# Profiling of proteins and targeting of myeloid mechanisms in atherosclerosis

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# Chapter 6

**General discussion and future perspectives**

## General discussion and future perspectives

### General discussion

Cardiovascular diseases (CVDs) are the main global cause of mortality. These diseases concern the heart and blood vessels, of which frequently occurring examples are coronary heart disease and cerebrovascular disease[1]. The main underlying cause of most CVDs is atherosclerosis, which is a lipid-driven inflammatory disease where lipids and immune cells accumulate in arteries to form atherosclerotic plaques[2]. While lipid-lowering therapy has been proven effective in limiting acute CVD[3–5], a residual inflammatory risk remains[6–8]. Anti-inflammatory therapy was proven to reduce the incidence of acute CVD in the CANTOS trial. In this clinical study, the administration of the anti-IL-1β antibody canakinumab reduced the incidence of secondary cardiovascular events in patients with previous myocardial infarction and high hsCRP levels (≥2 mg/L). However, canakinumab did not lower all-cause mortality and increased mortality due to infection[9]. The anti-inflammatory agent colchicine also reduced the occurrence of cardiovascular events in coronary disease patients[10,11] and lowered the risk of cardiovascular events in patients that had a recent myocardial infarction[12]. In a more recent trial however, colchicine had no effect on cardiovascular event risk[13]. Additionally, colchicine has also been shown to cause a higher incidence of pneumonia[12]. These studies clearly illustrate the potential of anti-inflammatory therapy in atherosclerotic cardiovascular disease, but also that further development of anti-inflammatory therapies for atherosclerosis is essential for clinical purpose.

In this thesis we therefore aimed to identify potential novel targets for atherosclerotic therapies, where we focused on the myeloid cell population. Macrophages are a predominant myeloid cell population in the plaque and are for example the main producers of proteases in atherosclerotic plaques[14–16], which are suggested to contribute to plaque rupture via amongst others induction of ECM degradation[17–22]. Additionally, mast cells are suggested to contribute to atherosclerosis through the release of the proteases tryptase and chymase[23,24]. Profiling of active enzymes and the cell types expressing these enzymes in human atherosclerotic plaques would aid in further elucidating the underlying mechanisms via which these cells contribute to atherosclerotic plaque destabilization. We thus profiled the active serine hydrolases in human atherosclerotic plaques and determined which cell types express these serine hydrolases.

Furthermore, we performed pre-clinical experiments to determine the anti-atherosclerotic potential of interventions in myeloid cell related processes.

We focused on inflammatory mechanisms in atherosclerosis, in particular effector mechanisms through the NLRP3 inflammasome, which is expressed by macrophages and foam cells in human carotid plaques[25,26]. The potential of this route has been established, as colchicine for example prevents NLRP3 inflammasome assembly[27], which may be one of the working mechanisms. In addition, the NLRP3 inflammasome mediates IL-1β activation[28,29], which is the cytokine inhibited by canakinumab in the CANTOS trial. This pathway is therefore of interest for atherosclerosis therapy development.

### Novel enzyme activity lead identification and myeloid-related pre-clinical testing

To identify novel active enzyme targets, in particular active serine hydrolases, in human atherosclerotic plaques, we performed activity-based protein profiling (ABPP) on human carotid plaques, described in **Chapter 2**. Subsequently, human atherosclerotic plaque single cell RNA sequencing (scRNA-seq) data was used to determine which cell types express the identified serine hydrolases. Serine hydrolases are enzymes that use a base-activated serine nucleophile to break substrate ester, thioester or amide bonds[30]. Enzymes of this class have been studied in atherosclerosis. Vulnerable human plaques were found to contain more of the serine hydrolase Lipoprotein-associated phospholipase $A_2$ (Lp-$PLA_2$) compared to fibroatheromas, determined with an immunohistochemical antibody staining[31]. Atheromatous plaques were shown to contain more neutrophil elastase than fibrous plaques as determined with Western blot[15]. Inhibiting neutrophil elastase in *Apoe*$^{-/-}$ mice stabilized plaques[32] and inhibition of Lp-$PLA_2$ in coronary heart disease patients inhibited the growth of the necrotic core[33], illustrating that such enzymes may contribute to lesion development and progression. To identify active serine hydrolases in human atherosclerotic plaques in an unbiased fashion, we performed ABPP on human carotid plaques.

We identified forty-five active serine hydrolases in human carotid atherosclerotic plaques, including the previously identified Lp-PLA2. Utilizing human plaque scRNA-seq data revealed that *Pla2g7* (or Lp-PLA2[34]) was relatively highly expressed by specific myeloid cell clusters as indicated in **Chapter 2**. Also the enzymes *Lipa*, *Nceh1*, *Pla2g15*, *Cpvl*, *Vnn1*, *Ppt1* and *Abhd12* were relatively highly expressed on mRNA level by myeloid cell clusters.

Next to Lp-PLA2, we identified active Liver carboxylesterase 1 (CES1) and Lysosomal acid lipase/cholesteryl ester hydrolase (LIPA) as active enzymes in the human plaques. These enzymes are interesting in relation to atherosclerosis due to their described involvement in foam cell formation. When macrophages have taken up lipoproteins, these cells store cholesteryl esters which can then be hydrolyzed for cholesterol efflux. This hydrolysis can be mediated by both LIPA and CES1[35–37]. In a study performed by Dubland et al., LIPA mRNA and protein expression was higher in macrophages compared to smooth muscle cells[38], which is in line with our findings as we found that *Lipa* expression was relatively high in two myeloid clusters compared to all other clusters. In contrast, *Ces1* was relatively higher expressed in smooth muscle cells, plasmacytoid dendritic cells and fibromyocytes compared to myeloid cells. Smooth muscle cells store cholesteryl esters at higher levels in lysosomal compartments compared to macrophages and in macrophages, the cholesteryl esters seem primarily stored in the cytosol[38]. These findings may suggest that macrophages mainly hydrolyze cholesteryl esters by LIPA in the lysosome and store cholesteryl esters in the cytosol and that smooth muscle cells mainly hydrolyze cholesteryl esters by CES1 in the cytosol and store cholesteryl esters in the lysosomes. This may point to a difference in mechanism leading to foam cell formation between these cell types, which remains to be determined. Specific overexpression studies of CES1 and LIPA in human plaque macrophages and smooth muscle cells may shed more light on the contribution of these enzymes to foam cell formation.

As we were interested to assess all plaque myeloid cell populations, we also focused on the expression of the identified serine hydrolases by mast cells. We found the enzymes *Abhd16a*, *Aldh1a1*, *Gbe1* and *Pafah1b3* to be relatively highly expressed by plaque mast cells on mRNA level. ABHD16A can mediate lysophosphatidylserine (lyso-PS) production[39] and lyso-PS can induce mast cell histamine release[40]. It remains to be determined whether mast cells can

contribute to atherosclerosis via these serine hydrolases and whether mast cells can exert a feedback loop for self-activation via ABHD16A.

Dipeptidyl peptidase 4 (DPP4) was also one of the active serine hydrolases identified in the human atherosclerotic plaques. In previous studies, proteomics analysis on human plaques showed the expression of proteins related to ferroptosis (a form of programmed cell death), which included DPP4, to be higher in unstable versus stable human plaques[41]. Additionally, DPP4 activity was found to be elevated in serum from *Ldlr*$^{-/-}$ mice given a high-fat diet compared to a normal diet and DPP4 inhibition inhibited this diet-induced effect. DPP4 inhibition also improved plaque stability as measured by a reduced necrotic core, an increased cap thickness and a reduced percentage senescent cells positive for the coagulation factors p21 and FX. This underpins their hypothesis that the improved plaque stability is a result of suppression of senescence-associated coagulation[42]. Furthermore, ferroptosis inhibition with ferrostatin-1 reduced atherosclerotic lesions and the formation of foam cells in *Apoe*$^{-/-}$ mice. Ferrostatin-1 also reduced iron content and accumulation of lipids *in vitro* in macrophages via AMP-activated protein kinase (AMPK) activation[43]. Combined, these findings suggest that DPP4 may indeed contribute to atherosclerotic lesion progression, which warrants further study.

In addition to the identification of active serine hydrolases in human atherosclerosis, we were also interested in associations between these serine hydrolases and plaque characteristics. We have not found significantly different serine hydrolase activities between plaques with a different instability index and neither between the subgroups of the individual plaque characteristics such as neovascularization and intraplaque hemorrhage, for example. Active serine hydrolase values also did not cluster the hydrolases based on stability parameters. The result thus far suggest that the active serine hydrolases do not associate with plaque instability, however this may be due to a low sample power in this pilot study. In addition, all plaques were advanced plaques which may limit the depth of the analysis. To gain more insight in the proteolytic activity during plaque development and different stages of disease, ABPP could be applied to mouse plaques from early to late atherosclerotic stages. Measuring ECM proteins in and performing ABPP on the same human plaque samples would in addition give insight into potential degradation processes of active serine hydrolases. A study by Lorentzen et al. indicates the promise of obtaining both protease activity and target protein information. They performed N-terminal proteomics, which maps N-terminal peptides made by protease cleavage and identified proteases and target proteins. To study the proteases that cleaved these peptides, they used a database to search for interactions between proteases and substrates. This enabled them for example to determine that chymase substrates had a significantly increased abundance in soft and mixed plaques compared to hard plaques[44]. Combining this method with ABPP would help to confirm which enzymes are active in the plaques and help elucidate proteolytic mechanisms in the plaques.

In summary, we identified active serine hydrolases in human atherosclerotic plaques with ABPP. Combined with scRNA-seq data analysis we obtained more insight in which cells would be most likely to be the main producers of these active serine hydrolases. These active serine hydrolases could be interesting leads for future atherosclerosis research concerning advanced atherosclerotic plaques.

The effect on coagulation factors of the serine hydrolase DPP4 inhibition[42] is in line with the concept that thrombotic factors are involved in both the initiation of plaques and the formation of a thrombus upon plaque rupture[45,46]. Targeting the thrombotic aspect of atherosclerosis is possible with a bioconjugate antiplatelet and anticoagulant (APAC). The structure of APAC is based on heparin proteoglycan (HEP-PG). These HEP-PGs or chondroitin sulfate PGs are present in granules of mast cells[47–50]. Mast cells are suggested to have pro-atherosclerotic effects via the release of tryptase and chymase, but may possess antithrombotic effects through the release of these HEP-PGs[51]. Therefore, in **Chapter 3**, APAC was studied for its anti-atherosclerotic properties in a collar-induced atherosclerosis mouse model. We tested these properties in a prevention and a treatment setting. In the prevention setting, initial atherosclerotic lesion formation was seen to be inhibited by APAC treatment, where we found a reduced collagen and macrophage content in the treated plaques. In the treatment setting, where APAC was administered when lesion formation had already been initiated, APAC tended to inhibit the progression of the lesions. Additionally, APAC treatment limited the macrophage content and tended to reduce the necrotic area in these pre-existing lesions.

In previous research in *in vivo* collagen exposing injury models, including femoral arteriovenous fistula by surgery and balloon angioplasty injury in the iliac and carotid artery, APAC colocalized with vWF and laminin at injury sites, after local administration[52]. Furthermore, in arterial thrombosis mouse models, APAC was able to extend the time to thrombotic occlusion and bind to collagen-exposing injury sites, thereby inhibiting *in situ* platelet accumulation[53]. These studies indicate that APAC can bind to these injury sites and attenuate the interaction between platelets and collagen.

The reduced macrophage content by APAC is suggested to be caused by a reduction in adhesion molecules and inflammatory mediators, as we did find a reduction in plaque TNFα mRNA expression in the therapeutic study. The tendency of APAC to limit the necrotic core in the treatment setting may be the result of APAC binding to the positive amino-acid residues of the apoB-component of LDL-particles. Modification of the lipoprotein particles happens upon binding to proteoglycans[54], but APAC might compete with the proteoglycans for binding and reduce the amount of modified LDL-particles. Modified lipoprotein particles are engulfed by macrophages forming foam cells, which contribute to the necrotic core formation by dying[55], which might thus be reduced due to APAC binding to LDL-particles. Lastly, previously the systemic anticoagulant effect of APAC has been confirmed by a prolonged activated partial thromboplastin time (APTT) after intravenous injection of APAC in rats and primates within 0.25 hours[56]. We found that one intravenous injection of APAC or unfractionated heparin (UFH) did not prolong the APTT or thrombin time in *Apoe*$^{-/-}$ mouse plasma. However, this was measured in plasma collected 15 minutes after injection and might be different when measured after a longer time period. Additionally, we did not determine the prolongation yet upon repeated dosing of APAC.

To conclude, we add the finding that APAC is able to inhibit atherosclerosis development and reduce inflammation in established lesions.

**NLRP3 inflammasome and related cytokines in preclinical atherosclerosis**

Targeting the NLRP3-inflammasome pathway as atherosclerosis therapy is of interest since the CANTOS trial. However, targeting this pathway in various clinical trials resulted in side effects, as mentioned before, which makes novel more specific therapeutic concepts necessary. A novel strategy to reduce the risk of side effects could be inhibition of the NLRP3 inflammasome in a specific cell type. For this purpose, a novel bispecific antibody InflamAb was investigated in our studies. InflamAb is designed to inhibit the NLRP3 inflammasome, but only in cells expressing the IL-1R1, which we found to be present in the human atherosclerotic plaque. This antibody first binds the IL-1R1, which results in its internalization and enables the antibody to inhibit the intracellular NLRP3 inflammasome. In these $NLRP3^{+}IL\text{-}1R1^{+}$ cells, IL-1β is able to induce an amplification loop of itself, because IL-1β can signal via the IL-1R1, which is a priming signal for the NLRP3 inflammasome[57–60]. In **Chapter 4**, we aimed to establish the efficacy of InflamAb in atherosclerosis inhibition.

We confirmed that cells targeted by InflamAb, positive for both NLRP3 and the IL-1R1, are present in the aortic arch, aortic root and PC of *apoE*$^{-/-}$ mice when fed a Western-type diet. In addition, we confirmed that InflamAb inhibits the production of IL-1β both *in vitro* and *in vivo* in *apoE*$^{-/-}$ mice upon hyperlipidemia.

Next, we showed that InflamAb inhibited plaque development, with a reduction in necrotic core and macrophage content in *apoE*$^{-/-}$ mice, similarly as we demonstrated previously with the small-molecule NLRP3 inflammasome inhibitor, MCC950[61]. In the peritoneal cavity of the InflamAb treated mice we found a reduction in myeloid cell populations such as the dendritic cells, nonclassical myeloid cells and $Ly6C^{mid}$ myeloid cells. Percentages of leukocyte populations in the circulation were not altered by InflamAb, suggesting that InflamAb predominantly acts at sites of local inflammation. This finding is in contrast with data from a study from Hettwer et al., where myeloid cells in the atherosclerotic aortas, but also leukocytes in blood, were reduced by MCC950 and a murine analogue of canakinumab[62]. The more local effects of InflamAb suggest its more specific nature compared to IL-1β or NLRP3 inflammasome inhibition. Hettwer et al. also found a reduction in protein levels of adhesion molecules and gene expression of chemokines in atherosclerotic aortas upon MCC950 treatment. Furthermore, they found that anti-IL-1β reduced the number of $GFP^{high}$ myeloid cells in the atherosclerotic aortas. These results indicate that IL-1β induces recruitment of leukocytes via adhesion molecules and chemokines to the atherosclerotic aorta, which can be inhibited by NLRP3-inflammasome or IL-1β inhibition[62].The mechanism of operation of InflamAb in reducing carotid plaque macrophages remains to be further studied. As a next step, local cytokine levels could be measured, as well as the expression of adhesion molecules and of chemo-attractants at the lesion site. Local cytokine levels may provide additional information on macrophage recruitment, because IL-1β is known to promote recruitment of macrophages[63]. Additionally, a study similar to the experiment from Hettwer et al. could be performed to determine whether InflamAb inhibits $GFP^{high}$ myeloid influx into the atherosclerotic aorta.

It is known that macrophages engulf modified LDL during atherosclerosis development leading to foam cell formation. Apoptosis or necrosis of these foam cells contributes to the necrotic core[64]. The reduction in the macrophage content upon treatment with InflamAb may thus have resulted in a reduced necrotic core content via a reduced foam cell content.

InflamAb treatment of mice with established atherosclerosis confirmed the reduction in peritoneal myeloid populations, while circulating leukocyte populations were again not affected. InflamAb reduced the macrophage and necrotic core content of pre-existing lesions, with a trend towards an increased collagen content. These stabilizing effects are in line with a study where lentivirus-mediated NLRP3 silencing prevented plaque progression in *apoE*$^{-/-}$ mice, and led to a lower amount of macrophages and lipids and a higher amount of smooth muscle cells and collagen[65]. Together, the improved stability parameters by InflamAb illustrate the potential for this strategy in the treatment of pre-existing lesions in humans.

Although we have not assessed this in our studies, IL-1β has been shown to upregulate MMP9 and 12 in macrophages[66,67] and MMP2 and 9 in monocytes[68]. Furthermore, macrophage-induced secretion of MMP3 and 9 from smooth muscle cells can be inhibited by an anti-IL-1β antibody[69]. Some of these MMPs are able to degrade collagen, with MMP2 degrading collagen type IV, V, VII, X, XI, MMP3 collagen telopeptides and MMP12 collagen type IV[70]. If we think along this line, InflamAb reduces IL-1β levels, which may in turn lead to lower protease levels and subsequently less collagen degradation. Measuring the protein levels of active enzymes, in addition to caspase-1, such as MMPs and serine hydrolases with for example activity-based protein profiling in lesions of mice treated with InflamAb would help to fully elucidate the underlying mechanism of InflamAb in these pre-existing lesions. We did find that InflamAb reduced the caspase-1 activity in the aortic root of these pre-existing atherosclerotic plaques, confirming the efficacy of InflamAb at the site we aimed to target. The efficacy of InflamAb at the target site in mice and the identification of a myeloid NLRP3$^{+}$IL-1R1$^{+}$ subset in human atherosclerotic plaques suggests that InflamAb has potential to locally induce its effects in human atherosclerotic plaques.

In conclusion, InflamAb inhibited plaque development and increased plaque stability markers in pre-existing plaques, rendering InflamAb a promising therapeutic lead for future tests with and developments based on this antibody.

The bispecific antibody InflamAb is one example of the current focus of inflammatory therapy development, which are targeting IL-1, IL-18 and IL-6 signaling in atherosclerosis. These therapies are mainly tested, similar to our study, in mouse models. However, whether animal sex is a factor important in measuring the therapeutic efficacy of such ant-inflammatory strategies in these preclinical studies is not known. Male mice seem to have smaller and more inflamed lesions compared to female mice[71], but whether this influences the efficacy of such therapies in preclinical studies is unknown. Therefore, we aimed to investigate the sex used in such preclinical studies, and study whether a difference exists in therapeutic efficacy between males and females. We did this by performing a systematic review described in **Chapter 5**. Studies that investigate the effect of interventions targeting IL-1, IL-6 and IL-18 signaling on atherosclerotic lesion size in animal models were identified. We show that inhibition of these cytokine signaling pathways collectively reduced plaque size, whereas stimulation increased plaque size. Plaque burden is a predictor of cardiovascular events in patients[72–75], making it a relevant outcome parameter for translation to human atherosclerosis. Therefore, the plaque reduction upon inhibition is in line with promising effects of clinical trials with colchicine[10–12] and an anti-IL-1β antibody[9].

We aimed to perform subgroup analyses for sex on a subset of papers, but we found that the majority (80 %) of the studies that specified the sex included males. The number of studies in

the female subgroups was too low to reliably determine the effect on plaque size for females. For male mice, the therapeutic efficacy was highly significant and thus, as the overall group effect, in line with results from human clinical trials. Despite that it is not possible to perform a formal calculation for female mice, a visual inspection of the forest plots suggests a similar effect in male and female mice. To enable future formal calculation for female mice are scientists encouraged to include both sexes in their studies.

In addition to plaque size, other characteristics may be of relevance to determine the need to include animal sex as a variable in these preclinical studies. These characteristics could be plaque composition, including inflammation, but also the age of the mice. It should be taken into account that the main conclusions of our systematic review are based on relatively young mice. Age is a variable that could affect lesion size differences between male and female *apoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. As discussed in **Chapter 5**, in general, the plaques of these female mice are larger compared to males, but there are exceptions, such as studies in *apoE*$^{-/-}$ mice, where a similar lesion size was found in male versus female mice[71,76–81].

Furthermore, we only evaluated the effect of animal sex in mice studies, as this was the main species resulting from our literature search. We thus did not include other species in our meta-analyses due to the limited number of studies that did not use mice. Lastly, important study details such as a clear and adequate description of the generation and application of the allocation sequence, a specification of the concealment of the allocation sequence, and a specification of random outcome assessment were not reported by most of the animal studies, impeding the analyses of the risks of bias, which affects our ability to draw reliable conclusions.

To conclude, we observed that predominantly male mice are used to study the effects of anti-inflammatory therapy in preclinical atherosclerosis models. We identified a significant inhibitory effect of anti-inflammatory therapy on plaque size overall, which we could see in the male subgroups as well and which are in line with promising effects of clinical trials targeting IL-1β.

## Future perspectives

We showed the potential of ABPP in discovering potential novel leads for advanced atherosclerosis research, preclinically confirmed the anti-inflammatory potency of two therapeutics and eventually studied the effect of the inclusion of sex as a variable in preclinical studies. The next step would be broadening our research with the aim of refinement/precision therapy. There are various important aspects to reach this refinement, such as sex, age, specificity of the therapeutic itself and combining techniques.

Considering differences between males and females is important in developing precision therapy[82]. It is expected by National Institute of Health (NIH) that sex is included as a biological variable in vertebrate animals and humans research. A strong justification is necessary if sex is proposed to be not included as a variable[83]. We focused with our systematic review on preclinical research and thus animal sex and not humans. In our systematic review we evaluated animal sex in preclinical research regarding the modulation of IL-1, IL-6 and IL-18 cytokine signaling. Systematic reviews on the effect of animal sex on outcomes in preclinical research should be performed more often. For now it is recommended to include animals of both sexes in research to enable researchers to determine with systematic reviews

whether we have to continue to include sex as a variable. If we enable the execution of these systematic reviews, there are multiple outcomes possible. The results could indicate that we need to include sex as a variable, which might, if we do, lead to better translation into the clinic. Another outcome could be that there is no need to include both sexes, which would lead to a reduction in animal use for such studies. To support this, a systematic review with a similar research question, but with a broader scope with regards to for example the signaling pathways or sites of atherosclerosis, may lead to the inclusion of more studies where female mice are used. This would enable subgroup analysis for sex for females as well.

In our InflamAb studies we used both male and female mice, but not in the same experiment. A study in *Ldlr*$^{-/-}$ mice investigated whether sex plays a role in NLRP3 inflammasome-mediated inflammation. The results suggest that sex hormones are involved in atherogenesis mediated by the NLRP3 inflammasome, with a suggested inhibitory effect of testosterone and stimulatory effect of estrogen[84]. Therefore, inclusion of both sexes in future preclinical research targeting this pathway would be interesting. Furthermore, estrogen can induce NLRP3 expression in mast cells[85]. However, we did not observe an effect of InflamAb on perivascular mast cell numbers or their activation status in our InflamAb studies. It would be of interest to study whether hormones affect the effects of InflamAb treatment. In the APAC experiments male mice were used, therefore performing these experiments in mice of both sexes would also be a useful addition.

Additionally, we could think about precision in the therapeutic itself. An example is our antibody InflamAb, as it targets the NLRP3 inflammasome only in cells expressing the IL-1R1. This cell specificity is expected to lead to less systemic effects, as we showed that InflamAb did not affect the leukocyte populations in the circulation, while Hettwer et al. found a reduction in leukocytes in the blood upon treatment with MCC950 or a murine analogue of canakinumab in *apoE*$^{-/-}$ mice. They also found that treatment with canakinumab in the CANTOS trial reduced blood monocyte counts among participants[62]. It would be interesting to determine whether patients that had side effects from canakinumab, and colchicine for example, had a stronger reduction in blood leukocyte levels compared to patients without side effects. This would give insight into the risks of these lower leukocytes levels. However, the difference in effect on blood leukocytes already suggests a lower side effect risk of InflamAb compared to the other therapeutics.

Our ABPP experiment could be performed with a larger amount of human atherosclerotic plaque samples with a balanced number of male and female patient samples. For example, a proteomics study on human plaque samples revealed that the proteoglycans versican and aggrecan were more abundant in females[86], which adds to reaching precision therapy. Furthermore, combining various techniques, such as combining ABPP with ECM protein analysis on human plaque samples will enable associating ECM protein abundance with the activity of particular enzymes. Moreover, analyzing the enzyme activities and ECM proteins in different plaque regions with spatial proteomics would give more insight in which processes the enzymes are involved, such as plaque destabilization[20]. Obtaining knowledge on these local enzyme activities in human plaques with different stabilities with spatial ABPP could be valuable for precision therapy development for specific plaque types. Combining the spatial ABPP data with human plaque scRNA-seq data, which means proteomics and transcriptomics are combined resulting in multi-omics, would enable the identification of serine hydrolase targets in specific cell types. Subsequently, dual targeting therapeutics against serine hydrolases

in these specific cell types can be developed. Dual targeting is a promising strategy for atherosclerosis therapies, as we have shown for InflamAb. Dual therapeutics are specifically useful for innate immunity targeting, because of the less specific presentation when compared to adaptive immunity which includes specific T cell receptors, as mentioned by Gisterå[87]. A spatial multi-omics approach is recommended, with the incorporation activity-based: spatial activity-based multi-omics. The progression of techniques continues to enable researchers to investigate human disease more directly.

As inflammatory cells are a major source of enzymatic activity in atherosclerosis, applying ABPP to samples from anti-inflammatory therapy experiments to identify enzymatic changes due to an intervention would be recommended. This could be applied to *in vivo* experiments in mice but also in human atherosclerosis models. Combining *in vivo* enzymatic activity data with *in vivo* histology data, could help in defining the enzymatic mechanisms by which the therapeutics operate. Activity-based probes can be used on isolated plaque tissue, but also for *in vivo* imaging, possibly to determine the *in vivo* localization of active enzymes in anti-inflammatory therapy experiments to identify mechanisms. Furthermore, using ABPP to identify enzymatic activity changes upon anti-inflammatory treatment in human atherosclerosis models, such as human cells, *ex vivo* human plaques and models mimicking the human plaque with human cells, and combining this data with ABPP data from different unstable human plaque types, could help to indicate what types of plaques would benefit the most from the treatment, contributing to precision therapy and translation to the clinic.

To conclude, in this thesis the value of ABPP on human atherosclerotic lesions was demonstrated and identified leads for future atherosclerosis research. Two preclinical therapy studies showed promising results, where the InflamAb study demonstrated the potential of the novel bispecific antibody as anti-atherosclerosis therapeutic with local efficacy. Lastly, we discussed the importance of inclusion of sex as a variable in preclinical atherosclerosis research to enable establishment of the influence of animal sex in treatment efficacy. Broadening of the results by including sex as a variable and combining techniques will help in reaching precision therapy.

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