [63] and calcifications [71]. Our own efforts are focused on the development and evaluation of CD80-binding radiotracers such as the oxodihydropyrazolocinnoline derivatives $[^{11}C]AM7$ and $[^{18}F]AC74$. The expression of the costimulatory molecule CD80 which is expressed by activated antigen-presenting cells (APCs) is increased in vulnerable compared to stable human carotid plaques [83]. Both CD80-targeting radiotracer candidates bound in vitro to human atherosclerotic plaques, but were not yet evaluated in an animal model of atherosclerosis [83] (Chapter 5).

The preclinical evaluation of radiotracer candidates for atherosclerosis imaging relies on animal models of atherosclerosis with predominantly rodents used. Rodent atherosclerosis models are primarily based on genetically modified animals in combination with a diet enriched in cholesterol and fat to make the animals susceptible to atherosclerosis. Based on this principle is an approach presented by Cheng et al. taking additionally the impact of blood flow dynamics on plaque development into account [100]. In this shear stress-controlled murine atherosclerosis model, a flow constrictive device, referred to as a cuff, is placed around the common carotid artery of ApoE KO mice fed a HFD. The implanted cuff leads to defined altered flow conditions giving rise to the
formation of atherosclerotic lesions up- and downstream of the cuff. Histological and gene expression analyses revealed a predominant vulnerable phenotype in plaques upstream (US) of the cuff in artery segments subject to low shear stress conditions whereas downstream (DS) of the cuff in regions of oscillatory shear stress plaques of a stable phenotype prevailed [100-103]. Altogether that renders the ApoE KO-cuff mouse model an interesting model to investigate radiotracer accumulation in vivo having plaques of a stable and a vulnerable phenotype in one mouse. Numerous studies have been performed with this mouse model of atherosclerosis [79, 100-103, 182-187].

We implanted into ApoE KO mice fed a HFD a flow-constrictive cuff around the right common carotid artery (cuff carotid) and a non-constrictive control (control carotid) around the carotid on the contralateral side. The goal of this study was to characterize the ApoE KO-cuff mouse model regarding its blood lipid profile, plaque formation and morphology. Gene expression of the macrophage marker CD68 and the radiotracer targets CD80 and MRC1 (encoding for the macrophage mannose receptor MMR) was determined by qPCR. Furthermore, two CD80-specific radiotracer candidates, [11C]AM7 and [18F]AC74, were evaluated in this mouse model and compared to the established gold standard for inflammation imaging [18F]FDG and its isomer [18F]FDM.
6.3 RESULTS

6.3.1 Characterization of the ApoE KO-cuff Mouse Model

6.3.1.1 Body Weight and Blood Lipid Levels

Mouse body weight and blood lipids were analyzed in wild-type C57BL/6-cuff mice on ND, ApoE KO-cuff ND and ApoE KO-cuff HFD mice (Figure 6.1). The first measurement was performed before onset of the HFD for one study group.

At the beginning of the analysis before diet onset, wild-type mice showed a significantly higher body weight than ApoE KO mice. Over time body weight of all three study populations increased similarly. During the analysis period, C57BL/6-cuff mice displayed a stable blood profile of the evaluated lipids total cholesterol (2.46 ± 0.15 mmol/L), triglyceride (1.75 ± 0.14 mmol/L), HDL (2.53 ± 0.19 mmol/L) and LDL (-0.84 ± 0.14 mmol/L) with low intra- and inter-individual variability. Compared to wild-type mice, ApoE KO-cuff animals fed a ND showed significantly increased total cholesterol (13.79 ± 0.49 mmol/L), HDL (6.69 ± 0.36 mmol/L) and LDL (5.88 ± 0.58 mmol/L) values whereas no significant increase was observed for the triglycerides (2.20 ± 0.19 mmol/L). The ApoE KO-cuff mice fed a HFD displayed a significant increase in all analyzed blood lipids compared to C57BL/6-cuff mice. In the ApoE KO-cuff mouse strain, the levels of total cholesterol (36.31 ± 2.89 mmol/L), triglycerides (2.90 ± 0.25 mmol/L) and LDL (27.33 ± 2.59 mmol/L) were significantly higher in animals fed a HFD than ND. Blood lipids increased in ApoE KO-cuff HFD animals right after the onset of the fat-rich diet.
Body weight and total cholesterol, triglycerides, HDL and LDL in C57BL/6-cuff mice (ND) and in ApoE KO-cuff mice fed a ND or HFD. Values indicate mean ± SD of n = 3 animals per time point per group (not identical animals at each time point). Dashed lines mark the onset of the HFD (ApoE KO HFD) and the cuff implantation surgery (all groups), respectively. ns: non significant, * \( p < 0.05 \), *** \( p < 0.001 \), **** \( p < 0.0001 \).

**Figure 6.1**

6.3.1.2 Visualization of Intravascular Lipid Depositions by Oil Red O Staining

Lipid staining was performed in ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice 17 or 18 weeks post-surgery (Figure 6.2) and additionally in ApoE KO-cuff HFD mice at defined time points after surgery (6, 8, 13 and 17 weeks) (Figure 6.3).

Positive lipid staining, an indicator for the presence of atherosclerotic plaques, was observed in ApoE KO-cuff HFD mice in the inner curvature of the aortic arch as well as branch points, defined locations in the descending aorta as well as US and DS of the implanted cuff (Figure 6.2). No staining was found in the control carotid on the contralateral side. ApoE KO-cuff mice fed a ND showed red staining in the aortic arch, in branch points of the aortic arch and bifurcations of the carotids DS of
cuff and control. A small lipid accumulation was noted US of the cuff. No lipid staining was observed in wild-type C57BL/6-cuff mice on ND.

Figure 6.2 Representative oil red o lipid staining images of the aorta and carotids of ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice. Tissues were dissected 17 weeks (ApoE KO-cuff HFD) or 18 weeks after surgery (ApoE KO-cuff ND and C57BL/6-cuff ND). Magnified images show the aortic arch, the cuff carotid and the control carotid. 1, cuff; 2, control; DS, downstream; US, upstream. Different colors of the cuff and control are caused by differences in the material composition of the implants. Cuff and control were fixed with a black suture. Scale bars 1 mm.

Over time, the plaque burden increased in ApoE KO-cuff mice on HFD as shown in representative oil red o staining images in Figure 6.3. Red staining in the aortic arch was present from the earliest time point of 6 weeks on. DS of the cuff and control, respectively, a time-dependent increase in lipid staining was observed in the bifurcation of the carotids and close to the implants. US of the cuff, red staining was only determined at late time points (Figure 6.3, 17 weeks). In some exceptional cases, the US cuff vessel segment remained non-pathological until the end of the study. On the control side
no red staining US of the control was observed in these samples. However, in approximately 32% of the animals, plaques were determined US of the control.

Figure 6.3 Representative oil red o lipid staining images of the aorta and carotids of ApoE KO-cuff HFD mice at 6, 8, 13 and 17 weeks after surgery. Magnified images show the aortic arch, the cuff carotid and the control carotid. 1, cuff; 2, control; DS, downstream; US, upstream. Scale bars 1 mm.
6.3.1.3 Histological Evaluation of the Aorta, Carotids and Heart of ApoE KO-cuff Mice on HFD

The development of atherosclerotic plaques in the vasculature of ApoE KO-cuff mice was investigated by hematoxylin/eosin (HE) staining and CD68 immunohistochemistry at defined time points post-surgery (6, 9, 16 and 18 weeks) (Figures 6.4, 6.5, 6.6, 6.7).

At all examined time points, vascular remodeling processes were observed in the aortic arch, initially appearing as fatty streaks and later as numerous plaques with CD68-positive foam cells (Figure 6.4). Small atherosclerotic plaques were found 16 weeks after surgery in the descending aorta. At 18 weeks post-surgery, the atherosclerotic plaques were larger with foam cells and intense CD68 immunohistochemical staining (Figure 6.4). Plaques covered large parts of the aorta thereby substantially narrowing the vessel lumen. Atherosclerotic plaque appearance was generally heterogeneous with focal accumulation of cholesterol crystals, foam cells and other immune cells as well as lipid droplets.

![Figure 6.4](image.png)

**Figure 6.4** Representative HE and CD68 immunohistochemistry images of the aortic arch and the descending aorta of ApoE KO-cuff HFD mice 6, 9, 16 and 18 weeks (wk) post-surgery. Higher magnification images show large lipid-rich plaques containing cholesterol crystals and inflammatory cells within the aortic arch (1, 2) or the descending aorta (3, 4).

In terms of plaque formation, three distinct regions were defined in the cuff carotid (Figure 6.5). DS of the cuff, plaque size was increasing over time and a circular plaque containing CD68-positive cells was observed after 18 weeks. Within the cuff no atherosclerotic plaques were observed at any time point. In the US segment, the initial non-pathologic vessel (6 weeks) was converted over time into a circular plaque containing CD68-positive cells. Plaques in the US and DS segments consisted...
of numerous inflammatory cells including large populations of foam cells that were located in superficial regions of the plaque towards the vessel lumen. In the analyzed plaque specimens, a tendency towards more cholesterol crystals and a slightly more heterogeneous plaque composition was observed in the US segment.

Figure 6.5 Representative HE and CD68 immunohistochemistry images of the segments DS, within and US of the cuff in ApoE KO-cuff HFD mice 6, 9, 16 and 18 weeks post-surgery. Higher magnification images show vascular regions with complex circular plaques (cuff DS 1, 2; cuff US 5, 6) or non-pathological arterial cross sections (cuff 3, 4).

Plaques in the contralateral control artery were morphologically similar to the lesions of the US/DS cuff segments (Figure 6.6).

Figure 6.6 Representative HE and CD68 immunohistochemistry images of the segments DS, within and US of the control in ApoE KO-cuff HFD mice 6, 9, 16 and 18 weeks post-surgery. Higher
magnification images show vascular regions with lipid- and cholesterol crystal-rich plaques (control DS 1, 2; control 3, 4; control US 5, 6).

Plaques were already present at the earliest analysis time point DS of the control. Within the control, no vascular remodeling was observed after 6 weeks. However at later time points, atherosclerotic plaques containing CD68-positive foam cells were noted. In the US control segment, the initially healthy carotid transformed into a pathologic vessel with fatty streaks (week 16) or large plaques (week 18). These plaques contained populations of CD68-positive cells. The US plaques and the DS plaques were histologically comparable.

Arterial vessels originating from the heart of ApoE KO-cuff mice were heavily affected by atherosclerotic lesions featuring large cholesterol crystal clusters and foam cell accumulations (Figure 6.7, A, D). Moreover, vascular remodeling was observed in the aortic valves (Figure 6.7, B, E), but no atherosclerotic plaques were found in the coronary arteries (Figure 6.7, C, F).

**Figure 6.7** Representative HE and CD68 immunohistochemistry images of the heart of an ApoE KO-cuff HFD mouse 18 weeks post-surgery. (A/D) large arterial vessel, (B/E) aortic valve, (C/F) coronary vessel in the left ventricle. Note the intense CD68 background signal in the myocardium.

In general, the plaques formed in the ApoE KO-cuff mouse model displayed extensive foam cell clusters, focal accumulations of cholesterol crystals, multiple enclosed lipid depositions, and a heterogeneous plaque structure. Histological sections of the cuff and control segments at late time points contained almost exclusively circular plaques occupying large parts of the vessel lumen. Plaque burden in the adjacent segments of the cuff were larger than on the contralateral side with a smaller preserved lumen. Plaques in the ApoE KO-cuff mouse model were classified by a pathologist (N. B.) into the categories fatty streak, stable and vulnerable plaques according to the adapted classification criteria of Table 2.1. At early time points, fatty streaks and stable plaques were identified in the cuff and control segments. At later time points, almost exclusively plaques of a vulnerable phenotype were identified in the US and DS segments of both carotids and the aorta. A perivascular inflammation due to the implantation of the cuff and control, respectively, was present in all examined sections with numerous CD68-positive inflammatory cells around the vessel, cuff and control. CD68 immunohistochemistry was performed in two separate experiments resulting in different staining intensities of the DS segments compared to the rest (Figures 6.5, 6.6).
6.3.1.4 Influence of the Mouse Strain, Diet and Time Post-Surgery on CD68, CD80 and MRC1 Gene Expression

The gene expression of the inflammatory markers CD68, CD80 and MRC1 was investigated in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus of ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice by qPCR. Gene expression between the different mouse strains and diet groups was evaluated 18 weeks after cuff and control implantation (Figures 6.8, 6.10, 6.12). Furthermore, a change in expression over time was investigated in ApoE KO-cuff HFD mice 9, 12, 15, 17 and 18 weeks post-surgery (Figures 6.9, 6.11, 6.13).

For the macrophage marker CD68, a significantly higher mRNA expression in ApoE KO-cuff HFD mice than in wild-type mice was observed for the aortic arch, descending aorta, cuff US, cuff DS, control US, control DS, heart and thymus (Figure 6.8). In all these regions except the thymus, ApoE KO-cuff mice on ND displayed an intermediate CD68 mRNA expression in between the two other study groups. A significantly higher relative CD68 expression in ApoE KO-cuff HFD than ND mice was observed for the descending aorta, cuff US and control US. Similar expression levels in all animal groups were found in the cuff, control and spleen. Moreover, a significant increase in relative CD68 levels from week 6 to week 18 post-surgery was recognized for the aortic arch, descending aorta, cuff US, cuff DS and control DS with a pronounced elevation in the descending aorta (Figure 6.9). For all other regions, no consistent change in expression was noted.

The relative expression of the costimulatory molecule CD80 in ApoE KO-cuff HFD mice was significantly higher in the aortic arch, descending aorta, cuff DS and heart than in wild-type mice (Figure 6.10). A diet-related change in expression was observed in the cuff US segment in ApoE KO-cuff mice. CD80 expression levels were unaffected by mouse strain and dietary conditions in the cuff, control US, control DS, control and spleen. No time-dependent significant differences in relative CD80 expression was detected in all analyzed regions in ApoE KO-cuff HFD mice indicating that between 6 and 18 weeks after surgery the expression remained stable (Figure 6.11).

MRC1 mRNA expression was significantly higher in the aortic arch of ApoE KO-cuff HFD mice than wild-type mice (Figure 6.12). Furthermore in the descending aorta, a significantly higher MRC1 level was observed in ApoE KO-cuff ND than C57BL/6-cuff ND mice. No differences in expression were detected for all other regions. MRC1 levels showed solely a time-dependent increase in the control segment of ApoE KO-cuff HFD animals, whereas a decrease was observed for the descending aorta and the control DS segment over time (Figure 6.13). Expression in all other examined regions was independent of the time after surgery.

Overall, the expression of the inflammatory markers CD80, CD68 and MRC1 were affected by genetic and dietary factors as well as the time after surgery. The most pronounced expression changes were noted for CD68.
**Figure 6.8** Relative CD68 mRNA expression in ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice 18 weeks after surgery. Expression was analyzed in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus. Each symbol represents the mean value of one animal per experiment \((n = 2\) independent experiments\). Lines indicate mean values per group. \(^* p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001, ^{****} p < 0.0001\).
Figure 6.9 Relative CD68 mRNA expression in ApoE KO-cuff HFD mice 9, 12, 15, 17 and 18 weeks (wk) after surgery. Expression was analyzed in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus. Each symbol represents the mean value of one animal per experiment (n = 2 independent experiments). Lines indicate mean values per group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Note that data of ApoE KO HFD 18 weeks are in addition shown in Figure 6.8.
Figure 6.10 Relative CD80 mRNA expression in ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice 18 weeks after surgery. Expression was analyzed in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus. Each symbol represents the mean value of one animal per experiment (n = 2 independent experiments). Lines indicate mean values per group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Figure 6.11 Relative CD80 mRNA expression in ApoE KO-cuff HFD mice 9, 12, 15, 17 and 18 weeks after surgery. Expression was analyzed in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus. Each symbol represents the mean value of one animal per experiment (n = 2 independent experiments). Lines indicate mean values per group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Note that data of ApoE KO HFD 18 weeks are in addition shown in Figure 6.10.
Figure 6.12 Relative MRC1 mRNA expression in ApoE KO-cuff HFD, ApoE KO-cuff ND and C57BL/6-cuff ND mice 18 weeks after surgery. Expression was analyzed in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus. Each symbol represents the mean value of one animal per experiment (n = 2 independent experiments). Lines indicate mean values per group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Figure 6.13 Relative MRC1 mRNA expression in ApoE KO-cuff HFD mice 9, 12, 15, 17 and 18 weeks after surgery. Expression was analyzed in the aortic arch, descending aorta, cuff segments, control segments, heart, spleen and thymus. Each symbol represents the mean value of one animal per experiment (n = 2 independent experiments). Lines indicate mean values per group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Note that data of ApoE KO HFD 18 weeks are in addition shown in Figure 6.12.
6.3.1.5 Fluorescence Activated Cell Sorting (FACS) Analysis of CD80 in ApoE KO Mice

FACS analysis was performed with single cell suspensions derived from the aorta and carotids of ApoE KO HFD or ApoE KO-cuff ND animals. Animals fed a ND displayed a significantly higher number of isolated CD45-positive leucocytes (Figure 6.14, A, B) than HFD mice. In ND animals, the leucocyte population showed a homogeneous appearance based on light scattering, whereas the population in ApoE KO HFD mice was broader distributed indicating a heterogeneous cell population (Figure 6.14, A). Within the leucocyte population, ApoE KO HFD mice presented a significantly higher expression of CD80 than ND animals (Figure 6.14, C).

Figure 6.14 FACS analysis of the aorta and carotids of ApoE KO HFD (n = 4) and ApoE KO-cuff ND animals (n = 3). (A) Representative FACS plots of an ApoE KO HFD mouse (top) and an ApoE KO-cuff ND mouse (bottom). Values represent the percentage of CD45-positive cells. SSC-A: side-scatter area. CD45: leucocyte marker. (B) Number of CD45-positive cells in ApoE KO HFD and ApoE KO-cuff ND animals. (C) Difference in median fluorescence intensity (ΔMFI) of CD80 in CD45-positive cells. * p < 0.05, *** p < 0.001.

The CD80 expression in the dendritic cell population of different lymph nodes was determined by FACS analysis in the same animals (Chapter 5).
6.3.2 Radiochemistry and Radiotracer Evaluation in the ApoE KO-cuff Mouse Model

6.3.2.1 Radiosyntheses

The precursor compound for $^{18}$F-FDM radiolabeling, 4,6-O-benzylidene-3-O-ethoxymethyl-2-O-trifluoromethanesulfonfyl-1-O-methyl-b-D-glucopyranoside was prepared according to the literature [188]. The radiosynthesis of $^{18}$F-FDM was performed as recently reported [189] but with a slight modification in the process. In brief, $^{18}$F-FDM was obtained in a two-step reaction sequence involving nucleophilic fluorination of fully protected triflate precursor and the subsequent deprotection under acidic conditions (Scheme 6.1). In a typical experiment, 62% radiochemical yield (decay corrected) was achieved with a radiochemical purity of greater than 95%. Radiochemical purities were determined by radio HPLC and radio–thin-layer chromatography (TLC). No free fluoride in the formulated product was detected by radio-TLC. $^{18}$F-FDM was stable in formulated solution up to 5 h determined by HPLC analysis (Figure 6.15).

![Scheme 6.1 Radiosynthetic scheme for the preparation of $^{18}$F-FDM.](image)

![Figure 6.15 HPLC chromatogram of $^{18}$F-FDM directly after radiotracer production for quality control (A) and after 5 h for stability evaluation (B).](image)
The CD80-specific radiotracers \[^{11}\text{C}]\text{AM7} and \[^{18}\text{F}]\text{AC74} were produced according to Scheme 6.2 [83] (A. Chiotellis et al., manuscript in preparation).

![Scheme 6.2 Radiosynthetic schemes for the preparation of \[^{11}\text{C}]\text{AM7} and \[^{18}\text{F}]\text{AC74}.](image)

### 6.3.2.2 Ex Vivo PET Studies in ApoE KO-cuff HFD Mice with \[^{18}\text{F}]\text{FDG} and \[^{18}\text{F}]\text{FDM}

*Ex vivo* PET/CT imaging was carried out with ApoE KO-cuff HFD mice 18 weeks after surgery to investigate tracer uptake in atherosclerotic plaques. Animals were sacrificed 1 h after i.v. tracer administration. The sample preparation included perfusion and dissection of the vasculature prior to the imaging. Oil red o staining was performed with the scanned tissues.

In the dissected blood vessels both radiotracers \[^{18}\text{F}]\text{FDG} and \[^{18}\text{F}]\text{FDM}, accumulated predominantly in the aortic arch, parts of the descending aorta and in the carotid segments close to the implanted cuff and control (Figure 6.16). Radiotracer binding co-localized with the lipid staining. Tracer accumulation in this study depended mainly on the extent of atherosclerotic plaques in the vasculature rather than the radiotracer. Plaque free segments displayed low radiotracer signals. For the tissue scanned with \[^{18}\text{F}]\text{FDG} a higher tracer uptake was observed in the cuff carotid than the control carotid correlating with the oil red o staining. In the \[^{18}\text{F}]\text{FDM} scan a slightly larger area of accumulation was determined in the control than the cuff segments. This is in line with the red staining DS and US of both implants and an extensive lipid staining in the DS control segment.
Figure 6.16 Representative PET images (maximum intensity projections) of the aorta and carotids of ApoE KO-cuff HFD mice scanned with either $[^{18}\text{F}]$FDG (A) or $[^{18}\text{F}]$FDM (B) with corresponding oil red o staining. Scale for minimum to maximum signal. Scale bars 1 mm.

6.3.2.3 In Vivo Radiotracer Evaluation in PET/CT Scans of ApoE KO-cuff HFD Mice

PET/CT scans of ApoE KO-cuff mice were acquired 9, 12 and 15 weeks after cuff implantation. The time points for imaging were chosen based on the plaque development in this mouse model and the inflammation resolution after surgery. A preliminary experiment with C57BL/6-cuff ND mice showed a gradual decrease in post-surgical inflammation visualized by $[^{18}\text{F}]$FDG over time (Figure 6.17). The initially significantly higher $[^{18}\text{F}]$FDG uptake in the cuff/control segments than the background diminished over time and no significant difference was determined after 50 days.
ApoE KO-cuff HFD mice were injected with either $[^{11}\text{C}]\text{AM7}$, $[^{18}\text{F}]\text{AC74}$ or $[^{18}\text{F}]\text{FDM}$ and every mouse was additionally scanned within one week time with $[^{18}\text{F}]\text{FDG}$. Quantitative data analysis of volumes of interest (VOI) US and DS of the cuff and additionally a background value derived from the muscular neck region was performed. The standardized uptake value (SUV) was calculated for every VOI.

For the CD80-specific tracer $[^{11}\text{C}]\text{AM7}$ a significantly higher SUV in the US and DS plaques of the cuff than the background was observed 9 weeks after surgery, indicating an accumulation in atherosclerotic plaques (Figure 6.18). Differences were not significant at later time points. For the CD80 radiotracer candidate $[^{18}\text{F}]\text{AC74}$, a similar tracer accumulation was observed in all evaluated VOIs and at all time points. $[^{18}\text{F}]\text{FDG}$ at any and $[^{18}\text{F}]\text{FDM}$ at the last two time points displayed significantly higher SUV values in the cuff US and DS segments than the background. A significant difference in SUV values between the US and DS segments was only determined for $[^{18}\text{F}]\text{FDG}$ at week 12 after surgery. Note the different animal numbers scanned per radiotracer and that significances were not corrected for multiple comparisons.

Figure 6.17 (A) $[^{18}\text{F}]\text{FDG}$ PET/CT coronal images of a female wild-type C57BL/6 mouse at day 1, 8 and 14 post-surgery. White arrows point at the implanted cuff and control visible in black. PET averaged from 30 – 60 min. (B) $[^{18}\text{F}]\text{FDG}$ radiotracer accumulation in the cuff region, control, scar and background (muscle) at defined time points after surgery.
Figure 6.18 Radiotracer accumulation (SUV ± SD) in the US and DS plaques of the cuff and in the background (muscle neck) in ApoE KO-cuff HFD mice at defined time points after cuff implantation. Evaluated were the CD80-binding tracers $[^{11}C]$AM7 and $[^{18}F]$AC74 as well as the metabolic tracers $[^{18}F]$FDG and $[^{18}F]$FDM. n: number of animals scanned. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. 
6.4 DISCUSSION

Preclinical radiotracer evaluation relies on in vivo studies in animal models of disease. There is currently no consensus about the most appropriate rodent model of atherosclerosis, however several models have been presented and validated [91, 93]. To generate atherosclerosis in rodents, genetically modified animals are fed an atherogenic diet characterized by a high content of saturated fat and cholesterol, but still these models lack an important characteristic of human atherosclerosis namely vulnerable plaques. To overcome this limitation, Cheng et al. presented a novel approach based on the implantation of a shear stress modifier resulting in altered hemodynamics [100]. In this way, stable and vulnerable plaque phenotypes were generated under controlled shear stress conditions in the same animal. To further evaluate and characterize the ApoE KO-cuff mouse model, we performed blood lipid analysis, oil red o staining, histology and expression profiling on mRNA and protein level.

Hyperlipidemia, a pathological condition associated with atherosclerosis, was induced in ApoE KO-cuff mice by feeding a modified Western type diet. In the setup of our study, total cholesterol levels in ApoE KO-cuff mice under HFD or ND were similar to values reported previously [100]. We did not observe a time-dependent change in blood lipids except for HDL with initial fluctuations in the evaluated study groups. The HFD led to an instant increase in total cholesterol and LDL levels which remained high throughout the analyzed period of time. This was in contrast to Cheng et al. who observed a gradual increase in total cholesterol over time [100]. Monitoring blood lipids in mouse models of atherosclerosis is essential since cholesterol levels and endothelial shear stress synergistically influence endothelial dysfunction resulting in plaque growth and the formation of lesions with a vulnerable phenotype [190].

Extensive vascular remodeling processes and plaque development in parallel with changes in inflammatory markers were observed in this mouse model. Plaque burden in ApoE KO-cuff HFD mice increased over time affecting predominantly the aortic inner curvature and branch points, the US/DS segments of the cuff/control as well as branch points further DS the carotid arteries. At the natural predilection sites of the aorta, an increasing plaque load and complexity was found over time. The plaques displayed characteristics of vulnerability including inward remodeling and a lipid-rich core with numerous inflammatory cells. Atherosclerotic plaques in the aorta of ApoE KO-cuff HFD mice were associated with a significant increase in CD68, CD80 and MRC1 mRNA levels compared to wild-type mice that are unsusceptible to atherosclerosis.

The implanted constrictive cuff as well as the non-constrictive control led to significant plaque formation in the adjacent vessel regions. Possibly owing to the proximity of the DS cuff segment to the arterial branch point, lesions in the DS region were present already at early time points, whereas plaques US of the cuff evolved over a longer timeframe.

The non-constrictive control implanted around the contralateral carotid artery was intended as internal control. However, several atherosclerotic plaques were formed in the control segment potentially triggered by a perivascular inflammation with cytokine release, vascular damage or
altered laminar blood flow as previously described in ApoE*3Leiden mice [191]. Plaques developed in the DS control segment in short periods of time, whereas US of the non-constrictive control in only one third of animals plaques were determined at late time points. At 9 weeks post-surgery, plaques were recognized within the control segment possibly due to the downstream growth of the US plaques since plaques generally expand towards the direction of lower shear stress [192].

Histological analysis of the heart of ApoE KO-cuff HFD mice in this study furthermore demonstrated that coronary vessels were not affected by atherosclerosis. However, large arterial vessels originating from the heart and surprisingly even aortic valves displayed numerous lesions. This might explain the higher CD68 and CD80 expression in the heart of ApoE KO-cuff HFD than C57BL/6-cuff ND mice.

By measuring the combined CD80 protein expression of the aorta and the carotids in CD45-positive leucocytes by FACS, ApoE KO HFD mice displayed a significantly higher average expression than ApoE KO-cuff ND animals. This finding indicates a major influence of the diet on the expression of the costimulatory molecule. Since the feeding of a HFD to ApoE KO mice accelerates atherosclerosis development, we postulate that in this mouse model CD80 expression correlates with plaque burden and vulnerability as observed in vitro in humans [83]. Since the implants did not promote plaque development considerably in ApoE KO ND mice, the different surgery status of the study groups in the FACS experiment might be of minor relevance.

Based on human autopsy studies, vulnerable atherosclerotic plaques that are prone to rupture are characterized as lesions with a thin fibrous cap, a large and soft lipid-rich core and numerous inflammatory cells [23]. Previous publications described that the plaques generated in this mouse model could be differentiated based on histology and gene expression profiling into stable and vulnerable plaques [100-103]. Differences in plaque size, smooth muscle cell number, collagen and lipid content, hemorrhage and remodeling were described whereas macrophage content was similar in US and DS plaques in previous studies [100, 185]. In our hands, atherosclerotic plaques in this mouse model did not differ significantly neither depending on the location relative to the implant nor on the flow hemodynamics controlled by the different implants. The lesions featured cholesterol crystals, immune cell infiltration with CD68-positive macrophages, focal foam cell accumulations, a large lipid-rich core and a constrictive vascular remodeling of heterogeneous organization. Hence histopathologically, these lesions were classified as vulnerable plaques. Based on macrophage number, lipid content and general plaque morphology, no distinct difference of the US compared to the DS segments was observed with two exceptions. First, a slightly more heterogeneous plaque composition was noted US than DS of the cuff. And second, the plaques adjacent to the control were smaller in size than those adjacent to the cuff. Our findings stand in contrast to previous reports about different plaque phenotypes in this mouse model. However, differences in experimental setup, the age of the animals at study beginning, analysis time points, the specific formulation of the diet and a generally high variance in this mouse model could explain the discrepancy.

Given the uniform plaque phenotype in this mouse model, a similar change in mRNA expression of CD68, CD80 and MRC1 US and DS of the cuff and control is anticipated as it was observed in this
study. Major differences in expression were only detected between the different animal populations reflecting the influence of the diet and the genetic background of the mice. Surprisingly, the expression within the implants was comparable or even higher than in the US/DS segments. A potential reason for the increased expression of the inflammatory markers is the plaque development within the control segment, however this is not applicable to the cuff segment. The high expression of the inflammatory markers within the implants could be explained by substantial perivascular inflammatory reactions caused by the foreign objects. The inflammatory cells were predominantly located between the vessel and the implants as well as surrounding the implants. However, only the latter could be removed completely prior to RNA isolation. Other limitations of the mRNA expression profiling experiments is the small number of samples per time point and a potential dilution of RNA of small cellular subsets. Overall, an increase in the expression of the inflammatory markers and in particular CD68 was in agreement with the plaque burden visualized by oil red o staining.

In the early stage in the ApoE KO-cuff mouse model, local hemodynamic conditions and shear stress in the straight carotid vessel are defined by the implanted cuff. Once atherosclerotic plaques are formed, these lesions alter local hemodynamics drastically thereby influencing plaque progression and composition [193]. Even within a single atherosclerotic plaque differences in composition were described. It was reported that the US plaque shoulder contains more macrophages and less smooth muscle cells than the DS shoulder which leads to a destabilization of the plaque and thus plaque rupture occurs more frequently in the US area [194–196]. Therefore, even the sampling location for histological analysis affects the classification of the atherosclerotic plaques in this mouse model. Furthermore, a synergistic effect of cholesterol levels and local endothelial shear stress was described in pigs [190]. Differences in plaque histomorphologic characteristics could therefore additionally reflect inter-individual differences in the plasma lipoprotein profile. These factors could explain large inter-individual differences in the ApoE KO-cuff mouse population thereby also negatively affecting reproducibility and reliability of this mouse model.

However, an important feature of human atherosclerosis, the rupture of vulnerable plaques and thereby the occlusion of arterial segments, was not observed in this study in accordance with previous reports [100, 101]. Nevertheless, this mouse model is still of interest to investigate vulnerable plaques in vivo and evaluate molecular imaging strategies [102].

Owing to the elevated CD80 levels in plaques, the ApoE KO-cuff mouse model was considered a promising animal model for the evaluation of two CD80-specific radiotracers, [11C]AM7 and [18F]AC74 developed in our group. Binding affinity of the radiotracer candidates was tested in an assay with human recombinant CD80 revealing an IC50 value of 2.0 ± 0.2 nM for [11C]AM7 and 40.1 ± 20.9 nM for [18F]AC74 (manuscript in preparation by A. Chiotellis et al., Chapter 5). So far, [11C]AM7 was evaluated in vitro and in xenograft bearing mice revealing an accumulation in CD80-positive tissue in vivo [83], but not yet in any animal model related to atherosclerosis. In this study, [11C]AM7 and [18F]AC74 were compared to the well-characterized imaging agent [18F]FDG and its stereoisomeric counterpart [18F]FDM. Both of them are metabolic tracers that are transported into
the cell by glucose transport proteins (GLUT). Subsequently, $^{18}$FFDG and $^{18}$FDM are phosphorylated by hexokinase and thereby intracellularly trapped since the backward reaction is considerably slower and no further conversion along the glycolytic pathway is feasible due to the lack of the 2-OH group [189, 197]. $^{18}$FDM additionally binds to the macrophage mannose receptor (MMR, CD206) that is a surface molecule of alternatively activated macrophages (M2a) [12]. The macrophages found in atherosclerotic plaques are highly heterogeneous. So far, controversial statements were published regarding the association of MMR-expressing macrophages and plaque phenotype. Chinetti-Gbaguidi et al. described that CD68$^+$ MMR$^+$ macrophages were localized in stable cell-rich areas of human atherosclerotic plaques [198]. Cho et al. reported that anti-inflammatory M2 macrophages were more frequent in stable plaques and in asymptomatic patients [199]. On the contrary, Tahara et al. stated that M2 and MMR-expressing macrophage subpopulations were more abundant in human thin-cap fibroatheroma and scarcely detected in stable plaques [200].

Radiotracer accumulation was investigated in ApoE KO-cuff HFD mice by in vivo and ex vivo PET/CT imaging. $^{18}$FFDG and $^{18}$FDM accumulated ex vivo in atherosclerotic plaques of the aorta and carotids in ApoE KO-cuff HFD mice co-localizing with the lipid staining. These ex vivo studies were performed in perfused arterial vessels to exclude any blood-related radiotracer binding. No ex vivo imaging studies were performed with the CD80-specific radiotracers due to the short half-life of carbon-11 and the plaque-independent distribution of $^{18}$FAC74 determined by in vivo PET scans.

In vivo radiotracer uptake was analyzed in the cuff segment only since plaque formation was more reproducible and reliable in neighboring cuff regions than on the contralateral common carotid artery. PET scans revealed that the high-affinity CD80 radiotracer $^{[11]}$CAM7 displayed a higher accumulation in the carotid segments US and DS of the cuff than the background at all examined time points with a significant difference 9 weeks after cuff placement. The low-affinity CD80 radiotracer $^{[18]}$FAC74 showed an unspecific distribution in vascular regions around the cuff comparable to the background. For both glucose analogs, a significantly higher radiotracer uptake was observed in the cuff regions than the background. For $^{[18]}$FDG, we observed a trend towards higher accumulation US than DS which is in line with a study by Wenning et al. [103]. They observed significant differences at all examined time points in contrast to our results. This discrepancy is probably due to the higher animal number used in the study of Wenning et al., different imaging time points and differences in plaque phenotype. Based on our histological analysis revealing a similar plaque phenotype US and DS of the cuff, the minor difference in accumulation between these segments is not unexpected. In conclusion, this PET study implies that $^{[11]}$CAM7 accumulates in atherosclerotic plaques in vivo. The detected difference between plaque-to-background accumulation for $^{[11]}$CAM7 is smaller than for $^{[18]}$FDG or $^{[18]}$FDM. However, this could be critically influenced by the different animal number used with approximately three times more mice undergoing $^{[18]}$FDG scans. The better in vivo plaque uptake of $^{[11]}$CAM7 compared to $^{[18]}$FAC74 reflects the in vitro binding affinity data.
The glucose analogs, $^{18}$FFDG and $^{18}$FDM, showed *in vivo* comparable results and both accumulated in atherosclerotic plaques US and DS of the cuff. Tahara *et al.* observed a similar accumulation of $^{18}$FFDG and $^{18}$FDM in atherosclerotic regions of a rabbit atherosclerosis model [200]. The impact of $^{18}$FDM binding to MMR may be negligible since MRC1 gene expression levels were not elevated in ApoE KO-cuff mice reflecting theoretically also MMR protein expression. Therefore, it can be assumed that $^{18}$FFDG and $^{18}$FDM accumulate both predominantly in macrophages via intracellular trapping which would explain the similar *in vivo* results. Based on this *in vivo* data, the use of the mannose isomer $^{18}$FDM seems not to present an advantage over $^{18}$FFDG for atherosclerosis imaging.

The used animal model in combination with the radiotracers included in this study imply that animal number is in general a critical factor in studies with ApoE KO-cuff mice. For future studies, appropriate animal numbers should be used to test specific hypotheses allowing sufficient statistical power. For CD80 imaging, future studies aim at optimizing the current lead structure of $^{11}$C]AM7 by maintaining the high binding affinity but at the same time improving pharmacokinetic compound characteristics [83].

Overall, the ApoE KO-cuff mouse model is a suitable model to investigate atherosclerosis development *in vivo* and to evaluate novel diagnostic or therapeutic interventions. However, some limitations apply by performing PET imaging in this mouse model. Murine atherosclerotic plaques are small with a size around the resolution limit of current PET systems. Therefore, it can be assumed that the partial volume effect influences the detected PET signal and the measured activity in the plaques might be underestimated. Furthermore, the extensive perivascular inflammation reactions in the cuff and control carotid segments could affect radiotracer accumulation. Nevertheless, the ApoE KO-cuff mouse model is an interesting tool to investigate vulnerable atherosclerotic plaques. Furthermore, this model is well suited for the evaluation of molecular imaging strategies *in vivo* after a thorough expression analysis of the targets of interest.
6.5 CONCLUSIONS

A substantial development of atherosclerotic plaques in the arterial tree of ApoE KO-cuff mice and changes in the mRNA and/or protein expression of the inflammatory markers CD68 and CD80 were observed. Plaques generated in this mouse model were of a vulnerable phenotype, however in contrast to previous publications, no differences in plaque phenotype between cuff US and DS regions were observed. In PET/CT imaging studies, the CD80-specific radiotracer $[^{11}\text{C}]\text{AM7}$ and the metabolic tracers $[^{18}\text{F}]\text{FDG}$ and $[^{18}\text{F}]\text{FDM}$ accumulated at sites of atherosclerotic plaque development in the US and DS segments of the cuff whereas no accumulation was observed for $[^{18}\text{F}]\text{AC74}$. Overall, ApoE KO-cuff HFD mice are a valid model to evaluate CD80-targeting PET tracers in vivo.
6.6 EXPERIMENTAL SECTION

6.6.1 General
All reagents and solvents were purchased from Sigma-Aldrich Chemie GmbH (Germany), Merck (Germany), Acros Organics (Belgium), ABCR GmbH (Germany) or Fluka (Switzerland) and were used without further purification.

6.6.2 Animals
Animal care and experiments were approved by the Veterinary Office of the Canton Zurich (Switzerland) and were conducted in accordance with the Swiss animal Welfare legislation. Four-week old male ApoE KO mice (B6.129P2-Apoetm1Unc/J) and five-week old male C57BL/6J mice were supplied by Charles River Laboratories (Sulzfeld, Germany). ApoE KO mice (n = 51) were fed a modified Western type diet containing 21% fat, 0.25% cholesterol and 19.5% casein ad libitum starting when they were five weeks old (Kliba Nafag, Kaiseraugst, Switzerland). For blood lipid analysis, nine ApoE KO and six C57BL/6 mice were fed a standard chow diet ad libitum.

6.6.3 Implantation of a Shear Stress-Modifying Cuff and a Non-Constrictive Control
The implantation of a constrictive cuff around the right common carotid artery and a non-constrictive control around the left common carotid artery was performed according to previous reports [100, 102]. Four weeks after HFD onset, cuff and control implantation surgery was carried out. In brief, premedication consisting of 0.04 mg/kg fentanyl and 4 mg/kg midazolam were administered intraperitoneally (in 0.9% NaCl). Animals were kept under isoflurane anesthesia during the whole surgery lasting approximately 30 min. Mice were placed in supine position on a heated surgical plate and the operating field was shaved and disinfected. A small medial incision in the skin between the mandible and sternum was applied and the common carotid arteries were exposed in succession. The two halves of the constrictive cuff (US inner diameter 500 µm, DS inner diameter 250 µm) were placed around the artery and fixed with a silk suture. On the contralateral side, a polyethylene tube (PE10, Smith Medicals) was implanted. After surgery, 5 mg/kg carprofen was administered subcutaneously to the mice and if required repeated once. The constrictive cuff was supplied by Promolding BV (Den Haag, the Netherlands) and consisted of polymethylpentene.

6.6.4 Blood Collection and Blood Lipid Analysis
C57BL/6-cuff and ApoE KO-cuff animals were anesthetized by isoflurane inhalation prior to blood sampling. Blood (50 – 200 µL) was collected from the sublingual vein into heparinized Microvette 200 LH tubes (Sarstedt, Nümbrecht, Germany) after incision with a 24 gauge needle. Samples were centrifuged at 2000 x g for 20 min (4 °C), the plasma was transferred to a new tube and stored at -80 °C up to one month. The blood lipid parameters total cholesterol (TCHO), triglyceride (TG) and high-density lipoprotein (HDL) were determined using a Fuji Dri-chem 4000i analyzer (Polymed
Medical Center AG, Glatthoeg, Switzerland). The corresponding low-density lipoprotein level (LDL) was calculated based on the Friedewald equation [201]:

\[
[\text{LDL}] = [\text{TCHO}] - [\text{HDL}] - 0.45 \cdot \text{TG}.
\]

Blood collection was performed every two weeks in alternating groups of three out of six ApoE KO-cuff HFD mice, ApoE KO-cuff ND mice and C57BL/6-cuff ND. For statistical analyses, mean values of three animals per time point and group were used.

### 6.6.5 Intracardiac Perfusion and Tissue Dissection

Animals were kept under isoflurane inhalation during the whole procedure and reflexes were tested frequently. Intracardiac perfusion was carried out with 0.9% NaCl for 6 min followed by 4% paraformaldehyde (PFA, in 0.9% NaCl) for 8 min. Tissue samples for RNA isolation were only perfused with 0.9% NaCl and after dissection stored at -80 °C. Dissected tissues included the heart, spleen, thymus, aorta and carotids. Dissected samples for immunohistochemistry and autoradiography were post-fixated with 4% PFA for 24 h at 4 °C. Samples for cryo-sectioning were additionally transferred to 30% sucrose solution for 72 h at 4 °C and thereafter stored at -80 °C except for the aorta and the carotids which were transferred to PBS (pH 7.4) for long-term storage (4 °C). The dissection of the aorta and carotids was performed using a SMZ1000 microscope (Nikon, Melville, NY, USA) and pictures were taken by a Nikon 1 J3 camera.

### 6.6.6 Oil Red O Lipid Staining of Aorta and Carotid Arteries

The procedure of the oil red o lipid staining was adapted from [155] and all steps were performed at room temperature. In brief, fixation of the aorta and carotids was done with 4% PFA for 10 min, followed by two washing steps with PBS (pH 7.4) and a preincubation in 60% 2-propanol for 5 min. The staining was performed with 0.3% (w/v) oil red o in 60% 2-propanol for 30 min on a shaking platform. In a final washing step under running tap water (2 min) unbound dye was removed.

### 6.6.7 Total RNA Isolation, Reverse-Transcription and Quantitative Polymerase Chain Reaction

Total RNA was isolated from the aortic arch, the descending aorta, right carotid artery, left carotid artery, heart, thymus and spleen. Each carotid artery was segmented into a piece upstream, within and downstream of the implanted cuff or control. For RNA isolation the Isol-RNA Lysis reagent (5 PRIME, USA) and the TissueLyser bead-mill system (Qiagen, Germany) was used and cDNA was generated by the QuantiTect Reverse Transcription Kit (Qiagen, Germany). Primers were custom-made oligonucleotides supplied by Microsynth (Balghac, Switzerland): murine actin β (ACTB, NM_007393.3) (forward 5'-AGACCTCTATGCACACATG-3', reverse 5'-TGCTAGGA-GCCAGACAGAA-3'), murine CD68 (NM_009853) (forward 5'-CTGTTACCTTGACCTGCTT-3', reverse 5'-AACATGGCGAAGGTGCT-3'), murine T-lymphocyte activation antigen CD80 (NM_009855.2) (forward 5'-TGGCTCTGATTCCTTCTAC-3', reverse 5'-GAGGAGGTTGTA-
CGGCAAG-3’), murine macrophage mannose receptor 1 (MRC1, NM_008625.2) (forward 5’-CTGGTCTCCTCCTGATTGTGA-3’, reverse 5’-CTTGAGGTATGTGCAACGCA-3’). qPCR was performed on an AB7900 HT Fast Real-Time PCR system (Applied Biosystems) with SDS software (Applied Biosystems, Carlsbad, USA) using the GoTaq qPCR Master Mix (Promega, Dübendorf, Switzerland). Relative gene expression with murine β-actin as reference gene was calculated based on the 2-ΔΔCt quantification method [110]. Reactions were performed in duplicates in two independent experiments and dissociation curve analysis was applied to verify the specificity of the amplification product. Log-transformation of the relative expression values was performed to assure uniform variances between groups. Statistical data analysis per evaluated region was carried out with mean values of one animal per experiment.

6.6.8 FACS Analysis of the Aorta and Carotids of ApoE KO Animals

Mouse aortas and carotids of ApoE KO animals fed a HFD (n = 4) and ApoE KO-cuff animals fed a ND (n = 3) were dissected after perfusion with PBS (pH 7.4). Tissues were cut into small pieces and incubated at 37 °C in Liberase III and DNase (Roche) according to the manufacturer’s protocol for 45 min in an overhead shaker. Tissue suspensions were passed through a 40 µm cell stainer (BD Bioscience) with PBS containing 2.5% fetal bovine serum (FBS) and 2 mM EDTA. Subsequently, cell suspensions were centrifuged for 5 min at 1200 rpm (4 °C) and resuspended in PBS with 2.5% FBS and 2 mM EDTA. Cell suspension were stained with the following antibodies: rat anti-mouse CD4-APC (clone GK1.5, Biolegend), rat anti-mouse CD45-PerCP (clone 30-F11, Biolegend), rat anti-mouse CD4-PE (clone GK1.5, Biolegend), hamster anti-mouse CD80-PE (clone 16-10A1, Biolegend), hamster IgG isotype control (eBioscience), mouse anti-mouse CD45.2-FITC (clone 104, BD Pharmingen), rat anti-mouse CD16/32 (clone 93, Biolegend), hamster IgG isotype control APC (clone HTK888, Biolegend), rat IgG2a κ isotype control (BD Pharmingen), rat anti-mouse I-A/I-E-PerCP (clone M5, Biolegend), mouse anti-mouse IgG1 κ isotype control FITC (clone MOPC-21, Biolegend). FACS analysis was performed on a BD FACSCanto instrument with FACSdiva software (BD Bioscience) and data was analyzed with the software FlowJo (Treestar). The number of CD45-positive cells and the difference in median fluorescence intensity (ΔMFI) between CD45 and isotype control was determined.

6.6.9 Histology and Immunohistochemistry

For histological analysis, aorta and carotid samples of ApoE KO-cuff mice 6, 9, 16 and 18 weeks post-surgery and the heart of an ApoE KO-cuff HFD mouse (18 weeks) were used. Tissues were paraffin-embedded and 2.5 µm serial sections were prepared. Histological sections were routinely stained with hematoxylin and eosin (HE). Sections for immunohistochemistry were labeled with rabbit anti-mouse CD68 (1:100, ab125212, Abcam). Antigen retrieval was performed by heat treatment for 20 min at 98 °C. The detection system consisted of the Omni Map anti-Rb HRP Kit (Roche, Rotkreuz, ZG, Switzerland) on the Discovery XT instrument (Roche). Sections were scanned with a digital slide scanner (Pannoramic 250, Sysmex, Horgen, Switzerland). HE and IHC staining were analyzed by a pathologist (N.B.) considering plaque composition and in particular the
localization and amount of CD68-positive cells. Lesions were classified as fatty streaks, stable and vulnerable plaques according to the adapted criteria of Table 2.1.

6.6.10 Radiotracer Production

In-house radiotracer syntheses of $[^{11}C]$AM7 and $[^{18}F]$AC74 were performed according to [83] (for $[^{18}F]$AC74: manuscript in preparation A. Chiotellis et al.). $[^{18}F]$FDG was supplied by the University Hospital of Zurich. $[^{18}F]$FDM was produced according to the following procedure. No-carrier-added $[^{18}F]$fluoride was produced via the $^{18}$O(p,n)$^{18}$F nuclear reaction by irradiation of enriched $^{18}$O-water in an IBA cyclotron (Cyclone® 18/9). $[^{18}F]$Fluoride was immobilized on an anion-exchange cartridge (QMA Light, Waters) which was preconditioned with K$_2$CO$_3$ (5 mL, 0.5 M), followed by 5 – 10 mL water. The activity was eluted with a solution of Kryptofix (5 mg), potassium carbonate (1 mg) in water and MeCN (1.1 mL, water/MeCN: 1/2 (v/v)). The solution was dried under vacuum with a stream of nitrogen at 110 °C. Azeotropic drying was repeated with 0.8 mL MeCN to afford dry KF$[^{18}F]$-Kryptofix complex. To the dried $[^{18}F]$fluoride complex (typically 20 GBq), precursor (5 mg in 1.0 mL CH$_3$CN) was added and the reactive vial was heated at 110 °C for 10 min. Then the reaction mixture was evaporated to dryness under reduced pressure with a slight inflow of nitrogen. To the crude material HCl (1 mL, 5 M) was added, and the reaction mixture was heated at 110 °C for 20 min. The mixture was neutralized by the addition of NaOH (4 M, 1 ml) and Na$_2$HPO$_4$ (400 mM, 1 mL) and purified by semi-preparative HPLC (HPLC; column: YMC Pack ODS-A, 250 x 20 mm, 5 μm; elute: 0.9% aqueous NaCl; flow rate: 3.0 mL/min). Radiochemical purity was determined by analytical radio HPLC (column: YMC-Pack Polyamine II, 250 x 4.6 mm, 5 μm; elute: isocratic 75% MeCN in water; flow rate: 1.0 mL/min) and radio TLC (60 F254; Merck, elute: MeCN/H$_2$O, 95/5 (v/v)).

6.6.11 Ex Vivo PET/CT Imaging

Animals were intraperitoneally injected with 50 MBq $[^{18}F]$FDG (n = 2) or 50 MBq $[^{18}F]$FDM (n = 2) 120 min prior to PET/CT imaging. One hour after injection, 4.5 mg pentobarbital in 0.9% NaCl was intraperitoneally administered and mice were perfused with PBS (pH 7.4). The aorta and the carotids were dissected and a static PET scan was performed over 90 min followed by CT acquisition (100 μA, 30 kV, 4 shots, 360 projections). After PET/CT scans, the tissues were stained with oil red o.

6.6.12 In Vivo PET/CT Imaging

ApoE KO-cuff mice were kept throughout the entire PET/CT acquisition under isoflurane anesthesia (1.5 – 5%) and respiratory frequency as well as body temperature were monitored. Animals were scanned with a VISTA eXplore small animal PET/CT camera (Sedecal/GE, Madrid, Spain). PET data reconstruction was performed in user-defined frames by 2-dimensional-ordered subsets expectation maximization (2D-OSEM) with a voxel size of 0.3875 x 0.3875 x 0.775 mm$^3$ applying random and scatter correction. Anatomical information was obtained by CT following PET
acquisition with optimized parameters to visualize the cuff and control (140 µA, 35 kV, 8 shots, 360 projections). Detailed PET protocols are delineated in Table 6.1.

<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>Administration route</th>
<th>Injected activity [MBq]</th>
<th>Scan start p.i. [min]</th>
<th>Scan time [min]</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>[11C]AM7</td>
<td>i.p.</td>
<td>9 – 20</td>
<td>1</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>[18F]AC74</td>
<td>i.p.</td>
<td>8 – 19</td>
<td>1</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>[18F]FDG</td>
<td>i.v.</td>
<td>9 – 20</td>
<td>30</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>[18F]FDM</td>
<td>i.v.</td>
<td>10 – 18</td>
<td>30</td>
<td>30</td>
<td>8</td>
</tr>
</tbody>
</table>

PET scans were analyzed with the PMOD biomedical imaging software (3.6, PMOD Technologies Ltd., Zurich, Switzerland). In total, seven volumes of interest (VOI) (1 mm³ each) were placed based on the CT location of the cuff in one animal. Three VOIs US and DS of the cuff and one in the neck region (background). Radioactivity per VOI was expressed as standardized uptake value (SUV). For the US and DS cuff region, averaged SUV values of the VOIs were used for statistical analyses.

To quantify the post-surgical inflammation, ~10 MBq of [18F]FDG was injected into the tail vein of female C57BL/6-cuff mice (n = 6). Dynamic PET scans were acquired from 30 – 60 min post injection, followed by a CT scan. VOIs for the cuff, control, scar and background (muscle) were manually defined and used for SUV calculation.

6.6.13 Statistics

Statistical data evaluation was performed with GraphPad Prism (GraphPad, La Jolla, CA, USA). Outliers in the gene expression data were identified by the ROUT method (Q = 1%) and grouped values were tested for Gaussian distribution with the D’Agostino-Pearson omnibus normality test. For gene expression analysis and PET quantification, intergroup significance was tested by a one-way ANOVA with a Tukey's multicomparison test after a multiple ANOVA test. For FACS data analysis, a two-tailed unpaired student’s t-test was performed. A p-value < 0.05 was considered significant (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Acknowledgements

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This work was financially supported by the Clinical Research Priority Program (CRPP) of the University of Zurich on Molecular Imaging (MINZ). The authors acknowledge support of the Scientific Center for Optical and Electron Microscopy (ScopeM) of the ETH Zurich.
7 EVALUATION OF THE RADIO-LABELED BORONIC ACID-BASED FAP INHIBITOR MIP-1232 FOR ATHEROSCLEROTIC PLAQUE IMAGING

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AUTHOR CONTRIBUTIONS

R. Meletta planned and coordinated the study, established and performed radiolabeling, qPCR and autoradiography, analyzed the data and wrote the manuscript. A. Müller Herde supervised the study, experimental planning and data interpretation, contributed to and supervised the manuscript writing. A. Chiotellis supervised organic synthesis, contributed to manuscript writing; M. Isa performed organic synthesis of precursor and reference compound and qPCR experiments, assisted in establishing the radiolabeling. N. Borel supervised and analyzed the immunohistochemistry experiments. Z. Rancic was responsible for collection of human carotid material at the University Hospital Zurich and was involved in project planning and plaque characterization. S. M. Ametamey was involved in the discussion of the results and revision of the manuscript. S. D. Krämer contributed to the supervision and planning of the experiments and revised the manuscript. R. Schibli initiated the project and was involved in the discussion of the results and revision of the manuscript.
Chapter 7

7.1 ABSTRACT

Research towards the non-invasive imaging of atherosclerotic plaques is of high clinical priority as early recognition of vulnerable plaques may reduce the incidence of cardiovascular events. The fibroblast activation protein alpha (FAP) was recently proposed as inflammation-induced protease involved in the process of plaque vulnerability. In this study, FAP mRNA and protein levels were investigated by quantitative polymerase chain reaction and immunohistochemistry, respectively, in human endarterectomized carotid plaques. A published boronic-acid based FAP inhibitor, MIP-1232, was synthetized and radiolabeled with iodine-125. The potential of this radiotracer to image plaques was evaluated by in vitro autoradiography with human carotid plaques. Specificity was assessed with a xenograft with high and one with low FAP level, grown in mice. Target expression analyses revealed a moderately higher protein level in atherosclerotic plaques than normal arteries correlating with plaque vulnerability. No difference in expression was determined on mRNA level. The radiotracer was successfully produced and accumulated strongly in the FAP-positive SK-Mel-187 melanoma xenograft in vitro while accumulation was negligible in an NCI-H69 xenograft with low FAP levels. Binding of the tracer to endarterectomized tissue was similar in plaques and normal arteries, hampering its use for atherosclerosis imaging.
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7.2 INTRODUCTION

The concept of plaque vulnerability has changed the understanding of the pathogenesis of atherosclerosis and has led to novel perspectives for diagnostic and therapeutic interventions. The development of diagnostic methods to assess plaque vulnerability is considered an urgent priority in clinical and basic research [23]. The assessment of plaque vulnerability in patients at risk for cardiovascular disease would allow an adequate pharmacological and/or surgical treatment already in the asymptomatic stage and, therefore, reduce atherosclerosis-associated disability and mortality. Molecular imaging with suitable tracers has the potential to non-invasively identify molecular processes providing functional information about disease progression. In the asymptomatic stage, functional imaging may thus provide more specific information on plaque vulnerability than morphology-based imaging modalities [35]. Several imaging targets and the respective tracers are under investigation with the goal to image plaque vulnerability. The most prominent tracer is [18F]fluorodeoxyglucose, which accumulates in cells with high glucose consumption, including activated macrophages. However, the unspecific mechanisms of accumulation and the high uptake in myocardium limit its applicability [202].

Nowadays, plaque progression is regarded as a dynamic and complex process with stabilizing and destabilizing components involved. If destabilizing plaque components prevail over stabilizing factors an atherosclerotic plaque may eventually rupture leading to often severe or even fatal complications. Stabilizing components include an intact and thick fibrous cap that is formed by smooth muscle cells (SMCs) embedded in an extracellular matrix rich in collagen. On the contrary, plaque vulnerability is related to a thinning of the fibrous cap facilitated by the gradual loss of SMCs and the degradation of the collagen-rich fibrous cap [203]. The digestion of the extracellular matrix is caused by proteases in the atheroma which include matrix metalloproteinases (MMPs), cathepsins S/K and as recently proposed the fibroblast activation protein alpha (FAP, seprase) [75, 88, 204, 205]. FAP is a type II membrane-bound serine protease belonging to the subfamily dipeptidyl peptidase IV N-terminal (DPP IV, S9B) within the prolyl oligopeptidase family (POP, S9) [206-208]. In contrast to other members of the DPP IV subfamily, FAP displays endo- besides exopeptidase activity [209]. FAP is capable of cleaving peptide bonds between proline and another amino acid [209]. FAP has gelatinase activity and is involved in the further digestion of degradation products of type I collagen [210-213]. The endo- and exopeptidase enzymatic activity requires homodimerization and glycosylation of the protease [207, 211, 214].

FAP was initially identified as a pivotal component of the tumor microenvironment expressed by reactive stromal fibroblasts in over 90% of common human epithelial carcinomas and may serve as a therapy target in oncology [86, 87, 215]. Furthermore, an association of FAP expression with inflammatory processes was described [86] and in line with this finding is emerging data by Brokopp et al. indicating an involvement of FAP in the pathogenesis of atherosclerosis [88]. In detail, Brokopp et al. showed that FAP is expressed by SMCs in human aortic plaques and confirmed its involvement in type I collagen degradation in aortic fibrous caps. Moreover, an association between tumor necrosis factor alpha (TNFα) secretion by macrophages with FAP expression in
cultured human aortic SMCs and additionally a positive correlation of FAP-expressing SMCs with the macrophage burden in human aortic plaques was described [88]. The extent of FAP expression at different stages in atherosclerotic plaque progression was evaluated and revealed an increased FAP expression in advanced aortic plaques and in thin-cap versus thick-cap coronary atheroma by immunohistochemistry and immunofluorescence [88]. These findings indicate that FAP expression is related to plaque vulnerability with FAP representing an inflammation-induced protease in atherosclerosis. In this respect, FAP could serve as a promising target for non-invasive atherosclerotic plaque imaging.

The goal of this study was to evaluate FAP as a target for atherosclerosis imaging. Imaging FAP density requires a FAP-selective ligand with high binding affinity. Several research groups have pursued to design small inhibitors with high specificity and selectivity towards individual serine proteases in the POP family. To selectively target FAP over other peptidases, its dual enzymatic activity as endo- and exopeptidase has to be considered. Identifying inhibitors with high selectivity for FAP over other DPPs and the most closely related prolyl endopeptidase PREP is challenging due to the 48% amino acid sequence identity of FAP and DPP-4, analogous substrate preferences and the ubiquitous expression of many proteases of the POP family [206, 208]. Most FAP inhibitors share the pyrrolidine-2-boronic acid moiety as a common structural motif. The first boronic acid inhibitor reaching phase II clinical trials in the field of cancer treatment was ValboroPro (talabostat, PT-100), however due to missing selectivity clinical evaluation was terminated [216-218]. ValboroPro displayed IC$_{50}$ values in the nanomolar range to several prolyl peptidases [219]. The introduction of a blocked N-terminus in the dipeptidyl boronic acid structure led to novel inhibitors that were evaluated regarding binding affinity and selectivity [220-224] with the advantage of impeded intra-molecular cyclization reactions mediated by the electrophilic boron and an increased selectivity over DPPs that lack endopeptidase activity [225].

Marquis et al. presented a para-iodine substituted benzamido-glycine-boronoproline analog, MIP-1232, with an IC$_{50}$ of 0.6 nM as determined in an enzyme inhibition assay with human recombinant FAP [224]. MIP-1232 was 32-fold more potent in inhibiting FAP than PREP. The corresponding K$_d$ value of $[^{123}$I]MIP-1232 in stably FAP-transfected human embryonic kidney cells (HEK-293) was 30 nM and different FAP-positive cell lines showed a markedly reduced enzymatic activity under MIP-1232 treatment compared to baseline conditions [224, 226]. The high binding affinity to FAP and the selectivity profile in combination with the possibility to radiolodinate MIP-1232 without altering its structure make this compound a promising molecule to assess the potential of FAP as an imaging target for the staging of plaque vulnerability and to detect FAP-positive tumors that may respond to FAP-targeted therapy. In this study, we investigated FAP expression in human carotid specimens by quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC). Furthermore, we synthesized MIP-1232 and subsequently radiolabeled this compound with iodine-125. Its accumulation in human atherosclerotic plaques was evaluated in vitro by autoradiography. A FAP-positive SK-Mel-187 melanoma xenograft and an NCI-H69 xenograft with low FAP levels, both grown in mice, were used as controls.
7.3 RESULTS

7.3.1 Gene Expression Analysis of FAP and SMA in Human Carotid Plaques

Quantitative expression analysis of FAP and alpha smooth muscle cell actin (SMA) by qPCR was performed with β-actin as reference gene (Figure 7.1). For FAP, a similar average gene expression was determined in normal arteries, stable plaques and vulnerable plaques (Figure 7.1, A). The average SMA gene expression was not significantly different comparing vulnerable and stable plaques (Figure 7.1, B). No significant correlation between the SMA and FAP gene expression in human endarterectomized plaques was observed (Figure 7.1, C).

![Figure 7.1](image)

**Figure 7.1** Relative mRNA expression levels of FAP (A) and SMA (B) in normal arteries (n = 2), stable plaques (n = 11) and vulnerable plaques (n = 9). For both proteins no significant difference was detected between stable and vulnerable plaques. (C) Comparison of the relative mRNA expression levels of FAP and SMA. mRNA expression was quantified by qPCR, shown are averages of three independent analyses. Lines indicate mean values. The square bracket indicates an outlier that was excluded from statistical analyses.

7.3.2 Immunohistochemical Staining of Human Carotid Plaques for FAP and SMA

The expression of FAP and SMA was further investigated by immunohistochemistry in consecutive sections of human atherosclerotic plaques (Figure 7.2). Normal arteries were FAP negative. In plaques, a focal FAP expression in macrophages and giant cells located in the superficial regions of the fibrous cap was observed with the most pronounced focal signals in vulnerable plaques (Figure 7.2, C2, D1, D2). SMA was strongly expressed in the tunica media in all three classification categories with the highest expression in the vasa vasorum of normal arteries (Figure 7.2, A1). The distribution pattern of SMA within atherosclerotic plaques was generally focal with major clusters in the cap or shoulder region. No distinct co-localization of the two expression markers was found in all examined carotid plaques (Figure 7.2, B1, B2).
Figure 7.2 Hematoxylin/eosin (HE; A, B, C) and immunohistochemical (A, B, C, D) staining for FAP and SMA of representative 2 μm paraffin-embedded sections of a normal artery (A), a stable plaque (B) and vulnerable plaques (C, D). Boxed higher-magnification images show a small blood vessel (normal artery A1), regions in the fibrous cap (stable B1, B2 and vulnerable plaque C1) and FAP-positive macrophages (C2, arrows). (D) High magnification images show FAP-positive giant cells (D1, arrowheads) and macrophages (D1, D2, arrows) in a vulnerable plaque. The endarterectomized plaques are composed of tunica intima and part of the media. Lu: lumen. Scale bar, low magnification 2000 μm; A1, B1, B2, C1, 200 μm; C2, D1, D2, 50 μm.
7.3.3 Chemistry and Radiochemistry

Reference compound and precursor were synthesized from commercially available 4-iodobenzoic acid and glycine ethylester hydrochloride, as shown in Scheme 7.1. The synthetic scheme followed was the one reported by Zimmerman et al. [226] with some distinct modifications. For the reference compound, glycine ethylester was efficiently coupled to 4-iodobenzoic acid with HBTU as the coupling agent to afford compound 1 in 79% yield. The ethylester was then cleaved under basic conditions (aq. KOH/MeOH) to give the free acid 2 in moderate yield (55%) [227]. Reaction of compound 2 with (R)-boroPro-(+)-pinanediol-HCl using the EDC/HOBt coupling system afforded dipeptide 3 in excellent yield (93%). Deprotection of the boronic ester to the free boronic acid, proved challenging. The proposed transesterification method [226] with phenylboronic acid was not efficient in our hands. Apart from solubility problems the reaction was sluggish producing many byproducts. Thus, an alternative method was applied, which involved oxidative cleavage of the pinanediol protecting group using sodium metaperiodate [228]. This procedure was more compatible with our substrate, yielding cleanly and relatively fast a new more polar product as revealed by HPLC monitoring. After workup, the crude was purified with preparative RP-HPLC to provide 4 in moderate yield (45%).

For the synthesis of the precursor, a similar procedure was followed. Stannylation of the iodinated compound 2 was achieved by reacting it with hexamethylditin and Pd(PPh₃)₄Cl₂ in refluxing dioxane, which yielded compound 5 in a good yield (81%). 5 was then coupled to the boronic ester (R)-boroPro-(+)-pinanediol with EDC/HOBt in DCM and the crude was purified with RP-HPLC to provide the precursor 6 in satisfactory yield (59%).
Scheme 7.1 Synthesis of reference compound 4 and corresponding precursor 6. Reagents and conditions: (a) HBTU, DIPEA, DMF, rt, 3 h, 79%; (b) KOH, MeOH/H₂O, rt, 1 h, 55%; (c) EDC, HOBt, (R)-BoroPro-(+)-Pinanediol-HCl, DCM, 0 °C to rt, 16 h, 93%; (d) NH₄OAc, NaIO₄, acetone, rt, 17 h, 45%; (e) hexamethylditin, Pd(PPh₃)₂Cl₂, dioxane, rt, 3 h, 81%; (f) EDC, HOBt, (R)-BoroPro-(+)-Pinanediol-HCl, DCM, 0 °C to rt, 16 h, 59%.

[¹²⁵I]MIP-1232 was produced in a one-step reaction by electrophilic radioiodination of the corresponding trimethylstannyl precursor (Scheme 7.2). The procedure was performed according to Zimmerman et al. [226] with some modifications since the order of addition of the reagents critically affected the outcome of the reaction. The experimental protocol was optimized so as to yield a reliable and robust radiolabeling procedure. Briefly, precursor 6 was incubated with Na[¹²⁵I] under oxidative conditions to achieve electrophilic radioiodination and simultaneously cleaving the boronic acid protecting group. After quenching with Na₂S₂O₃, the reaction mixture was purified by analytical HPLC to yield [¹²⁵I]MIP-1232 in 10 – 12 % radio-chemical yield (decay-corrected; n = 3) and radiochemical purity ≥ 90%.

The stability in acetonitrile/water/TFA (as eluted from the HPLC column) was investigated by HPLC with reference compound 4. The compound was stable with > 96% intact compound present after 110 h storage. [¹²⁵I]MIP-1232 was stored under identical conditions and all experiments were performed within this time period after purification.
Scheme 7.2 Radioiodination scheme of precursor 6 to $^{[125]}$I-MIP-1232. Reagents and conditions: Na$^{[125]}$, H$_2$O$_2$, H$_2$SO$_4$, CH$_3$COOH, MeCN, rt, 10 min then Na$_2$S$_2$O$_3$.

### 7.3.4 In Vitro Autoradiography

Radiotracer binding was evaluated by in vitro autoradiography with human carotid plaques and xenograft tissue, as shown in Figure 7.3. $^{[125]}$I-MIP-1232 binding was higher in atherosclerotic plaques than normal arteries. Vulnerable plaques showed a slightly higher radioactivity signal integrated over the tissue slice than stable plaques. However, after correction for the size of the tissue samples, average total binding was similar for the three categories (Figure 7.3, A, B). Radiotracer binding was reduced under blockade conditions with an excess of unlabeled MIP-1232 indicating displaceable (specific) binding of $^{[125]}$I-MIP-1232 (Figure 7.3, A). No significant difference was detected comparing the specific binding of the three groups (Figure 7.3, B). In a proof-of-principle study, target specificity of $^{[125]}$I-MIP-1232 was evaluated in an autoradiography assay with xenograft tissue (Figure 7.3, C). FAP-positive SK-Mel-187 melanoma xenografts displayed a markedly higher radioactivity signal than NCI-H69 lung small cell carcinoma xenografts and radiotracer binding was blocked completely by excess of MIP-1232 in both xenografts. IHC experiments confirmed high FAP levels in the SK-Mel-187 xenograft and low levels in the NCI-H69 xenograft (Figure 7.3, C).
Figure 7.3 (A) *In vitro* autoradiogram of representative sections of human carotid plaques under baseline ([125]I-MIP-1232) and blockade condition ([125]I-MIP-1232 with excess unlabeled MIP-1232). Hematoxylin/eosin (HE) staining below represents plaque morphology. Scale bar 3 mm. (B) Quantified total and specific binding of [125]I-MIP-1232 to normal arteries (n = 5), stable plaques (n = 16) and vulnerable plaques (n = 15) determined by autoradiography and corrected for tissue size. No significant intergroup differences were determined. Lines indicate mean values, diamonds indicate the specimens shown in A. (C) *In vitro* autoradiography with xenografts under baseline and blockade conditions. IHC staining for FAP of the SK-Mel-187 and the NCI-H69 xenograft (20 µm cryosections). Scale bar 3 mm for autoradiography; 50 µm for IHC images. Color scales for minimal to maximal binding.
7.4 DISCUSSION

Recent studies suggest that inflammation-related processes provide promising targets for the non-invasive imaging of plaque vulnerability [62, 73, 230]. We lately identified the co-stimulatory molecule CD80 involved in T cell activation as a promising imaging target since its expression is increased in vulnerable plaques. A radiolabeled specific inhibitor accumulated in human vulnerable plaques in vitro [83]. Moreover, we evaluated a F-18 labeled folate derivative targeting activated macrophages that accumulated stronger in atherosclerotic plaques than normal arteries [80]. Brokopp et al. presented another inflammation-related target, FAP, that displayed increased levels in advanced plaques indicating an association with the process of plaque destabilization [88]. The mechanistic role of FAP in atherosclerosis remains vague. Collagen, thereof 70% collagen type I, is a primary component of the extracellular matrix in atherosclerotic plaques [231]. Synergistically with matrix metalloproteinases, FAP is capable of degrading type I collagen and these proteases therefore have a destabilizing effect on atherosclerotic plaques [212]. The involvement of serine proteases, in particular of the DPP IV subfamily, in atherosclerosis and its clinical adverse events certainly warrants further investigations.

In this study, we analyzed plaque specimens obtained from the carotid artery that showed similar FAP mRNA levels as normal artery segments, independent of plaque vulnerability. In agreement with Brokopp et al., FAP protein levels as determined by IHC correlated with plaque progression, with the highest focal staining in vulnerable plaques. The discrepancy between mRNA and protein levels could indicate lower degradation of FAP in vulnerable than stable plaques, in line with differences in protease and protease inhibitor levels in the two lesion types [75]. Overall, the difference in FAP expression between normal arteries and plaques was modest in our study. In contrast to the publication of Brokopp et al., we found FAP protein in macrophages and giant cells within the plaque and found no co-localization with SMCs. However, we want to point out that we evaluated artery segments of a different location and used different tissue preparations than Brokopp and colleagues. The localization of FAP in macrophages in our study is in agreement with recent reports showing FAP expression in M2 macrophages [232, 233].

The FAP inhibitor MIP-1232 was successfully synthesized and radiolabeled with iodine-125, a long-lived gamma-emitting nuclide. The synthesis and radiolabeling were accomplished in reasonable yields and purity. In a proof-of-principle study with a FAP-positive SK-Mel-187 xenograft [229], a high and displaceable binding of the radiotracer was observed, whereas binding to the NCI-H69 xenograft with low FAP levels was negligible. This indicates binding of $^{125}$I MIP-1232 to FAP-positive tissue in vitro.

The potential of this radiotracer for atherosclerotic plaque imaging was investigated by in vitro autoradiography with human carotid plaques. Here, we found a pronounced binding to carotid plaques, however with no difference in average specific binding between stable and vulnerable plaques and between plaques and normal arteries, after correction for the size of the tissue samples. However, 3 of the 16 stable and 4 of the 15 vulnerable plaques showed several-fold higher
specific accumulation than normal arteries. Only a prospective study would show whether this is of clinical relevance.

Based on our data we cannot conclude on the selectivity of $^{125}$I-MIP-1232 for FAP. In the absence of a known selective inhibitor, we investigated specificity by blocking with the unlabeled compound itself. The relatively high amount of remaining radiotracer after blocking must, therefore, accumulate with low affinity. Lipophilicity is most probably not involved as clogP of MIP-1232 is about 0.5. The non-specific accumulation may result from interactions with highly abundant hydrolases or other proteins with affinities in the high micromolar range, considering that our blocker concentration was 100 µM. Specificity analysis of MIP-1232 was performed exclusively with FAP and PREP [224]. A conclusive evaluation of the binding affinity to dipeptidyl peptidases such as DPP-2, DPP-4, DPP-8 and DPP-9 would be required, irrespective of the fact that DPPs display in general low affinities for N blocked peptides [234, 235]. As $^{125}$I-MIP-1232 did not selectively accumulate in the atherosclerotic tissue and as its low FAP/PREP affinity ratio is already known we did not further investigate its selectivity profile. For future studies more selective inhibitors are needed to reduce non-specific tissue accumulation. To overcome limitations in specificity, novel lead structures and the use of antibodies and fusion proteins was proposed to minimize off-target effects [219, 229, 235].

Our findings with the tumor xenografts are of interest in oncology [229, 236, 237]. Although FAP as a target may be of little relevance for tumor imaging in general considering the high diagnostic value of $^{18}$F-fluorodeoxyglucose; FAP imaging with a selective ligand would enable the identification of FAP-positive tumors sensitive to a FAP-targeted radiotherapy [229].
7.5 CONCLUSIONS

Target expression analysis by IHC revealed moderately higher levels of FAP in plaques than normal arteries. The radiolabeled boronic acid-based inhibitor, \([^{125}\text{I}]\text{MIP-1232}\), was successfully produced. The radiotracer displayed displaceable binding to FAP-positive xenografts \textit{in vitro} and accumulation in human carotid plaques \textit{in vitro}. However, binding was similar in plaques and normal arteries and was independent of plaque vulnerability. Targeting FAP by \([^{125}\text{I}]\text{MIP-1232}\) may, therefore, be of low relevance for atherosclerosis imaging. The high binding of \([^{125}\text{I}]\text{MIP-1232}\) to a FAP-positive SK-Mel-187 xenograft but low binding to a xenograft with low FAP levels is promising towards the imaging of FAP to support FAP-targeted therapy in oncology.
7.6 EXPERIMENTAL SECTION

7.6.1 Patient Characteristics and Human Carotid Tissue Banking

Human atherosclerotic plaque tissues were excised during carotid endarterectomy (CEA) surgery at the University Hospital Zurich using the bifurcation advancement technique [28]. The atherosclerotic material was removed from the common, external and internal carotid artery. Before surgery, written informed consent was obtained from all patients. A total of 25 patients were included in this study with an average age of 73.1 years (73.1 ± 6.6 y) at surgery and 84% of them male. After CEA, the tissue was transferred to RNA later® solution (Sigma, St. Louis, USA) and stored at -80 °C until further use. Excised material was classified into the categories "normal artery", "stable plaque" and "vulnerable plaque" based on a macroscopic visual inspection and a histological examination. The histological analysis was performed with standard staining methods (e.g. hematoxylin and eosin) and according to the classification system of the American Heart Association as previously described [83, 106]. Plaques were classified as stable if there was a lipid core separated to the blood stream by an intact fibrous cap with a representative cap thickness > 500 μm and a minimum cap thickness > 200 μm [109]. Vulnerable plaques were lesions with a large necrotic core, a thin or ruptured fibrous cap, high infiltration of inflammatory cells and neovascularization. The microscopic characterization of all plaques used in this study was in agreement with the macroscopic evaluation. In total 7 normal arteries, 25 stable plaques and 23 vulnerable plaques were used for gene expression analysis (normal n = 2, stable n = 11, vulnerable n = 9), immunohistochemistry (normal n = 1, stable n = 4, vulnerable n = 4) and autoradiography (normal n = 5, stable n = 16, vulnerable n = 15), respectively. Classified normal arteries were redundant segments from the A. iliaca or A. thyroidea. Note the limited availability of normal arteries.

7.6.2 RNA Isolation, Reverse-Transcription and Real-Time Polymerase Chain Reaction

Total RNA was isolated from human atherosclerotic plaque segments according to the protocol of the Isol-RNA Lysis reagent (5 PRIME, Gaithersburg, USA) using the bead-mill TissueLyser system (Qiagen, Hilden, Germany). cDNA was generated by the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Used primers were custom-made oligonucleotides from Microsynth (Balgach, Switzerland): human actin beta (ACTB) (forward 5’-CATGTACGTTGCTATCCAGGC-3’, reverse 5’-CTCCCTTAATGTCACGCAGAT-3’, NM_001101), human fibroblast activation protein alpha (FAP) (forward 5’-TGAACGAGTATGTTTGCAGTGG-3’, reverse 5’-GGTCTTTGGAC-AATCCCATGT-3’, NM_004460), and human alpha smooth muscle cell actin (SMA) (forward 5’-GCTGGCATCCATGAAACCAC-3’, reverse 5’-TGCCCCCTGATAGGACATTG-3’, NM_001613). Quantitative polymerase chain reaction (qPCR) was performed with the DyNAmo™ Flash SYBR® Green PCR System (Applied Biosystems, Carlsbad, USA) using a AB7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, USA). Quantification was performed by the 2^ΔΔCt quantification method with β-actin as a reference gene [110]. All reactions were conducted in
duplicates in three independent experiments. Specificity of the amplification products was assured by dissociation analysis. SMA is specifically expressed in SMGs of different origin.

### 7.6.3 Histology and Immunohistochemistry

Plaques were paraffin-embedded and serial sections of 2 μm were prepared for further histological and immunohistochemical investigations. Hematoxylin and eosin (HE) staining was performed according to routine procedure to classify plaques into the categories "stable" and "vulnerable". For immunohistochemistry, primary antibodies for FAP (anti-FAP, 1:50, rabbit, polyclonal antibody directed against the Fibroblast activation protein, NB100-91763, Novus Biologicals, Littleton, CO, USA) and SMA (anti-SMA, 1:400, mouse, monoclonal antibody directed against anti-human alpha smooth muscle actin, M0851, Dako, Baar, ZG, Switzerland) were used. Antigen retrieval for the anti-FAP antibody was performed using acid buffer (pH 6.0), whereas no antigen retrieval was performed for the anti-SMA antibody. The detection system included the OmniUltraMab Kit (Roche, Rotkreuz, ZG, Switzerland) for the anti-FAP antibody on the Discovery XT instrument (Roche) and the Dako RealKit (Dako) for the anti-SMA antibody on the immunostainer (Dako). FAP IHC staining of a SK-Mel-187 and a NCI-H69 xenograft was performed with 20 μm frozen sections according to the above specified procedure without antigen retrieval. Sections were scanned by a slide scanner (Pannoramic 250, 3D Histech, Sysmex, Horgen, Switzerland). HE and IHC staining were analyzed by a pathologist (N. B.).

### 7.6.4 Chemicals and Reagents

All reagents and starting materials were purchased from commercial suppliers and used without further purification. All solvents used for reactions were obtained as anhydrous grade from Acros Organics (puriss., dried over molecular sieves, H₂O < 0.005%) and were used without further purification unless otherwise stated. Solvents for extractions, column chromatography and thin layer chromatography (TLC) were purchased as commercial grade. All non-aqueous reactions were performed under an argon atmosphere using flame-dried glassware and standard syringe/septa techniques. In general, reactions were magnetically stirred and monitored by TLC performed on Merck TLC glass sheets (silica gel 60 F₂54). Spots were visualized with UV light (λ = 254 nm) or through staining with anisaldehyde solution or basic aq. KMnO₄ solution and subsequent heating. Chromatographic purification of products was performed using Fluka silica gel 60 for preparative column chromatography (particle size 40 – 63 μm). Reactions at 0 °C were carried out in an ice/water bath. Nuclear magnetic resonance (NMR) spectra were recorded in CDCl₃, CD₃OD or DMSO-d₆ on a Bruker Av-400 spectrometer at room temperature. The measured chemical shifts are reported in δ (ppm) and the residual signal of the solvent was used as the internal standard (CDCl₃: ¹H: δ = 7.26 ppm, ¹³C: δ = 77.0 ppm; CD₃OD: ¹H: δ = 3.31 ppm, ¹³C: δ = 49.15 ppm; DMSO-d₆: ¹H: δ = 2.50 ppm, ¹³C: δ = 39.51 ppm). All ¹³C NMR spectra were measured with complete proton decoupling. Data of NMR spectra are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, br = broad signal. The coupling constant J is reported in Hertz (Hz). Electrospray (ES) mass spectra
(HRMS) were obtained with a Bruker's maXis (ESI-Qq-TOF-MS) spectrometer. Analytical HPLC was performed with a reverse phase column (Ultimate® XB-C18 column 4.5 × 250 mm, 5 μm) with the following solvent system: water/0.1% TFA (solvent A), acetonitrile (solvent B); 0 – 30 min: 25% B (system 1) or 0 – 30 min: 75% B (system 2). The flow rate was 1 mL/min and UV detection at 254 nm. Preparative HPLC was performed with a reverse phase preparative column (Ultimate® XB-C18 column 21.2 × 150 mm, 5 μm) using the above mentioned isocratic conditions for analytical HPLC at a flow of 20 mL/min and UV detection at 254 nm.

In vitro stability evaluation of the reference compound (4) in formulation (30% acetonitrile, 70% water/0.1% TFA) was performed with a reverse phase column (LUNA® Phenomenex C18 4.5 × 250 mm, 5μm). The following solvent system was applied: water/0.1% TFA (solvent A), acetonitrile (solvent B); flow 1 mL/min; 0 – 13 min: 30% B, 13 – 18 min: 30 – 80% B, 18 – 36 min: 80% B, 36 – 38 min: 80 – 30% B, 38 – 40 min: 30% B; UV = 254 nm. Stability was assessed up to 110 h.

Purification and analytics of the radiolabeled material was performed on a Merck Hitachi D-6000 system equipped with multi-UV-wavelength and Raytest Gabi Star detectors and HSM software. A reverse phase column was used (LUNA® Phenomenex C18 4.5 × 250 mm, 5 μm) according to the above specified solvent system and conditions for the stability evaluation.

7.6.5 Chemistry

ETHYL-2-(4-iodobenzamido)acetate (1)

To a solution of 4-iodobenzoic acid (1.5 g, 6.05 mmol) and N,N-diisopropyl-ethylamine (2.1 mL, 12.1 mmol) in DMF (23.6 mL), O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (2.75 g, 7.26 mmol) was added portionwise at room temperature. After stirring for 15 min, a solution of glycine ethyl ester hydrochloride (0.748 g, 7.26 mmol) and N,N-diisopropylethylamine (2.1 mL, 12.1 mmol) in DMF (10 mL) was added dropwise and the reaction mixture was stirred for 3 hours. The mixture was then diluted with ethylacetate and washed successively with 0.5 M HCl, 5% NaHCO₃, H₂O and brine. The organic layer was dried over MgSO₄ and concentrated in vacuo. After evaporating, the residue was purified by flash column chromatography on silica gel (hexane/AcOEt 7:3) to afford compound (1) (1.59 g, 79%) as light yellow solid. NMR data were in accordance with previously published data [227].

Rf: 0.24 (hexane/AcOEt 7:3). ¹H-NMR (400 Hz, CDCl₃); δ = 7.82 – 7.77 (m, 2H), 7.56 – 7.50 (m, 2H), 6.68 (br, 1H), 4.26 (q, J = 7.2 Hz, 2H), 4.21 (d, J = 4.9 Hz, 2H), 1.31 (t, J = 7.2 Hz, 3H).

2-(4-iodobenzamido)acetic acid (2)

To a solution of ethyl 2-(4-iodobenzamido)acetate (1.4 g, 4.2 mmol) in MeOH (35 mL) and H₂O (35 mL), KOH (707 mg, 12.61 mmol) was added with continuous stirring and was kept for 1 h at room temperature, at which point TLC confirmed the complete consumption of the starting material (1). The reaction was then diluted with H₂O (42 mL) and the pH of the solution was adjusted to 2 with
HCl 1 M. The precipitate was filtered off and washed with cold H$_2$O. The compound was dried under high vacuum over P$_2$O$_5$ for 3 h to afford (2) as a white solid (707 mg, 55%).

$\text{Rf : 0.48 (DCM/MeOH/AcOH 9:1:0.02).}$  
$^{1}$H-NMR (400 Hz, DMSO): $\delta = 8.88$ (t, $J = 5.6$ Hz, 1H), 7.89 - 7.85 (m, 2H), 7.67 - 7.62 (m, 2H), 3.89 (d, $J = 5.6$ Hz, 2H). $^{13}$C-NMR (100 Hz, DMSO): $\delta = 171.2$, 165.7, 137.2, 133.3, 129.2, 99.1, 41.3.

4-IODO-N-(2-OXO-2-((R)-2-((3AS,4S,6S,7AR)-3A,5,5-TRIMETHYL-HEXA-HYDRO-4,6-METHANO-N-039;BENZO[039;D][1,3,2]DIOXABOROL-2-YL)PYRROLIDIN-1-YL)ETHYL]BENZAMIDE (3)

To an ice-cooled solution of 2-(4-iodobenzamido)acetic acid (300 mg, 0.983 mmol) in DCM (5.6 mL), hydroxybenzotriazole (151 mg, 0.98 mmol) was added, followed by 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (245 mg, 1.28 mmol) and the resulting mixture was stirred at room temperature for 30 min. N-Methylmorpholine (0.2 mL, 1.967 mmol) and (R)-boroPro-(+)pinanediol HCl (245 mg, 0.98 mmol) were then added and stirring was continued for 16 h. The reaction mixture was diluted with DCM, washed successively with 1 M KHSO$_4$, 10% Na$_2$CO$_3$, H$_2$O and brine. The organic layer was dried over MgSO$_4$, filtered and concentrated in vacuo. After evaporating, the residue was purified by passing quickly through a short plug of silica gel, eluting with AcOEt, to afford compound (3) (489 mg, 93%).

$\text{Rf : 0.52 (hexane/AcOEt 3:7).}$  
$^{1}$H-NMR (400 Hz, CDCl$_3$): $\delta = 7.82$ - 7.77 (m, 2H), 7.59 - 7.53 (m, 2H), 6.65 (br, 1H), 4.33 (br, 1H), 4.15 (d, $J = 3.8$ Hz, 2H), 3.51 - 3.41 (m, 2H), 3.24 - 3.17 (m, 1H), 2.39 - 2.30 (m, 1H), 2.22 - 1.96 (m, 5H), 1.94 - 1.78 (m, 4H), 1.46 (s, 3H), 1.29 (br, 3H), 0.84 (s, 3H). $^{13}$C-NMR (100 Hz, CDCl$_3$): $\delta = 166.5$, 166.1, 137.9, 133.7, 128.9, 128.5, 98.8, 86.4, 78.1, 51.5, 45.9, 42.5, 39.7, 38.5, 35.7, 28.7, 27.6, 27.3, 27.2, 26.5, 24.3. ESI-QTOF MS (DCM/MeOH) m/z calculated for C$_{23}$H$_{31}$BIN$_2$O$_4$ [M+H]+ 537.1420, measured 537.1414.

(R)-(1-((4-IODOBENZOYL)GLYCYL)PYRROLIDIN-2-YL)BORONIC ACID (4)

To a stirred solution of 3 (150 mg, 0.28 mmol) in acetone (7.4 mL) was added 0.1 M NH$_4$OAc (6 mL, 0.60 mmol) and NaIO$_4$ (189 mg, 0.88 mmol). The mixture was stirred at room temperature for 17 h, the acetone was removed in vacuo and the aqueous phase was turned basic with 2 M NaOH (9 mL), washed with DCM and acidified cautiously to pH 2 with 2 M HCl. The acidic solution was extracted with DCM (4x), dried over MgSO$_4$, filtered and evaporated to dryness. The crude was purified with preparative HPLC using system 1 to afford compound 4 (45 mg, 45%).

$\text{Rf : 0.19 (AcOEt).}$  
$^{1}$H-NMR (400 Hz, MeOD): $\delta = 7.88$ - 7.83 (m, 2H), 7.65 - 7.59 (m, 2H), 4.42 - 4.08 (m, 2H), 3.60 - 3.48 (m, 2H), 3.15 - 3.07 (m, 1H), 2.22 - 1.86 (m, 4H), 1.76 - 1.63 (m, 2H). $^{13}$C-NMR (100 Hz, MeOD): $\delta = 139.1$, 130.3, 129.5, 47.0, 46.8, 43.1, 41.9, 36.5, 28.8, 27.6, 21.9. HRMS C$_{14}$H$_{17}$BIN$_2$O$_3$ [M+H-H$_2$O+CH$_3$] 399.0377, measured 399.0365.
2-(4-(TRIMETHYLSTANNYL)BENZAMIDO)ACETIC ACID (5)

To a solution of 2-(4-iodobenzamido)acetic acid (331 mg, 1.085 mmol) in dry dioxane (6.7 mL), hexamethylditin (0.4 mL, 1.93 mmol) was added, followed by Pd(PPh₃)₂Cl₂ (43.5 mg, 0.062 mmol) and the reaction mixture was heated under reflux for 3 h. After this time, the mixture was filtered through a pad Celite and the solvent was removed under reduced pressure. The crude was purified by flash column chromatography on silica gel (hexane/AcOEt/AcOH 60:40:0.4) to afford 5 (301 mg, 81%) as a clear colorless oil [18].

Rf: 0.21 (hexane/AcOEt/AcOH 60:40:0.4). \(^1\)H-NMR (400 Hz, MeOD): \(\delta = 7.81 - 7.77 \text{ (m, 2H)}, 7.61 - 7.57 \text{ (m, 2H)}, 4.01 \text{ (s, 2H)}, 0.37 - 0.21 \text{ (br, 9H)}\).

\(^{13}\)C-NMR (100 Hz, MeOD): \(\delta = 172.4, 170.1, 148.5, 136.5, 134.6, 127.2, 43.0, -2.2\).

MALDI MS (3-HPA) m/z calculated for C₁₂H₁₈NO₃Sn \([M+H]^+\) 344.0305, measured 344.0304.

N-(2-OXO-2-((R)-2-((3AS,4S,6S,7AR)-3A,5,5-TRIMETHYLHEXAHYDRO-4,6-METHANOBENZO[D][1,3,2]DIOABO-2-YL)PYRROLIDIN-1-YL)ETHYL)-4-(TRIMethylSTANNYL)BENZAMIDE (6)

To an ice-cooled solution of 2-(4-(trimethylstannyl)benzamido)acetic acid (220 mg, 0.643 mmol) in DCM (3.7 mL), hydroxybenzotriazole (99 mg, 0.64 mmol) was added followed by EDCI (160 mg, 0.84 mmol) with continuous stirring and was kept for 30 min at room temperature. N-Methylmorpholine (0.15 mL, 1.287 mmol) and (R)-boroPro-(+)-pinanediol-HCl (160 mg, 0.64 mmol) were then added and the reaction mixture was stirred for 16 h. The reaction mixture was diluted with DCM and washed with 1 M KHSO₄, 10% Na₂CO₃, H₂O and brine. The organic layer was dried over MgSO₄, filtered and concentrated in vacuo. The crude was purified with preparative HPLC using system 2 to afford compound (6) (216 mg, 59%) as a white solid.

Rf: 0.56 (AcOEt). \(^1\)H-NMR (400 Hz, CDCl₃): \(\delta = 7.80 - 7.76 \text{ (m, 2H)}, 7.58 - 7.54 \text{ (m, 2H)}, 6.64 \text{ (br, 1H)}, 4.33 \text{ (br, 1H)}, 4.18 \text{ (d, } J = 3.5 \text{ Hz, 2H)}, 3.54 - 3.41 \text{ (m, 2H)}, 3.24 - 3.18 \text{ (m, 1H)}, 2.37 - 2.29 \text{ (m, 1H)}, 2.22 - 2.08 \text{ (m, 5H)}, 1.94 - 1.77 \text{ (m, 4H)}, 1.46 \text{ (s, 3H)}, 1.29 \text{ (s, 3H)}, 0.85 \text{ (s, 3H)}, 0.39 - 0.23 \text{ (s, 9H)}\).

\(^{13}\)C-NMR (100 Hz, CDCl₃): \(\delta = 167.5, 166.4, 136.2, 132.3, 128.6, 126.5, 98.7, 86.4, 78.1, 51.5, 45.9, 42.5, 39.8, 38.5, 35.7, 28.6, 27.6, 27.4, 27.2, 26.5, 24.3, -9.3\).

ESI-QTOF MS m/z calculated for C₂₆H₄ₒBN₂O₄Sn \([M+H]^+\) 575.2106, measured 575.2102.

7.6.6 Radiochemistry

MeCN (500 µL), 50% H₂SO₄ (50 µL) and freshly prepared oxidant (100 µL, 4% CH₃COOH and 6.7% H₂O₂) were added to a sealed reaction vial. Na[I²⁵I] (1.3 – 34.6 MBq, PerkinElmer, Waltham, USA) was diluted with water \(\text{ad} 50 \mu\text{L}\) and added simultaneously with the precursor (100 µL, 1 mg/mL in MeCN) to the reaction mixture. The mixture was incubated for 10 min at room temperature with intermittent shaking. After this time, the reaction was quenched by the addition of 0.1 M Na₂S₂O₃ (200 µL). The product was purified by analytical RP-HPLC. The purified product was obtained in 30% acetonitrile and 70% water/0.1% TFA and was stored at 4 °C until \textit{in vitro} experimentation.
Product identification was confirmed by co-injection of the reference 4. Radiochemical purity was determined by analytical HPLC.

7.6.7 In Vitro Autoradiography

For in vitro autoradiography, cryosections and paraffin-embedded sections of human atherosclerotic plaques (20 µm and 5 µm, respectively) and paraffin-embedded sections (5 µm) of a FAP-positive melanoma xenograft (human skin melanoma cell line SK-Mel-187) and a FAP-negative xenograft (human lung small cell carcinoma cell line NCI-H69) were used. A SK-Mel-187 xenograft was kindly provided by Dr. E. Fischer (Paul Scherrer Institut, Villigen, Switzerland). Cryosections were thawed and dried at room temperature for 30 min and all sections were subsequently incubated in HEPES buffer (50 mM HEPES, 5 mM MgCl₂, 125 mM CaCl₂, 0.1% BSA, pH 7.4) for 15 min on ice. The slices were incubated with [¹²⁵I]MIP-1232 solution (2.95 nM in HEPES buffer, 0.1% BSA) or for blockade conditions with [¹²⁵I]MIP-1232 solution containing additionally 100 µM unlabeled MIP-1232 for 60 min at room temperature in a humidified chamber. After incubation, the slices were washed in HEPES buffer supplemented with 0.1% BSA (5 min), three times in HEPES buffer (3 min each) and distilled water (1 min) on ice. For quantification of the radiotracer signal a calibration curve of a serial dilution of the tracer solution on Whatman filter paper (Whatman, Bottmingen, Switzerland) was used. Dried slides and the filter papers were exposed to a BAS-MS 2025 phosphor imaging plate (Fuji Film, Dielsdorf, Switzerland) for 19 h. The plate was scanned in a BAS-5000 bio-imaging analyzer (Fuji Film, Dielsdorf, Switzerland). Data analysis and quantification was performed with the AIDA 4.5 software (Raytest, Sprockhövel, Germany). Background values were subtracted from sample values and it was assured that all samples were within the linear range of the calibration curve. The spatially integrated signal intensities were divided by the plaque size to correct for heterogeneity in tissue size. Displaceable binding was calculated by subtraction of the radioactivity signal under blockade conditions from the baseline signal.

7.6.8 Statistical Analysis

Differences in mean values were evaluated by an unpaired two-tailed student's t-test (GraphPad Prism 6.0 software). A p-value < 0.05 was considered significant.

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8 CONCLUSION AND OUTLOOK

In this thesis, several potential targets for the imaging of atherosclerosis were identified. Among these targets, CD80 has a good prospect as its expression is related to plaque vulnerability and inflammation which are central determinants of the cardiovascular risk. Moreover, target expression is limited to restricted activated cellular subpopulations and defined stages in the inflammatory process. On the other hand, the expression of FAP is not restricted to atherosclerotic plaques since a basal expression in normal arteries was identified. The use of FAP-specific radiotracers for atherosclerosis imaging is, therefore, limited.

For radiotracer evaluation, candidates were tested in vitro with human carotid plaque tissue and/or in a shear stress-induced mouse model of atherosclerosis. This model was implemented and characterized and is applicable for future research related to atherosclerosis. The ApoE KO-cuff mouse model is one of the few animal models that offers the possibility to investigate vulnerable atherosclerotic plaques in vivo. For imaging purposes, the implanted cuff facilitates the localization of vascular segments affected by atherosclerosis considerably without the need for the administration of contrast agents. However, the model presented in this thesis did not enable us to investigate stable and vulnerable plaques at the same time which would be a great asset in radiotracer development. In addition, the plaque development in the control carotid and therefore the lack of a suitable internal control complicates the estimation of radiotracer potency. Nevertheless, this mouse model is a valid system for the investigation of vulnerable-like plaques and reflects partially advanced human atherosclerosis. Most important for this study, our imaging target CD80 was elevated in murine plaques, similar to human vulnerable plaques.

The CD80/CD86-specific $^{[111]}\text{In}$DOTA-belatacept had among the evaluated tracers the best in vivo properties as it accumulated in Raji xenografts and murine atherosclerotic plaques with a generally low background radioactivity. However, the pharmacokinetic profile of this fusion protein in particular the long biological half-life restricts its application to preclinical studies. In vitro, a specific radiotracer binding to human plaques correlating with key features of plaque vulnerability was determined. Overall, this proof-of-principle study with $^{[111]}\text{In}$DOTA-belatacept demonstrated that the imaging of CD80 and CD86 is feasible and that this tracer might be of interest not only in atherosclerosis but also other disease areas such as oncology and autoimmune disorders.

The CD80-specific low molecular weight tracers $^{[14]}\text{C}$AM7 and $^{[18]}\text{F}$AC74 bound to human vulnerable atherosclerotic plaques in vitro, however only $^{[14]}\text{C}$AM7 accumulated in murine plaques in vivo. The pharmacokinetic profile of both tracers is similar with a rapid hepatobiliary excretion potentially via transport proteins, a high plasma protein binding and as a consequence a low tissue distribution. Moreover, for both compounds, the distinct difference in binding affinity between
human and murine CD80 complicates preclinical *in vivo* studies. This might cause the significantly lower plaque uptake of the CD80 tracers than the metabolic tracers $[^{18}\text{F}]$FDG and $[^{18}\text{F}]$FDM in mice. Despite the good target attributes of CD80, the evaluated radiotracer candidates in this thesis do not fulfill the numerous requirements for plaque imaging for instance a high selectivity, a high binding affinity, a balanced pharmacokinetic profile in combination with a high plaque uptake.

Further tracers used in this thesis were the glucose isomer $[^{18}\text{F}]$FDM and the FAP-binding $[^{125}\text{I}]$MIP-1232. In the atherosclerosis mouse model, $[^{18}\text{F}]$FDM proved to have similar *in vivo* characteristics as $[^{18}\text{F}]$FDG with a comparable uptake in murine atherosclerotic plaques in PET/CT scans. The FAP inhibitor $[^{125}\text{I}]$MIP-1232 did not bind specifically to human vulnerable plaques possibly due to a lack in target specificity and the absence of a distinct connection of FAP expression and vulnerability.

In future research, central attention should be focused on the identification of novel targets, the enlargement of the radiotracer library, animal models of atherosclerosis and the investigation of the available tracers in other disorders.

The biobank of human carotid atherosclerotic plaques is an excellent basis for the identification and validation of targets relevant in atherosclerosis. This valuable resource of human tissues should be further explored in target expression analysis studies. In addition, since preclinical radiotracer evaluation relies on animal models of atherosclerosis, target expression analysis preferably on protein level should be performed with any mouse model used. Thereby, human and murine protein expressions can be compared allowing to address specific research questions *in vivo*. The presented shear stress-induced mouse model in this thesis, may serve as a model for therapy studies with statins or other pharmaceuticals. Thereby, the generation of stable plaques could be feasible. Besides the ApoE KO-cuff mouse model, the most promising radiotracer candidates could be further characterized in mouse models of CVD such as in SR-BI/ApoE dKO or SR-BI KO/ApoER61h/h mice [98, 99]. In addition, the underlying mechanisms and the similarities of human and murine atherosclerosis should be further elucidated.

Future synthesis efforts should aim at enlarging the current set of CD80-specific radiotracers to obtain novel imaging agents with an improved pharmacokinetic and binding affinity profile. Novel low molecular weight ligands should present a lower clearance via hepatobiliary transport systems, the amenability for fluorine-18 labeling, while maintaining high affinity and specificity for CD80. A higher binding affinity to murine CD80 is desirable for *in vivo* evaluations, but if unachieved the adaption of the *in vivo* model should be considered since for a clinical application a high affinity to human CD80 remains the highest priority. Evidence suggests that with the currently employed lead structures a high affinity to the targets of both species cannot be realized and therefore other classes of lead structures should be evaluated [164, 165, 167].

Given the promising results with indium-111 labeled belatacept, the investigation of abatacept (Orencia®) featuring a higher specificity and affinity to murine CD80 and truncated versions of these fusion proteins are straightforward options [145]. In particular, these strategies could be successful in preclinical studies. However, for a clinical translation of a CD80-binding tracer,
macromolecules are not the first choice due to their potential immunogenicity, a prolonged \textit{in vivo} circulation unnecessary for an imaging approach and high requirements for a clinical approval.

The targeting of CD80 is not only an interesting strategy in atherosclerosis, but there are several other pathological conditions with involvement of costimulatory pathways. Conceivable diseases in which patients might profit from CD80 imaging include cancer and autoimmune disorders [160-162]. Thereby, CD80 could be used as an approximation for the immunogenic level during ongoing inflammation and provide a means for disease identification and monitoring as well as support the treatment decision making and therapy control.

To resolve technical issues of current PET/CT imaging systems, the use of PET/MRI hybrid systems could be of advantage for plaque imaging and should be explored in future studies. Thereby, a higher resolution of anatomical structures together with a better soft tissue contrast might facilitate plaque identification and classification. This would enable us to combine the strengths of PET and MRI, the excellent sensitivity and resolution, respectively, thus opening up new possibilities for radiotracer development in atherosclerosis.
9 REFERENCES


References


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