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Apoptosis inducing factor 1 and its role during plaque stabilization in carotid artery disease and ischemic stroke

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Contents

Abstract

Introduction: Whereas cerebrovascular diseases, most and foremost ischemic events, incorporate the second deadliest disease today in the Western world, the process behind carotid artery plaque (CAP) vulnerability remains understood insufficiently. We could determine proteins of potential relevance to plaque stability through proteomic analysis of blood serum from patients treated for atherosclerotic lesions in carotid arteries. One identified target is mitochondrial apoptosis-inducing factor 1 (AIFM1), which we found upregulated in serum profiles from patients with vulnerable atherosclerotic lesions.

Methods and Results: The study was conducted with human vascular tissue and blood samples from the 'Munich Vascular Biobank'. Proteomic profiling of serum was performed using the platform OLink (Uppsala, Sweden) taken from patients with either stable (n=53) or unstable (n=53) CAPs. Furthermore, gene expression of AIFM1 in stable (n=5) and unstable (n=3) CAPs was assessed via RT-qPCR. Via Immunostaining, the regions showing higher expression of AIFM1 within the plaques (n=10) were determined and put in perspective with markers of cell types predominantly accumulated in those regions. In addition, Peripheral Blood Mononuclear Cell (PBMC)- derived macrophages were stimulated with different factors, and changes in the expression of AIFM1 were assessed. With the proteomic profiling of blood sera, higher levels of AIFM1 in patients with unstable versus stable CAP were determined. This trend was also seen assessing the gene expression of AIFM1 in human vascular tissue. Significant enrichment of AIFM1 staining positivity was observed in regions where apoptosis occurred, such as the necrotic core and the shoulder regions of advanced plaque. Furthermore, AIFM1-enriched regions were identified as areas infiltrated by immune cells, especially macrophages. Macrophages of healthy donors showed an increased expression of AIFM1 when compared to the unstimulated PBMCs from the same donor.

Conclusion: In conclusion, AIFM1 was identified as a potential novel marker for advanced, unstable lesions in carotid artery plaques. Further studies (in vitro and in vivo) will need to be performed to reveal the role of AIFM1 in processes that trigger plaque destabilization and atheroprogression. Especially cell type-specific expression patterns will be of great importance.

Zusammenfassung

Einleitung: Obwohl Cerebrovaskuläre Erkrankungen, allen voran ischämische Insulte, an zweiter Stelle der Todesursachen in der heutigen Westlichen Welt stehen, so verbleiben die Prozesse der Pathogenese von atherosklerotischen Ablagerungen in den Halsschlagadern (*Arteriae Communis Internae*) und die Entwicklung einer Vulnerabilität dieser nicht ausreichend verstanden. Mit der Sequenzierung des Proteoms von Blutserumproben, gewonnen von Patienten bei Zustand nach Karotisendarteriektomie, war es uns ermöglicht Proteine als potentielle Biomarker für die Stabilität atherosklerotischer Plaques zu bestimmen. Unter den Resultaten wurde das Protein AIFM1 als signifikant überexprimiert in Blutserumproben von Patienten mit vornehmlich instabilen, vulnerablen atherosklerotischen Läsionen identifiziert.

Methoden und Resultate: Zur Durchführung der Studie wurde humanes Gewebe und Blutproben aus der 'Munich Vascular Biobank' verwendet. Von Patienten mit stabilen (n=53) und instabilen (n=53) Plaques wurde eine Sequenzierung des Proteoms mithilfe von OLink (Uppsala, Schweden) durchgeführt. Zusätzlich wurde die Genexpression von AIFM1 in stabilen (n=5) und instabilen (n=3) Plaques via RT-qPCR experimentell bestimmt. Durch Immunohischochemische Analysen konnte in definierten Regionen von Plaques (n=10) eine ausgeprägtere Expression von AIFM1 gezeigt werden und mit zellspezifischen Biomarkern in diesen in Relation gesetzt werden. Zusätzlich wurden aus peripheren, mononucleären Blutmonozyten (engl. PBMC) - entwickelten Makrophagen die Veränderung der AIFM1 Expression im Vergleich zu den nicht stimulierten PBMCs des selben Spenders aufgezeigt. Die Durchführung der Proteomsequenzierung zeigte höhere Serumlevel von AIFM1 in Blutproben von Patienten mit instabilen versus stabilen Plaques auf. Gewebeanalysen von humanen Karotisproben bestätigten diesen Trend der erhöhten Genexpression von AIFM1 in instabilen Plaques mit signifikanten Anreicherungen in von Apoptose geprägten Regionen, wie den Nekrotischen Kern und die Schulterregion des fortgeschrittenen Plaques. Zusätzlich wurden die AIFM1 markierten Regionen mit der Infiltration von Immunzellen, speziell Makrophagen, in Verbindung gebracht. Von gesunden Probanden gewonnene Monozyten präsentierten eine Steigerung der AIFM1 Genexpression bei Entwicklung zu Macrophagen via M-CSF-Stimulation.

Schlussfolgerung: AIFM1 wurde als potentieller Biomarker für fotgeschrittene, instabile Läsionen innerhalb atherosklerotischer Ablagerungen in der Karotiden identifiziert. Zukünftige Studien (in vitro und in vivo) zur genaueren Entschlüsselung der Rolle von AIFM1 in der Entwicklung von instabilen Plaques können von erheblicher Bedeutung, spezielll, zelltypische Expressionsmuster sein.

1 Introduction

1.1 Ischemic Stroke and Carotid Artery Disease

Carotid Artery disease (CaAD) is defined as the extracranial internal carotid artery narrowing caused by the development of atherosclerotic lesions, so-called carotid artery plaques (CAP). Stroke, in general can be classified as a symptomatic neurological dysfunction brought from acute focal damage of the central nervous system caused by irregular and insufficient blood flow. Central nervous system damage includes brain, spinal cord, and retinal cell death. Alongside *hematoma intracerebrale* and *hematoma subarachnoidale* causing insufficient blood flow by ruptured vessels, the CNS infarction, so-called *ischemic stroke*, can be distinguished as such vascular cause of central nervous system damage. [Sacco et al., 2013]

1.1.1 Epidemiology

Stroke is a severe burden to the population and healthcare systems worldwide. Under the Global Health Estimates 2019 by the World Health Organization (WHO), Stroke has been ranking second in the leading causes of death globally over the last 20 years, exceeded only by ischemic heart disease as the leading cause of death worldwide [WHO, 2020]. Globally, those two cardiovascular diseases are the most prominent causes of death and together, they contributed to 15.2 million deaths worldwide in 2015 [Katan and Luft, 2018, 2015 Neurological Disorders Collaborator Group, 2017]. In the European Union (EU) stroke affects 1.1 million inhabitants yearly, resulting in 440 000 deaths [OECDPublishing, 2018, Béjot et al., 2016]. Studies like *The Framingham Study* illustrating more than half of the stroke survivors suffering from disabling remains [Party et al., 2011, Kelly-Hayes M et al., 2003] and the 7.06 million disability-adjusted life years lost due to stroke in Europe 2017, resulting in a stroke-associated cost of an estimated 45 billion Euros [Wafa et al., 2020, Wilkins et al., 2017] quantify the burden. Subclassifying, the hemorrhagic variants represent about 15 %, and ischemic stroke accounts for the remaining 85 % of strokes in total [MartInez-Coria et al., 2021a]. Within the ischemic stroke subclassification, at least 15 - 20 % can be aligned to thromboembolic infarctions caused by internal CAD [Mughal et al., 2011, Petty et al., 2000]. Amongst individuals >65 years of age, an estimated 5-10 % reveal at least moderate asymptomatic carotid artery stenosis (ACAS) of 50-70 % [Mineva et al., 2002, Group et al., 1995]. Further, 7,5% (95 % CI, 5,2 to 10,5 %) of male and 5 % (3,1 to 5,7 %) of female individuals aged >80 years present moderate ACAS, when in fact, 3.1 % (1.7 to 5.3 %) and 0,9 % (0,3 to 2,4 %) respectively present severe ACAS (>70 %) with a reported annual stroke risk of approximately 2 - 5 % [de Weerd

et al., 2010, Inzitari et al., 2000, Norris et al., 1991]. With the aging population worldwide leading scientists expect stroke numbers and the burden with it continuing to increase [Campbell and Khatri, 2020].

1.1.2 Risk Factors

As a noncommunicable disease Stroke develops promoted by various risk factors and is distinguished into modifiable and non-modifiable of such. Many risk factors are shared with various cardiovascular diseases, such as the non-modifiable risk factors age, male sex, and genetical disposition [Walli-Attaei et al., 2020]. Also, various modifiable risk factors, e.g., illustrated in *The INTERHEART Study* [McQueen et al., 2008, Teo et al., 2006, Yusuf et al., 2005, Rosengren et al., 2004, Yusuf et al., 2004] are shared with other cardiovascular diseases, but of different relative importance [Campbell and Khatri, 2020]. Modifiable risk factors, including hypertension, current smoking, diabetes mellitus, lack of physical activity, waist-to-hip ratio, excessive alcohol consumption, psychosocial stress, and depression account for 90 % of the population-attributable risk of stroke, all of them with high significance for ischemic stroke [O'Donnell et al., 2010]. Further, atrial fibrillation is seen as specifically potent risk factor for cerebrovascular infarctions resulting in severe strokes compared to strokes of other etiology due to wider ischemic areas and greater potential of hemorrhagic transformation [Tu et al., 2010]. Internal CaAD, causing nearly every fifth ischemic stroke [Mughal et al., 2011], can also be seen as a modifiable risk factor for these occurrences. For internal CaAD, risk factors go hand in hand with those leading to ischemic stroke. However, history of migraine and peridontitis were additionally characterized as risk factors for CaAD [Kaul et al., 2017], while the impact of atrial fibrillation on the development of CaAD may not be. Preventional regulation of modifiable risk factors remains clinical in eager to reduce stroke incidence and is to be discussed later on.

1.1.3 Pathogenesis

The ground principle leading to ischemic stroke manifestation can be insufficient arterial blood flow from occluded vessels. Most arterial occlusions are caused by embolism. Firstly, by extracranial originating embolism from, e.g., atrial fibrillation thrombosis or ruptured atherosclerotic plaques within the aortic arch and cervical arteries resulting in mostly territorial infarctions and secondly, by intracranial originating embolism from small vessel atherosclerosis mainly leading to smaller ischemic areas, so-called lacunar strokes [Maksimova and Gulevskaya, 2019]. Additionally, inflammatory causes, e.g., variants of vasculitis, virus infections, and arterial dissections lead to ischemic stroke, predominantly in patients <60 years of age. [Campbell and Khatri, 2020] Once occlusion occurs, interrupted blood flow and the resulting metabolite deficiency causes the neurons to rely on insufficient anaerobic metabolism. Consequently, within minutes an ischemic cascade including ionic imbalance, excitotoxicity, calcium overload, cytotoxic and vasogenic edema, peri-infarct depolarization, oxidative and nitrosative stress, blood-brain barrier

disruption, inflammation and consequently, cell death is set off [MartInez-Coria et al., 2021b, Maida et al., 2020]. Within the so-called ischemic core, cell damage progresses rapidly depending on the duration and widespread of the infarction [Deb et al., 2010]. Subsequently, an area of brain tissue around the ischemic core is weakened in its homeostasis by interrupted blood flow, although collateral blood supply by the circle of Willis ensures cell structure being kept for some time, the penumbra [Brouns and De Deyn, 2009]. This hibernating cerebral area can be saved by quick storage of blood flow to lower infarction-related disabilities, or else may incorporate into the ischemic core [MartInez-Coria et al., 2021b, Campbell and Khatri, 2020].
Carotid artery disease predominantly localizes in the carotid bifurcation due to the lower shear stress being experienced by the vessel wall and increased turbulences in this area[Litsky et al., 2014]. As for the process of internal CaAD as a variant of atherosclerotic lesions, its development follows the principles of atherosclerosis to be discussed later in section 1.3.

1.1.4 Clinical Expression

Within the group of patients diagnosed with internal CaAD, the discrimination between asymptomatic and symptomatic clinical expression is to be made. In general, patients are expected to suffer from symptomatic CaAD whenever they have experienced cerebrovascular events related to the ipsilateral cerebral hemisphere or retina within six months prior to medical examination and intervention; likewise, they are classified in the Munich Vascular Biobank [Wendorff et al., 2015, Litsky et al., 2014]. Further, cerebrovascular events may be distinguished by the duration of neurological deficit persisting. Whenever neurological deficits cease within the first 24 h, the cerebrovascular event is to be classified as transient ischemic attack (TIA) and as a stroke in case of neurological deficit outlasting the 24 h period. Prior studies have not only concluded a 5 % chance of stroke within 30 days and up to 25 % of a recurrent cerebrovascular event within one year after the occurrence of TIA, but also validated a score assessing the short-term stroke risk within the ipsilateral hemisphere after TIA [Mughal et al., 2011, Johnston et al., 2007]. The individual neurological deficits resulting from cerebrovascular events depend on the expansion of insufficiently perfused cerebral areas. Common neurological deficits may include limbal and facial unilateral sensomotoric malfunction, dysphasia, dysarthria, visual field failure including amaurosis fugax, as well as coordinative difficulties [Yew and Cheng, 2015, Litsky et al., 2014, Sobieszczyk and Beckman, 2006].
With the absence of cerebrovascular events and the stated neurological deficits, patients are to be diagnosed with asymptomatic CaAD. Patients may report subjective symptoms, including general weakness, dizziness and confusion, but in accordance to *Litsky et al.* shall be continued to be categorized as asymptomatic. However, patients with asymptomatic CaAD performed lower in various cognitive scores compared to non-stenotic patients with similar cardiovascular risk factors and thus, recently led to the conclusion of asymptomatic CaAD association with cognitive impairment independent of risk for cognitive impairment caused by conventional cardiovascular risk factors [Lal et al., 2017, Buratti

et al., 2016, Buratti et al., 2014]. Cerebral hypoperfusion, including decreasing cerebrovascular blood pressure due to inadequate collateral perfusion, initiating the mechanism of cognitive deterioration is to be discussed in current literature [Khan et al., 2021].

1.1.5 Diagnostics

The reported acute onset or waking up with focal neurological deficit is generally seen as the first step in ischemic stroke diagnostics and indicating further diagnostics. Since the time of stroke onset is not always possible to detect exactly, but clinical for determining suited therapeutic options (Section 1.2), the onset is generally classified as the time the patient was lastly seen well. From every fifth up to every fourth presented acute neurological deficit suspecting a stroke can be accounted to mimics including seizures, syncopes, sepsis, migraine and brain tumors as the five most frequent [Gibson and Whiteley, 2013, Fernandes et al., 2013]. Thus, together with the insufficient differentiation between intracerebral hemorrhage and ischemic infarction by clinical diagnostics, brain imaging is essential in complementing the assessment of stroke variants and mechanisms and shall only be delayed by capillary blood glucose determination, according to the American Stroke Association [Campbell and Khatri, 2020, Jauch et al., 2013]. With native CT and gradient-echo T2-weighted MRI scans intracerebral hemorrhage can be distinguished, while multimodal CT imaging and diffusion-weighted-imaging MRI (DWI-MRI) detect localization of the infarction core and magnitude of the ischemic area [Hurford et al., 2020, Hankey, 2017, El-Koussy et al., 2014]. Additionally, arterial tree imaging with CT- and MRI-angiography and penumbral imaging by mismatching perfusion and diffusion CT or MRI scans (e.g., PWI-DWI-MRI Missmatch) are crucial diagnostics increasing potential success of followed therapeutic pathways [Powers et al., 2019, El-Koussy et al., 2014].
Diagnosing CaAD various invasive and non-invasive imaging modalities have been successfully established. Since it is non-invasive and prior meta-analyses have confirmed 98 % of sensitivity and 89 % specificity identifying carotid stenosis >50 % with experienced operators, carotid duplex ultrasonography is the most frequently used first-line imaging technique assessing degree of lumen stenosis [Jahromi et al., 2005, Sabeti et al., 2004]. Measuring the exact percentage of lumen stenosis the European Carotid Surgery Trial (ECST) and the North American Symptomatic Carotid Endarteriectomy Trial (NASCET) differ within their methods, while the NASCET measurement has been adopted in the majority of European Society for Vascular Surgeries (ESVS) guidelines [Naylor et al., 2018, ECSTGroup et al., 1991, NASCTCollaborators, 1991]. Although its clinical relevance in first-line diagnosis seems relatively undisputed, duplex ultrasonography remains dependent on the operating physician and cannot sufficiently picture intracranial and intrathoracic arterial stenosis. Therefore, CT- and MRI-angiography, as well as Digital Subtraction Angiography (DSA) are being utilized highlighting extra and intracranial arterial stenosis in more detailed and enabling the identification of possible CaAD complications like pseudoaneurysm and dissections [Mortimer et al., 2018]. Additionally, recent analyses have illustrated contemporary MRI imaging to enable better evaluation of rupture-prone CAP by picturing characteristics correlating with CAP instability, like intraplaque hem-

orrhage, plaque ulceration and calcification, plaque neovascularity, fibrous cap thickness and presence of a lipid-rich necrotic core in higher resolution [Brinjikji et al., 2016].

1.2 Therapeutic Pathways of Ischemic Stroke and Carotid Artery Disease

With its immense impact on the population and health systems globally, therapy improvement of acute ischemic stroke remains of high contemporary interest and shall be illustrated briefly. Dealing with carotid artery disease as a classified major cause of ischemic stroke, multiple modalities for treatment and preventional management continue to be discussed to limit disease progression to a minimum.

1.2.1 Emergency Care

The ongoing incorporation of the hibernating penumbra into the ischemic core within hours and the resulting possible permanent secondary cerebral deficiency requires quick and efficient acute management. In dealing with acute ischemic stroke, the establishment of an interdisciplinary stroke unit has been clearly proven beneficial for patients' survival without disabilities independent of stroke type and severity [Langhorne et al., 2020]. Alongside sufficient imaging (Section 1.1.5), assessing reperfusion therapy eligibility and the usage of a stroke severity rating scale, preferably NIHSS, are recommended by current AHA guidelines [Powers et al., 2019].
Aiming for adequate revascularization, intravenous thrombolysis, and endovascular therapy can be used separately and in combination. On the one hand, patients can be treated with intravenous application of recombinant human tissue plasminogen activator (IV-tPA), and through plasminogen to plasmin conversion the thrombus causing the infarction can hereby be dissolved [Campbell and Khatri, 2020]. To be classified as eligible for this intervention, patients must be last seen well within the last 4,5 h [Del Zoppo et al., 2009, Hacke et al., 2008]. Even though patients age and stroke severity assessment remains strongly recommended for prognostics, meta-analyses have exemplified IV-tPA therapy to benefit chances of overall stroke outcome irrespectively of those factors, but with significantly greater relative benefit of treatment within the first 3 h compared to the following 1,5 h [Emberson et al., 2014, Lansberg et al., 2009]. Further, possible time expansion up until 9 h regarding IV-tPA eligibility assessment by additional usage of CT and perfusion-MRI imaging has been discussed in recent studies but not yet implemented in current guidelines [Ma et al., 2019, Campbell et al., 2019]. On the other hand, endovascular thrombectomy (EVT) has recently shown great efficacy in treating patients with acute ischemic stroke up to 12 h of symptoms onset, independent of combining it with IV-tPA [Campbell et al., 2015, Goyal et al., 2015, Jovin et al., 2015, Saver et al., 2015]. Additional emergency care modalities include management of blood pressure, cerebral edema, glycemia, temperature modulation, and ventilation [Herpich and Rincon, 2020].

1.2.2 Conservative Treatment

Dealing with CaAD pharmaceutical therapy remains clinical by modulating vascular risk factors as primary and secondary prevention and conservative CaAD treatment in general. Thus, conservative treatment predominantly involves antithrombotic treatment alongside antihyperlipidemic and antihypertensive treatment. Again, the discussed differentiation between symptomatic and asymptomatic CaAD patients may be made.
In accordance with the 2017 European Society of Cardiology (ESC) - in collaboration with the ESVS - guidelines, life-long antithrombotic treatment with low-dose Aspirin is recommended for every patient dealing with symptomatic CaAD, irrespectively of successful interventional revascularization and with ADP-receptor antagonist Clopidogrel as an alternative [Aboyans et al., 2017, Sacco et al., 2008]. Dual antiplatelet therapy (DAPT) is recommended for patients undergoing endovascular techniques [Aboyans et al., 2017]. However, due to prior analysis DAPT may be considered for up to one month following minor ischemic stroke and TIA with potential extension after myocardial infarction within the past 12 months and low bleeding risk [Aboyans et al., 2017, Udell et al., 2016, Wang et al., 2013b]. The singular antiplatelet therapy (SAPT) for asymptomatic CAD patients remains in conflict. Despite the lack of randomized controlled studies backing SAPT, benefit on stroke prevention in asymptomatic CAD patients (stenosis >50 %) is stated, ESC/ESVS guidelines recommend lifelong, low-dose Aspirin treatment [Naylor et al., 2018, Aboyans et al., 2017]. Antiplatelet therapy can be seen as an independent prognostic factor decreasing ischemic infarction risk in patients with asymptomatic 70-99 % carotid artery stenosis [King et al., 2013].
Further, ESC/ESVS guidelines recommend treating hyperlipidemia in all patients dealing with CaAD with targeted low-density lipoprotein (LDL) serum levels of <70 mg/dl or a minimum cutback of 50 % in case of LDL levels initially being between 70 and 135 mg/dl [Piepoli et al., 2016]. Such reduction can be realized by decreasing cholesterol synthesis with HMG-CoA reductase inhibition (statins), resulting in higher cellular LDL-uptake, alongside intestinal cholesterol resorption inhibitors (Ezetemib) and inhibition of proprotein convertase subtilisin/kexin 9 (PCSK9) downgrading cellular LDL-receptors (e.g., Evolocumab). With the significant reduction of stroke risk by regression of carotid intima-media-thickness and general cause mortality in CaAD patients being portrayed in recent studies [Huang et al., 2013, Taylor et al., 2011], statins remain gold standard in treating hyperlipidemia. Nevertheless, combination or substitution with Ezetemib has proven its potential benefit [Murphy et al., 2016] and has been included in current guidelines, while Evolocumabs advantage over statin therapy is being presently discussed [Aboyans et al., 2017, Sabatine et al., 2017].
Due to its undisputed association with CaAD, hypertension also is to be strictly regulated for primary and secondary preventive reasons. Adequate antihypertensive treatment has been known to slow down CaAD progression for decades [Sutton-Tyrrell et al., 1994]. Consequently, European guidelines recommend treating non-diabetic CaAD patients dealing with hypertonus, regardless of symptom presentation, with a targeted blood pressure <140/90 mmHg, while diabetic CaAD patients ought to reach blood pressure <140/85 mmHg; ACEIs and ARBs shall be considered first-line therapeutics [Mancia et al., 2013].

1.2.3 Interventional Treatment

In eager to treat CaAD interventionally, practitioners mainly rely on two modalities. On the one hand, patients can be treated surgically with carotid endarterectomy (CEA); on the other hand, an intravascular method can be used for carotid artery stenting (CAS). Regardless of the chosen method, ESVS guidelines recommend blood pressure to be maintained <180/90 mmHg peri-procedurally [Naylor et al., 2018].

Carotid Endarterectomy

Anesthesia for CEA can be performed locally and generally. The atherosclerotic plaque is removed through longitudinal arteriotomy, while common and external carotid arteries are clamped. Intraoperative DUS has shown association with lower rates of in-hospital stroke or death following CEA [Knappich et al., 2017]. In accordance with current ESVS guidelines, CEA is recommended for symptomatic patients with 70-99 % stenosis but only considered with conflicting evidence in 50-69 % symptomatic carotid stenotic patients and not recommended <50 % and complete stenosis [Eckstein, 2018, Naylor et al., 2018]. Although, American guidelines, considering greater benefit from intervention, favourably recommend CEA for symptomatic CaAD patients with 50-99 % stenosis [Kernan et al., 2014a]. Treatment within 14 days of symptom onset is considered to be safe and shall be operated in this time span by ESVS guidelines approval [Eckstein, 2018, Tsantilas et al., 2016].
As for asymptomatic carotid stenotic patients, contemporary imaging (Section 1.1.5) enables discrimination of characteristics leading to rupture-prone plaques. In addition to detailed MRI imaging, characteristics associated late ipsilateral stroke also include silent infarction on CT imaging, stenosis progression, impaired cerebral vasoreactivity, spontaneous embolization on transcranial Doppler ultrasonography, and history of contralateral TIA. With the detection of at least one of such characteristics, ESVS urge to consider CEA for asymptomatic patients with 60-99 % carotid artery stenosis [Naylor et al., 2018, Eckstein, 2018, Naylor et al., 2014].

Carotid Artery Stenting

The endovascular stenting revascularization is performed percutaneously. Common surgical complications like hematoma and nerve injury are less frequent, while surgical difficulties due to anatomic structures like high carotid artery bifurcation, recurrent stenosis, and previous surgeries and radiation in the cervical area can be circumnavigated [Gaba et al., 2018]. Therefore, in current ESVS guidelines, CAS may be considered for symptomatic patients with carotid artery stenosis of 50-99 % considered of 'high risk for surgery' due to severe cardiac or pulmonary co-morbidities and poorly accessible operating areas [Naylor et al., 2018, Aboyans et al., 2017].
However, rates of peri-procedural stroke have proven significantly higher in asymptomatic CaAD patients being stented than those being treated with CEA (2,6 % vs. 1,3 % respectively, p = 0,04) [Cui et al., 2018, Gaba et al., 2018]. Thus, CAS is not equally put

into consideration for asymptomatic patients with 60-99 % carotid stenosis in current guidelines [Naylor et al., 2018]. With the continuous improvement of CAS treatment regarding symptomatic and asymptomatic carotid stenosis, randomized controlled trials like CREST-2, ECTS-2, and ACTS-2 have been drawn up to deliver contemporary perspectives on CEA, CAS, and medical treatment in comparison. Consequently, less invasive treatment pathways seem plausible to be included in upcoming guidelines [Gaba et al., 2018].

1.2.4 Preventional Screening and Life-style Managements

The burden of CaAD and its resulting ischemic infarctions whenever symptomatic stands undeniably (Section 1.1.1). However, prevalence of moderate and severe ACAS in general population is low at 4,2 % and 1,7 %, respectively [de Weerd et al., 2009]. The lower the ACAS prevalence in the population to be screened is recorded, the greater the required benefit must be to justify the psychological and physiological distress caused by false positive results and the financial effort. With the prevalence of moderate ACAS assessed via DUS sinking <2 % in people potentially benefitting from CEA (aged <80), carotid screening is not generally recommended by ESVS guidelines [Naylor et al., 2018, de Weerd et al., 2010]. Estimating ACAS prevalence even lower (<1 %) and with great concern of potentially harming false positives with unnecessary surgery, the US Preventive Service Task Force (USPSTF) recommends against ACAS screening in general population [Force, 2021, Jonas et al., 2014]. Furthermore, ESVS and USPSTF guidelines did not conclude a reliable characterization for 'higher risk' ACAS patient subgroup potentially benefitting more from carotid screening and resulting interventional treatment [Naylor et al., 2018, Jonas et al., 2014]. Although with studies indicating a decreasing cardiovascular morbidity and mortality risk through preventional carotid screening in patients presenting numerous cardiovascular risk factors [Giannoukas et al., 2016], the ESVS guidelines recommend ACAS screening under such circumstances as may be considered [Naylor et al., 2018].
The preventional screening for ACAS remains recommended widely different, while lifestyle management changes, however, have proven their beneficial disease-modulating impact not only for CaAD but for stroke prevention in general. In recent analyses, the cardiovascular risk, even though it did not reach the level of never smokers, for heavy smokers dropped significantly within five years of smoking cessation compared to current smokers [Duncan et al., 2019]. Further, in studies like *The Northern Manhatten Study*, moderate and intense aerobic physical activity has been shown to have a protective effect on ischemic stroke risk independent from other modulated risk factors [Willey et al., 2009]. Additionally, metaanalyses of various randomized controlled trials have portrayed significant associations with stroke risk reduction for nutritional modalities, like the Mediterranean diet and salt intake reduction [Khan et al., 2019, Dehghan et al., 2012]. Regarding general overweight and obesity, cardiovascular risk has been suggested to be positively correlated with increasing Body Mass Index (BMI) values, while data for potential BMI correlation with cerebrovascular incidents remains less well determined, leading to uncer-

tain effectiveness of weight loss, especially in secondary preventive care [Kernan et al., 2014b].

1.3 Atherosclerosis

Atherosclerosis is a chronic disease of the vascular intima in arteries and the main pathological cause of cardiovascular diseases worldwide. It can be defined as the development of plaques consisting of accumulated lipids, cells, debris, and scar tissue in the inner layer of the arterial vessel wall. Advanced atherosclerotic lesions can narrow down arterial lumen, disrupt blood flow and thus, lead to ischemia in the areas supplied by the affected vessel. Ischemia can also result from atherosclerotic lesion disrupture following blood clot formation and thromboembolic infarctions. The two leading causes of death globally, myocardial infarction and ischemic stroke, are mainly results of atherogenic processes, and atherosclerosis, subsequently, is the main pathological process causing death and loss of productive life years in the world. [WHO, 2020, Basatemur et al., 2019]

1.3.1 Anatomical Composition of Arteries

The arterial vessel wall in humans is made out of three layers, the external *tunica adventitia*, the intermediate *tunica media*, and the internal *tunica intima*. The *adventitia* is made up of fibrous tissue, mostly collagen and elastin fibers, and fibroblasts, and contains mast cells, nerve endings, and vasa vasorum in bigger vessels. The *media* is architectured of extracellular matrix (ECM) as connective tissue and vascular smooth muscle cells (VSMCs) enabling blood pressure regulation through contraction and relaxation. The luminal side of the arterial wall is covered with the *intima*, built up by singularly layered, squamous endothelial cells (ECs) forming a semi-permeable barrier. The vessel communicates with the bloodstream via humoral and inflammatory messenger substances through this squamous epithelium.

1.3.2 Common Locations of Atherosclerotic Lesions

Specific arterial geometries are associated with hemodynamic flow disturbances leading to biomechanically induced endothelial dysfunction in certain defined areas down the arterial branches. This leads to predilection of arterial localities in which atherosclerotic plaque formation takes place predominantly. Those certain areas are preferentially located in the supplying main vessels of the arterial tree, more precisely in branching areas, curvatures, and bifurcations like the iliac bifurcation and the carotid sinus [Wang et al., 2013a, Asakura and Karino, 1990, DeBakey et al., 1985]. The theory about the impact of hemodynamic stresses on atherogenesis and the discussion of what level of vessel wall shear stress (VWSS) is to be classified as athero-prone dates back to the early 20th century [Anitschkow, 1933, Fry, 1969, Caro et al., 1971]. Currently, the hypothesis about low levels of VWSS in pulsatile and steady flow initiating atherogenesis and being enhanced by additional turbulences and oscillation in the direction of the VWSS is being widely

accepted [Mohamied et al., 2015, Chatzizisis et al., 2008, He and Ku, 1996, Ku et al., 1985], although not unquestioned [Peiffer et al., 2013]. In this, via mechanotransductional processes by low and oscillating VWSS in athero-prone areas ECs are put under oxidative and inflammatory stress [Ando and Yamamoto, 2009] and undergo change regarding their alignment and surface protein expression complimenting atherogenesis [Heo et al., 2011a, Heo et al., 2011b, Heo et al., 2013]. In contrast, undisturbed laminar flow with high VWSS is seen to be having an athero-protective impact down the vasculature, ensuring ECs survival [Heo et al., 2014, Kim et al., 2012]. Further studies have shown steady laminar flow in unbranched vessels, increasing ECs messenger release and subsequently downregulating thrombogenic and inflammatory processes [Abe and Berk, 2014, Garin et al., 2007, Diamond et al., 1989].

1.3.3 Risk Factors

With its exceptional great role in the pathogenesis of the world's two most common causes of death and further diseases, like peripheral artery disease and aneurysm formation, the determination and regulation of atherosclerotic risk factors remains of great interest. Risk factors may be distinguished into non-modifiable and modifiable risk factors.
On the one hand, non-modifiable risk factors regarding atherogenesis are age, male gender, black, Hispanic, and southeast Asian ethnicity, and further genetical disposition [Volgman et al., 2018, Carnethon et al., 2017, Rodriguez et al., 2014]. Further athero-prone genetic dispositions are considered cardiovascular events within blood-related family members, familial hypercholesterolemia or elevated lipoprotein(a) levels [Bouhairie and Goldberg, 2015, Nordestgaard et al., 2010]. On the other hand, the relevance of modifiable risk factors, labeled as such due to their possible regulation via behavioural alternation, to the process of atherogenesis has been described, including current smoking, calorie surplus, fatty diet, excessive alcohol consumption, waist-to-hip ratio and insufficient levels of physical activity [Herrington et al., 2016]. The previously discussed risk factors for cardiovascular disease mentioned in the *The INTERHEART Study*, e.g., arterial hypertension, diabetes mellitus, hypercholesteremia, hyperphosphatemia, elevated LDL/HDL ratio, low HDL serum levels and adipositas, are also characterized as modifiable risk factors for atherogenesis and provide defined starting points for possible prevention modalities [McQueen et al., 2008, Teo et al., 2006, Yusuf et al., 2005, Rosengren et al., 2004, Yusuf et al., 2004].
Additionally, various chronic diseases and their impact on atherogenesis have been portrayed in recent studies. The reduced glomerular filtration in patients suffering from chronic kidney disease (CDK) has been assessed to prosper atherogenesis through hypertension and dysregulated lipid blood levels caused by it [Schiffrin et al., 2007]. With CDK-associated mineral bone disease, the calcium-phosphate metabolism is dysregulated, also. Subsequently, higher atherosclerotic risk by hyperphosphatemia and general acceleration within calcification of plaques follow in accordance with recent literature [Kono et al., 2012, Palmer et al., 2011]. Analyzing chronic diseases' impact on atherogenesis on a psychological level, chronic stress is seen as an independent modifiable risk fac-

tor for atherosclerosis causing imbalanced regulation of inflammatory processes, lipid metabolism, and blood pressure [Yao et al., 2019]. This complements the requirement of well-defined risk factor regulation for psychological and physiological risk factors.

1.3.4 Pathogenesis and Morphology

Since Rudolf Virchow gave his lecture in cellular pathology about atheromatous affection on arteries at the University of Berlin in 1885, illustrating atherosclerosis' development by chronic inflammatory response to intimal mechanical forces in combination with disbalances blood composition [Virchow, 1989], pathogenesis of atherosclerosis has been continued to be a highly researched field. In recent times, subdivision of atherosclerotic pathogenesis into three stages, initiation, progression, and complications, with evolution in lesion morphology throughout the stages, has been established.

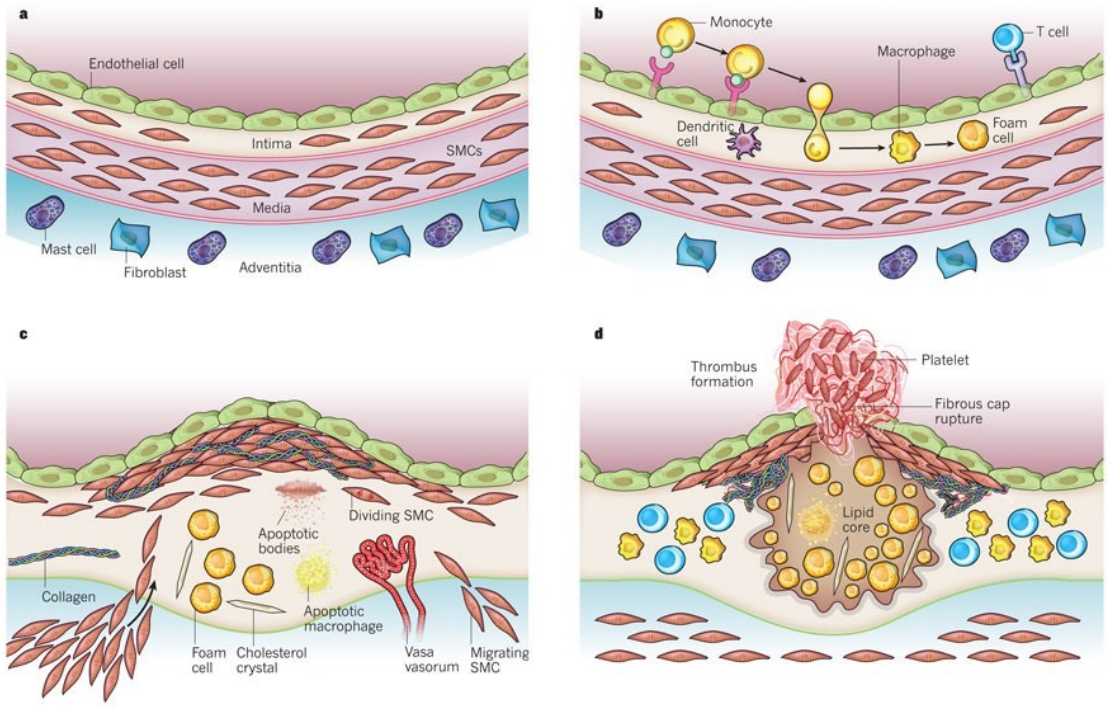

Figure 1.1: **Stages of atherosclerotic plaque formation [Libby et al., 2011]**
a) Healthy arterial state with intact vessel architecture consisting of intima, media, and adventitia. b) Initiation stage of atherogenesis with fatty streak formation by accumulating lipid particles and resulting inflammatory cell migration; foam cell formation. c) Progression stage of atherogenesis with plaque evolution, including fibrous cap formation, necrotic core expansion, ECM production by migrating VSMCs, and cell apoptosis. d) Complication stage of atherogenesis with plaque rupture and thrombus formation.

Firstly, the initiation, the local impact of low and oscillating VWSS on the vessel wall by imbalanced blood flow is leading to athero-prone regions in the vasculature, as previously discussed (Section 1.3.2), causing a heterogenous ECs morphology with increased permeability for macromolecules [Gimbrone, 1999]. The chronic inflammatory process of atherosclerotic plaque formation is initiated by trigger factors passing the functionally impaired ECs layer and accumulating in the subendothelial matrix. Predominantly, apolipoprotein B-100 (apo B) containing macromolecules like low-density lipoprotein (LDL) and lipoprotein(a) particles are considered to be such trigger factors, leading to fatty streaks in the intima, the initial atherosclerotic lesion [Lusis, 2000, Steinberg and Witztum, 1999]. This is favoured by more recent studies showing strong correlation between atherosclerosis onset and LDL serum levels with the assumption of possible complete atherosclerosis absence with LDL serum levels of 10-30 mg/dl [Goldstein and Brown, 2015, Nordestgaard et al., 2013, Cohen et al., 2006]. Separated from antioxidants in the arterial lumen, the lipid particles are oxidized and furtherly modified to pro-inflammatory factors, eventually causing expression of leukocyte adhesion molecules, like vascular cell adhesion molecule 1 (VCAM-1) and selectines, and pro-inflammatory chemokine release by activated ECs; chemoattracted monocytes from the bloodstream then migrate into the artery wall, subsequently maturing into tissue macrophages [Libby et al., 2011, Li et al., 1993, Cybulsky and Gimbrone, 1991]. The high-capacity scavenger receptor expressed on the macrophage surface and responsible for oxidized LDL phagocytosis is not being downregulated with the rise of cholesterol levels and, thus, enables cholesteryl ester overload within the macrophages and their transformation into foam cells, a hallmark for initiated atherogenesis [Libby et al., 2019, Li et al., 2009, Schrijvers et al., 2005].

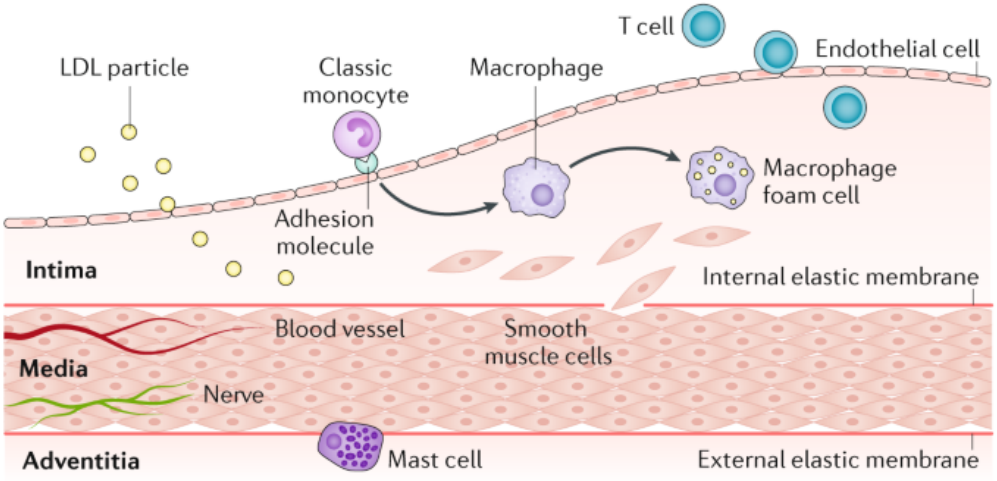

Figure 1.2: **Initiation stage of atherosclerosis with fatty streak formation [Libby et al., 2019]** Hallmarks of atherosclerosis initiation include subendothelial accumulation of LDL particles, monocyte attraction, and VSMCs migration through the internal elastic membrane

Further, T helper type 1 lymphocytes - although in vastly lower plentitude than monocytes - are chemoattracted into the subendothelial area, perpetuating the inflammatory process by reacting against the autoantigens of apo B on lipid particles, whereas regulatory T lymphocytes seem to inhibit atherogenesis [Gisterå and Hansson, 2017]. The accumulated leukocytes flourish VSMC migration and activation from the media in the subendothelial space, and with scavenger receptor-like members of the LDL receptor-related protein family, the migrated VSMC are then engorged with LDL particles bound to intimal proteoglycans [Libby et al., 2019, Nus and Mallat, 2016, Llorente-Cortés et al., 2000]. Other than the ones responsible for LDL imbalance, atherosclerosis risk factors remain less elucidated regarding their atherogenesis initiation. However, like hypertonus participant angiotensin II elaborates the master transcriptional regulator nuclear factor-kB (NF-kB) inflammatory pathway [McMaster et al., 2015] and smoking or insulin resistance lead to soluble, pro-inflammatory mediator release, they are generally considered on making their impact on atherogenesis initiation by prospering the inflammatory processes. [Libby et al., 2018, Libby et al., 2016, Rocha and Libby, 2009].

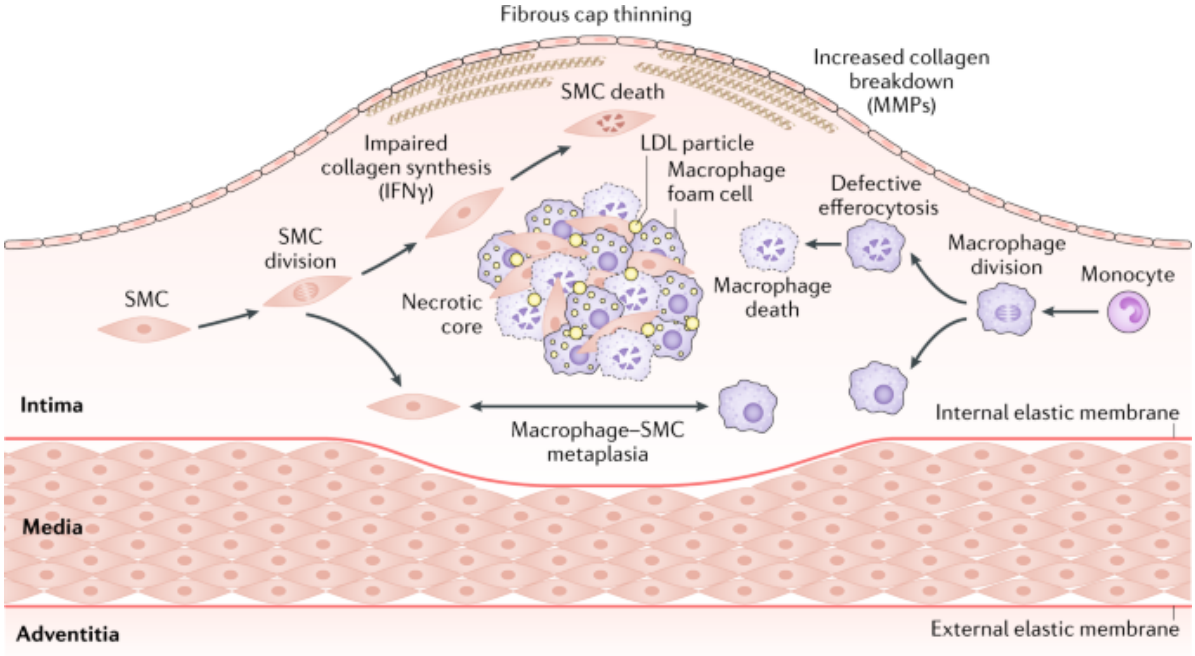

Figure 1.3: **Progression stage of atherosclerosis with fibroatheroma formation [Libby et al., 2019]** Featuring processes include macrophage-VSMC metaplasia with apoptosis and lipid-rich necrotic core formation, ECM production by VSMCs and collagen breakdown by macrophages secreting enzymes resulting in fibrous cap thinning

Secondly, with the migration of VSMCs into the initial fatty streak, the further progression of the atherosclerotic lesion is commenced. The participating cells, macrophages, lymphocytes, ECs, and VSMC stay activated, expressing pro-inflammatory messengers, like TNFα, IL-6, and MCP-1 [Glass and Witztum, 2001]. The ongoing accumulation of foam

cells and atherogenic lipid particles form an avascular, hypocellular, and soft-like mush that architectures a lipid core in the subendothelial area, lacking supporting collagen. [Falk, 2006, Guyton, 2001]. The migrated VSMCs of a synthetic type produce extracellular matrix within the lesion, consisting of interstitial collagen, elastin, proteoglycans, and glycosaminoglycans, thickening the intima-media layer [Bennett et al., 2016]. The ECM synthesized by intima and media-migrated VSMCs eventually forms the fibrous cap on the surface of the atherosclerotic lesion in combination with the ECs. However, VSMCs and macrophages are interchanging phenotypes via metaplasia as atherogenesis proceeds and with an increasing release of enzymes, like matrix metalloproteinases, thinning out the fibrous cap [Libby et al., 2019, Bennett et al., 2016]. During this further progression of atherosclerosis, ECs, macrophages, and VSMCs die by apoptosis, forming the nidus of the lipid-rich or necrotic core of the lesion [Falk, 2006]. The necrotic core formation is also perpetuated by impaired clearance of cell debris, also described as defective efferocytosis [Yurdagul et al., 2018, Tabas et al., 2015]. Local calcification takes place while the atherosclerotic lesion ages due to imbalanced regulation between calcium deposition and clearance [Ruiz et al., 2015, Hoffmann et al., 2003]. While microcalcifications scattered around the lesion seem to enhance plaque instability, broadly spread calcifications are considered to be protective regarding the risk of possible plaque rupture [Rocha and Libby, 2009, Huang et al., 2001].
Thirdly, possible complications, including luminal stenosis, plaque rupture, and thrombus release, occur in dependence on plaque stability. While the plaque evolves, the artery undergoes constant remodeling, resulting in either constrictive remodeling with severe luminal stenosis caused by predominating fibrous tissue produced by VSMCs, or expansive remodeling with the diminishing of the fibrous cap and, consequently, of lesion stability [Falk, 2006, Vink et al., 2001]. The formation of unstable lesions by a thin fibrous cap (<65 µm) in combination with a large necrotic core, great abundance of inflammatory cells, and defective efferocytosis is considered to be the clinically more significant predicting factor for plaque complications and commonly known as 'vulnerable plaque' formation [Bennett et al., 2016, Tabas et al., 2015, Bentzon et al., 2014]. A fissure of the fibrous cap and hereby enabled communication of the interior plaque with the arterial lumen is characterized as plaque rupture. Predominantly, this takes place in the lesion edges, or shoulder region, due to the great local abundance of foam and pro-inflammatory cells [Lusis, 2000]. While the development into foam cells enables VSMCs to keep the plaque skeleton alive with ECM production [Kolodgie et al., 2001], activated pro-inflammatory cells actively diminish the key structure of the lesion, predominantly the fibrous cap, with the release of proteolytic enzymes, like plasminogen activators, cathepsins, and matrix metalloproteinases [Libby et al., 2019, Bentzon et al., 2014]. Once communication with the arterial lumen is established, pro-thrombogenic factors, notably tissue factors, whose secretion is being enhanced by oxidative LDL or ligated CD40 effecting ECs, macrophages, and VSMCs, are released from the plaque interior into the vessel and thrombosis is triggered [Lusis, 2000, Schönbeck et al., 2000]. Over time, the thrombus, described by Libby et al. as 'a source of transforming growth factor-β (TGFβ) and platelet-derived growth factor elaborated by activated platelets' may lead to further VCMCs migration and ECM

production, eventually causing significant higher lumen stenosis with encapsulated red thrombi, a hallmark for plaque rupture [Libby et al., 2019].
On the contrary, encapsulated white thrombi detected in the atherosclerotic lesion are characteristic of another atherosclerosis complication, plaque erosion. Erosion hereby is classically characterized as endothelial denudation. In contrast to plaque rupture with its usually disrupted anatomic vessel structure, plaque erosion happens typically with the elastic laminas in the artery staying intact and the media being structurally organized with a great abundance of contractile VSMCs and is often localized within close range to ruptured plaque areas [Hao et al., 2006, Virmani et al., 2000b]. Prior studies on the exact pathogenesis of plaque erosion lack yet details, but blood flow disturbance and pro-inflammatory processes, like polymorphonuclear leukocytes enhancing local thrombosis activated via pattern-recognition receptors, e.g., Toll-like receptor 2, are suggested to be contributing to this modality [Franck et al., 2017, Quillard et al., 2015].

1.4 Cellular Protagonists in Atherosclerosis

In the development of atherosclerotic lesions, the cellular protagonists have each been seen of different relevance to the process over time. Endothelial cells, vascular smooth muscle cells, and immune cells, including monocytes, macrophages, as well as the previously mentioned lymphocytes (Section 1.3.4) are all of significant relevance to atherogenesis, which shall be further illustrated in the following.

1.4.1 Endothelial Cells

In a healthy environment, ECs form the monolayered tunica intima of the vessel wall, a semi-permeable barrier with tight junctions sealing the surface in between as communicating border between the intramural blood flow and the vessel itself and, thus, playing a clinical role in the homeostasis by coordinating leukocyte trafficking, preventing thrombosis, and regulating vascular tone. Exposed to atherogenic risk factor derived, pro-inflammatory modulators, like oxidated LDL, TNFα, IL-1β, angiotensin II and endothelin, ECM particles and imbalanced VWSS, phenotypic transformation and activation of ECs is initiated, resulting in increased permeability and chemoattraction of leukocytes [Funk et al., 2012, Hahn and Schwartz, 2009, Pober and Sessa, 2007]. The chemoattraction of leukocytes by activated ECs is a result of enhanced expression of P- & E-selectin, chaining and slowing down leukocyte velocity, and VCAM-1 as final adhesive ligand on the intramural surface [Ley et al., 2007, Pober and Sessa, 2007]. This is complemented by chemokine release (e.g., MCP-1) causing leukocyte VCAM-1 affinity augmentation by integrin expression in accordance to literature [Funk et al., 2012, Ley et al., 2007].
Increased permeability results from the additionally initiated endothelial dysfunction. The key aspect of endothelial dysfunction can be seen as the decreasing nitric oxide (NO) levels, the hallmark of endothelial regulation. In a healthy state, the endothelial semi-permeable barrier structure is being upheld, and platelet and leukocyte adhesion is being inhibited by NO signaling [Chigaev et al., 2011, Roberts et al., 2008, Draijer et al., 1995].

Whereas, under the influence of certain stimuli, like turbulent pressure and decreased insulin signaling, NO synthesis in ECs is downregulated, promoting permeability, lack of thrombosis, and inflammation prevention [Funk et al., 2012, Tai et al., 2004]. Downregulating NO synthesis leads to its uncoupling, producing superoxide, forming further oxidants with the remaining intracellular NO [Gimbrone and García-Cardeña, 2016].

1.4.2 Vascular Smooth Muscle Cells

In a healthy arterial environment, VSMCs have been identified as the main cellular component forming the media of the arterial wall in a closely arranged pattern and as loosely distributed cellular components in the intima layer [Pease and Paule, 1960]. Within their healthy morphology presenting as fusiform, often labeled contractile, VSMCs are characterized by high expression of contractile markers such as smooth muscle actin (alpha-SMA), transgelin (TAGLN), smoothelin (SMTN), and smooth muscle myosin heavy chain I & II (SMMHC I & II) [Shanahan and Weissberg, 1998, Chamley et al., 1977]. While VSMCs in the media are responsible for regulating vascular diameter via balanced contraction, they are believed to be the main connective tissue, including collagen, elastin, proteoglycans, producers in the healthy and diseased intima [Schwartz et al., 2000].
Although fully functioning and completely differentiated, contractile VSMCs uphold the ability of phenotypical de-differentiation and, consequently, great plasticity. By undergoing phenotypical de-differentiation, also known as phenotypic switch, VSMCs show decreasing gene expression levels of contractile markers previously mentioned, while levels of intracellular ECM particles, ECM synthesizing enzymes, and secretory modalities increase [Alexander and Owens, 2012, Clarke et al., 2008]. At the molecular level, the transcription factors Myocardin-serum response factor (SRF) and Krüppel-like factor 4 (KLF4) are considered as predominantly responsible for phenotypic switch regulation [Shankman et al., 2015, Pipes et al., 2006], alongside cell-cell interactions and epigenetic regulation. While SRF and its co-factor MYCOD bind on CArG-box modules in primer regions of contractile gene markers, KLF4 not only counteracts this by binding on neighbouring G/C repressor modules, but also promotes VSMCs proliferation directly, making phenotypic switching KLF4 dependant, in accordance to recent studies [Basatemur et al., 2019, Sheikh et al., 2018, Sheikh et al., 2015, Zhou et al., 2015]. Extracellular atherogenic stimuli, including platelet-derived growth factor (PDGF) via KLF4 expression enhancement and inflammatory cytokines and cholesterol via MYCOD downregulation by NF-kB and miR-143-miR-145 pathways, lead to enhanced phenotypic switching towards so-called synthetic VSMC [Basatemur et al., 2019]. However, surrounding ECM is considered to suppress phenotypic switching, keeping VSCMs in a contractile state and more resistant against atherogenic mitogens [Bennett et al., 2016].
Contractile type VSMCs with balanced ECM production mainly migrating from the media layer as the atherogenesis progresses have been shown to comprise the fibrous cap of the plaque with mainly collagen-based ECM production; they are considered protective regarding potential plaque complications [Jacobsen et al., 2017, Durgin et al., 2017]. On the contrary, synthetic VSMCs are believed to be of great pro-atherogenic impact. The

intima, synthetic VSMC-produced proteoglycans with negatively charged side chains enhance lipoprotein retention, primarily apo B, with positively charged side chains and, thus, direct atherosclerosis initiation [Tabas et al., 2007, Skålén et al., 2002]. Further, activation by oxidated lipid particles and pro-inflammatory cytokines present in the atherosclerotic initiation stage leads to VSMCs releasing monocyte chemoattractants, like CCL2, CCL5 and CXCL1 [Basatemur et al., 2019, Tabas, 2010]. Prior studies have also illustrated the relative incapability of adequate excess lipid release due to downregulated levels of efflux transporters (e.g., ABCA1) and hence, higher foam cell formation tendency for synthetic VSMCs in comparison to contractile VSMCs and immune cells [Allahverdian et al., 2014, Campbell et al., 1985, Campbell et al., 1983]. Throughout lipid overload and foam cell formation, expression levels of contractile markers (e.g., alpha-SMA) in VSMCs are further downregulated. In contrast, macrophage markers (e.g., CD68 and LGALS3) showed increasing expression, according to recent lineage studies [Chappell et al., 2016, Vengrenyuk et al., 2015, Shankman et al., 2015]. VSMC-derived cells are considered to account for around half of the foam cells and CD68+ cells [Shankman et al., 2015, Allahverdian et al., 2014]. However, the lack of the distinct macrophage-derived foam cell ability of sufficient autophagy, phagocytosis, and efferocytosis inevitably leads to inflational VSMCs-derived foam cell death [Osonoi et al., 2018, Allahverdian et al., 2014]. Thus, VSMCs would enhance necrotic-core growth and lipid particle release, subsequently contributing directly to atherosclerotic lesion progression and vulnerable plaque formation.
Furthermore, prior studies give reason to believe that VSMCs plasticity expands over the longtime considered binary phenotypic switch. On the one hand, the de-differentiation into mesenchymal stem-cell-like cells with VSMCs expression of stem cell antigen 1 (SCA1) and endoglin is being discussed as a potential foundation for VSMCs clonality within the plaque, according to mouse studies [Basatemur et al., 2019, Shankman et al., 2015]. On the other hand, VSMCs transformation into osteochondrogenic cells with expression of mineralizing ECM proteins and chondrocyte-related proteins transcription factor SOX9 and chondroadherin is considered to impact lesion calcification [Dobnikar et al., 2018, Durham et al., 2018, Alves et al., 2014]. This is backed by lineage tracing studies in a mouse model, illustrating the majority of osteochondrogenic precursor and chondrocyte-like cells being VSMCs-derived [Naik et al., 2012].

1.4.3 Myeloid Cells

Myeloid cells, also referred to as immune cells, show presence in all three stages of atherosclerosis described earlier. In contrast, to permanently present ECs and VSMCs, myeloid cell abundance is caused mainly by chemoattraction and migration (Section 1.3). The contribution to atherogenesis of the two predominant immune cell types, monocytes and macrophages shall be introduced in the following.

Monocytes

As a main part of the innate immune system and accounting for 3-8 % of the circulating blood leukocytes monocytes are critical for exogenous bacterial, viral, and fungal infection intervention via phagocytosis and are rarely present in the intima layer of the vessel wall [Shalhoub et al., 2011, Libby et al., 2002]. Nonetheless, monocytes are also widely known for their responsibility for endogenous inflammation, including rheumatic diseases, lung fibrosis, and atherosclerosis. Atherosclerosis initiation is characterized by monocytic rolling-adhesion and eventual diapedesis into subendothelial intima milieu, promoted by VSMCs and ECs expressing leukocyte chemoattractants VCAM-1, selectins and MCP-1 [Funk et al., 2012, Ley et al., 2007, Pober and Sessa, 2007, Glass and Witztum, 2001]. Monocytes express not only various CC chemokine receptors (CCR) necessary for such diapedesis but distinct surface receptors allowing monocyte subclassification. When activated via various stimuli present in the atherosclerotic milieu, including growth factors (e.g., PDGF & M-CSF), lipid particles (e.g., oxLDL & apoB), and cytokines (e.g., IL-1 & TNFα), monocyte subclasses impact atherogenesis differently [Ghattas et al., 2013]. Classical monocytes (Mon1) account for over 80 % of human monocytes and show high expression levels of CD14 and no CD16 expression; Mon1 distinct code can be classified as CD14++CD16-CCR2+ [Wong et al., 2011, Ernst, 2001]. The Mon1 subset predominantly executes phagocytosis and chemokine secretion leading to further leukocyte migration and plaque progression and hence, may be classified as pro-atherogenic [Jaipersad et al., 2014]. Monocytes portraying the distinct expression code CD14+CD16++CCR2- are defined as non-classical monocytes (Mon3) and considered a vasculature resident subset [Ghattas et al., 2013, Ernst, 2001]. Mon3 primarily promote anti-inflammatory processes and collagen deposition and thus impacts atherogenesis [Jaipersad et al., 2014]. More recently, an intermediate monocyte subset with a distinct expression of CD14++CD16+CCR2+ (Mon2) was identified with gene microarray analysis [Wong et al., 2011, SHANTSILA et al., 2011]. This intermediate subset is believed to promote neovascularization within the plaque primarily by expressing a certain tyrosine kinase (Tie-2) [Venneri et al., 2007, De Palma et al., 2005]. Significant data on which monocyte subset differentiates into distinct macrophage types in humans throughout the atherosclerotic process is lacking as of today. However, published studies in vitro and mouse models propose a possible linkage between classical monocytes and M1 macrophages and non-classical monocytes and M2 macrophages, respectively [Ghattas et al., 2013, Gratchev et al., 2012, Geissmann et al., 2010].

Macrophages

The presence of macrophages is crucial for the initiation, progress, and complications of atherosclerotic plaque formation. Macrophage marker-expressing cells in early atherosclerotic lesions are primarily derived from migrating blood monocytes, but secondly, they may also derive from proliferating resident macrophages [Nahrendorf, 2018, Ensan et al., 2016, Robbins et al., 2013]. There are two macrophage types with distinct phenotypic polarisation participating in atherogenesis, whose derivation is initiated by an in-

terplay between M-CSF and GM-CSF. On the one hand, the M1 phenotypically polarized macrophage subset (M1) is identified as atherogenic, primarily expressing pro-inflammatory cytokines (e.g., TNFα and IL-6) and impacting tissue destruction; M1 polarization results in a stimulating factor imbalance shifting towards GM-CSF in vitro. On the other hand, the M2 phenotypically polarized macrophage subset (M2) is identified as rather athero-protective, expressing primarily restructuring growth factors (e.g., IGF-1 and FGF-13) and regulating inflammatory processes; in vitro, M2 derive from a surplus of M-CSF. [Hamilton, 2008, Mosser and Edwards, 2008, Gordon, 2007, Martinez et al., 2006] However, macrophages retain certain plasticity enabling phenotypic swith between M1 and M2 and even intermediate phenotypes [Kadl et al., 2010, Gordon, 2003]. Both subsets, M1 and M2, also show different abundance within distinct areas of the atherosclerotic plaque. While M1 abundance can be mainly found in areas of unstable cell homeostasis, like the lipid-rich necrotic core and the shoulder region, M2 predominantly reside in more stable areas with arguably smaller intracellular lipid particles [Chinetti-Gbaguidi et al., 2011, Boyle et al., 2009, Bouhlel et al., 2007]. This leads to the hypothesis of greater phagocytic activity by M1 and hence, a considerable impact on foam cell mutation.
With the phagocytic overload of cholesterol and oxidated lipid particles via scavenger receptors, macrophages subsequently transform into foam cells. Due to the lack of scavenger receptor expression regulation by uprising intracellular lipid levels, the foam cells eventually undergo apoptosis [Libby et al., 2019, Li et al., 2009, Schrijvers et al., 2005]. With the death of macrophage-derived foam cells (MDFC), the necrotic core firstly progresses because of an increasing deficit of phagocytes eliminating further oxidated lipid particles [Falk, 2006] and secondly due to deficient efferocytosis of MDFC remnants and the previously phagocyted atherogenic particles causing secondary necrosis [Basatemur et al., 2019]. As atherogenesis proceeds, vulnerable plaque formation in advanced lesions is not only promoted by macrophages through necrotic core expansion but also by diminishing ECM. Prior studies have illustrated a high expression of matrix metalloproteinases (e.g., MMP14) - responsible for ECM destruction and fibrous cap thinning - and considerably lower expression of tissue inhibitor of metalloproteinase in M1 in vitro and in vivo [Johnson et al., 2008, Newby, 2005, Chase et al., 2002].

1.5 Proteomics

The rapidly advancing evolution of encoding biological processes leads to more detailed information about physiological and pathological processes. Nowadays, current technologies acquire information about singular interactions all the way down to the molecular level. With the consequent rising understanding of how proteins impact life, their distinct expression on the cellular level to be sequenced is of great contemporary interest.

1.5.1 Definition

'Proteomics' was firstly described in *Progress with Proteome Projects: Why all Proteins Expressed by a Genome Should be Identified and How To Do It* by Marc Wilkins in 1996

as the protein counterpart to genome [Wilkins et al., 1996]. Proteome may be defined after Aslam et al. in *Proteomics: Technologies and Their Applications* as 'the overall protein content of a cell that is characterized with regard to their localization, interactions, post-translational modifications, and turnover, at a particular time' fluctuating in distinct cells at certain times and impacted by external stimuli [Aslam et al., 2017]. Identifying the proteome, including expression, molecular structure and function, biochemical interactions, and modifications of proteins at any stage, is described as 'proteomics' [Domon and Aebersold, 2006].

1.5.2 Preliminary Transcriptomics

The transcriptome may be seen as the dynamic production protocol of the proteome and, therefore, a piece of valuable information to decipher in eager to modulate proteins once they have been detected in certain processes. It is the collection of transcribed RNA molecules within a single cell, distinct tissue, or up to an entire organism, including coding RNA (e.g., mRNA) and non-coding RNA molecules (e.g., long non-codingRNA) at a certain state at a particular time. The assessment of the transcriptome, so-called transcriptomics, is possible via microarray technology. However, high throughput DNA sequencing (NextGen seq.), leading to more and more systematical monitoring and comprehension of the biofunctional status, has been established over the last years as a more contemporary laboratory practice[Thompson et al., 2016]. NextGen seq. enables the determination of details on differential gene expression, variously spliced RNA molecules and gene alleles, post-transcriptional modifications, non-coding RNAs, alternative splicing, single-nucleotide polymorphism, and gene fusions with a clinical surplus in throughput [Morgensztern et al., 2018, Gupta and Gupta, 2014]. Since the transcriptome comprises the protein sequences and their regulatory and modifying elements, it can be identified as the precursor of the proteome [Kaur, 2013].

1.5.3 Proximity Extension Assay

One of the most recent additions to the arsenal of proteomic modalities is the Proximity Extension Assay (PEA) technology, developed and distributed by Olink Proteomics (Uppsala, Sweden). The PEA technology is a 96-plex immunoassay for high throughput and rapid expression analysis for protein biomarkers in complex samples like blood and serum, but also in xenografted mice and resuspended dried blood spots. With a minimum sample requirement of no more than 1 µl of a sample, the PEA portrays high specificity and high sensitivity in accordance with prior studies. [Assarsson et al., 2014]
On a molecular level, for every biomarker, two paired antibodies, either two matched monoclonal or one pool of polyclonal antibodies, are coupled with exclusive oligonucleotides, so-called proximity probes, on both ends each. The two proximity probes per antibody are architectured by a 40-mer oligonucleotide covalently linking on the 3'-end and 5'end, respectively, each containing binding sequences for distinct detection and various amplification primer. On the 3'-linked probe, an additional oligomer containing 40 complementary

nucleotides to the probe and a further 16 nucleotides, seven as a spacer and the final nine as a complimentary sequence to the corresponding 5'-end, are hybridized. When brought in solution with the antigen-containing sample, the antibodies link the antigen with their antigen-specific sections. Due to the proximity of the antigen, the complimentary probes on the antibodies hybridize, and with the addition of DNA polymerase, the overlapping probe arms are fully extended, and a complete qPCR template is formed. With unique detection primers, the qPCR templates are then preamplified and, subsequently, digested by Uracil-DNA glycosylase by removing the unbound primer. With each DNA sequence representing one biomarker per qPCR template, the proteome analysis is then performed by microfluidic RT-qPCR. [Assarsson et al., 2014, Lundberg et al., 2011] This extremely sensitive antibody-based technology enables precise quantitative determination of human plasma proteins in concentrations below picogram per milliliter.

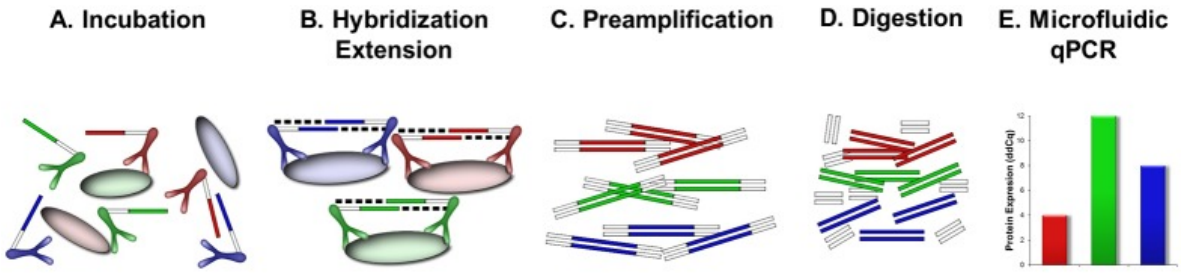

Figure 1.4: **Design and description of 96-plex PEA** [Assarsson et al., 2014]

1.5.4 Suitable Projects

The detailed results acquired with proteome analysis, especially PEA, can provide critical information helping with screening, monitoring, prognosis, and follow-up of disease expansion, resulting in a wide range of suitable projects. With the needed sample size reduced to a bare minimum, even projects with only a margin of resource samples are applicable. Unlike transcriptomics and genomics, proteomics distinctly confirms the qualitative presence of protein biomarkers in the assessed sample at the given time and state and additionally enables expression quantification. With the decoded evolution of biomarker expression throughout the disease development, the potential treatment of multiple pathologies, like atherosclerosis, via influencing exclusively responsible proteins comes within promising range.

2 Aim of Study

With this study, the aim of decoding a potential biomarker for worldwide challenging diseases caused by atherosclerosis was pursued with the help of a constantly updated and maintained human biobank. As for atherosclerosis being a major column in the pathogenesis of leading causes of death worldwide, the biological processes involved were targeted to be furtherly specified with contemporary identification technologies.
Firstly, the value of human biobanking was tried to be highlighted not only by illustrating the benefits of close linkage between clinical data-based studies and experimental studies on a molecular level and the additional research chances being created by it but also by the wide utilization range of the obtained human samples in experimental technologies possible. A possible resulting contribution to advancing personalized medical treatment was implied. Furthermore, as research modalities evolve, the adequately stored samples may be eligible for technologies not yet designed, as it has been with the constantly evolving sequencing methods.
Secondly, with atherosclerosis being the principal aetiological foundation of the leading causes of CaAD and stroke, this study aimed to provide supplementary viewpoints on stabilization and destabilization processes within atherosclerotic lesion development on a protein molecule level. Hereby, it was intended to characterize protein biomarkers for distinct developmental stages of the atherosclerotic lesion and their expression in plaque areas and the cellular components contributing to atherogenesis via various qualitative and quantitative analytical methods. Intentionally, a linkage between a certain protein target to a specific cell type, ideally as its producer, was aimed to achieve. Subsequently, identifying such biomarkers aimed at potential individualized therapeutic targets for atherosclerosis treatment while the ongoing importance of the interplay between conservative descriptive modalities and modern sequencing technologies was intended to be portrayed.
Lastly, from a more personal and practical point of view, the study aimed to create an advanced scientific understanding and build a foundation of essential experimental skillset. Weekly laboratory meeting participation, regular attendance to clinic seminars and journal clubs, and wide-ranging literature research, predominantly in the cardiovascular field, commenced and streamlined the preparation for the subject. The experimental skillset was intended to be primarily obtained by helping maintain the biobank with staining, characterization, and interpretation of CAPs, as well as isolating RNA and determining protein expression out of CAP samples and the cultivated cells obtained from them. Secondarily, by further investigating prior studies on downregulated genes and proteins initiated within the laboratory group and the further assessment of detailed immunohistochemical analysis of the CAP.

3 Material

3.1 Antibodies

Product (Clone) Dilution	**Article Nr.**(Lot Nr.)	**Supplier**
Monoclonal Mouse Anti-Human to AIFM1 (7F7AB10) 1:100	ab110327 (GR1018134)	Abcam
Polyclonal Rabbit Anti-Human to Galectin 3 1:100	ab53082 (GR5105817)	Abcam
Monoclonal Rabbit Anti-Mouse, Anti-Human to Myosin, smooth muscle heavy chain 1 and 2(EPR5335) 1:4000	ab124679 (GR2184587)	Abcam
Monoclonal Mouse Anti-Human to CD45 (2B11+PD7126) 1:500	M0701 (20053425)	Dako Denmark A/S
Monoclonal Mouse Anti-Human to CD68 (KP1) 1:1000	**M0814**(20053851)	Dako Denmark A/S
Monoclonal Mouse Anti-Human to SMA (HHF35) 1:200	**M0635** 10045990	Dako Denmark A/S

Table 3.1: **Antibodies**

3.2 Primer

Product	**Article Number**	**Supplier**
RPLPO	Hs00420895_gH	Thermo Fisher Scientific
ACT beta	Hs01060665_G1	Thermo Fisher Scientific
CD68	Hs02836816_G1	Thermo Fisher Scientific
AIFM1	Hs00377585_m1	Thermo Fisher Scientific
Cytochrome C	Hs01588974_g1	Thermo Fisher Scientific
Caspase 9	Hs00962278_m1	Thermo Fisher Scientific
GapDH	Hs03929097_G1	Thermo Fisher Scientific

3.3 Kits

Product (art.Nr.)	**Lot Number**	**Supplier**
High-Capacity RNA-to-cDNA Kit (438895)	00903832	Applied Biosystems by Thermo Fisher Scientific
Taq Man Geneexpression Master Mix (4369016)	00914665	Applied Biosystems by Thermo Fisher Scientific
Dako REAL™ Detection System (K5001)	2004246	Dako Denmark A/S
Pan Monocyte Isolation Kit, Human (130-096-537	5200601292	Miltenyi Biotec GmbH
Smooth Muscle Cell Growth Medium Kit Classic (PB-MH-200-2190)	QC01B20F07	PELO Biotech
miRneasy® Mini Kit (4388950)	166030842	QIAGEN Sciences
Avidin/Biotin Blocking Kit (SP-2001)	2D010SC	Vector Laboratories, Inc.

Table 3.3: **Kits**

3.4 Chemicals

Product (art.Nr.)	**Lot Number**	**Supplier**
Ethanol 70% (T 913.3)	350299773	Carl Roth GmbH & Co. Kg
Ethanol 96% (T 171.4)	219284289	Carl Roth GmbH & Co. Kg
Ethanol >99,5% (5054.1)	306247495	Carl Roth GmbH & Co. KG
Hämalaunlösung sauer nach Mayer (T 865.2)	376249538	Carl Roth GmbH & Co. KG
Xylol 98,5% (CN80.5)	239284216	Carl Roth GmbH & Co. KG
Milkpowder (T 145.1	478270656	Carl Roth GmbH & Co. KG
Sodiumhydroxide 2mol/l (T 135.1)	19009955	Carl Roth GmbH & Co. KG

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Hydrochloric Acid 2mol/l (T. 134.1)	20008105	Carl Roth GmbH & Co. KG
Dako REAL™ Antibody Diluent (52022)	20050373	Dako Denmark A/S
TRIS Base Molecular Biology Grade	D00137505	EMD Chemicals
Ficoll-Plaque™ PLUS (17-1440-02)	10291184	GE Healthcare Bio-Sciences AG
Hdrogen Peroxide 30%	K43900997249	Merck KGaA
Cytric Acid Monohydrate	K43900997249	Merck KgaA
M-CSF (130-093-963)		Miltenyi Biotec GmbH
Eosin 1%	19896-5	Morphisto GmbH
Resorcin-Fuchsin nach Weigert (1602)	190318	MRI TUM Pharmacy
Weigert'sche Lösung I (677)	190318	MRI TUM Pharmacy
Weigert'sche Lösung II (678)	030717	MRI TUM Pharmacy
Pikrofuchsinlösung (708)	300617	MRI TUM Pharmacy
QIAzol® Lysis Reagent (1023537)	56307131	QIAGEN Sciences
Eukitt®quick-hardening-mounting-medium (03989-100ML)	BCBZ6120	Sigma-Aldrich Chemie GmbH
Chloroform 99% (32211-1L-M)	STBJ4293	Sigma-Aldrich Chemie GmbH
2-Propanol (19516-500ML)	SHBD1467V	Sigma-Aldrich Chemie GmbH
RNase ZAP™ (R2020-250ML)	SLBW8968	Sigma-Aldrich Chemie GmbH
Poly-L-Lysine Solution 0,1% (P8920-100ML)	SLCC5591	Sigma-Aldrich Chemie GmbH
Bovine Serum Albumin Solution 30% (A9576-50ML)	SLCC6663	Sigma-Aldrich Chemie GmbH
Normal GOAT Serum (G62767-100ML)	19H092	Sigma-Aldrich Chemie GmbH
Dulbecco's Phosphate Buffered Saline (D8537-500ML)	RNBJ1283	Sigma-Aldrich Chemie GmbH
Trypan Blue Solution 0,4% (T8154-20ML)	RNBH2120	Sigma-Aldrich Chemie GmbH
RPMI-1640 Medium (R8758-500ML)	RNBJ4052	Sigma-Aldrich Chemie GmbH
0,2µm filtered Ambion™ Nuclease-Free Water (AM9930)	2007138	Thermo Fisher Scientific

table to be continued on next page

HEPES 1M (15630-080)		Thermo Fisher Scientific
Fetal Bovine Serum (FBS), Qualified (10270-106)	2088284	Thermo Fisher Scientific
0,25% Trypsin-EDTA (25200-056)		Thermo Fisher Scientific
Pen Strep Glutamine (10378-016)		Thermo Fisher Scientific
EDTA 0,5M sterile solution (E177-100ML)	19H2056255	VWR Life Sciences
Etahnol absolute	20821.296	VWR Life Sciences

Table 3.4: **Chemicals**

3.5 Laboratory Equipment

Product (art.Nr.)	**Serial Number**	**Supplier**
Zeiss Primo Vert	3842002903	Carl Zeiss Microscopy GmbH
GFL1052	10567413C	Gesellschaft für Labortechnik mbH
Kern ABJ220-4M	WB 1250960	Kern & Sohn GmbH
Leica RM2255 (14050237960)	6496/09.2013	Leica Biosystems
Leica EG 1150C (14038838037)	9921/08.2013	Leica Biosystems
Medax Slide Warmer (13801)	14414	MEDAX GmbH & Co. KG
QuadroMACS™	36362	Miltenyi Biotech GmbH
Bio Gen PRO200		Pro Scientific Inc.
Pipet Lite XLS SL10	B840540286	RAININ™
Pipet Lite XLS SL100	B838474937	RAININ™
Pipet Lite XLS SL1000	B849863315	RAININ™
Bonestead Gen Pure Pro UV/UF (50131950)	41463230	Thermo Fisher Scientific
Heraeus Fresco21 Centrifuge	41508250	Thermo Fisher Scientific
ClickSeal Biocontainment Rotor (75003424)		Thermo Fisher Scientific
Quant Studio™3 Real-Time PCR Instrument (A28131)	272311593	Thermo Fisher Scientific
Veriti 96Well Thermo Cycler (4375786)	2990244096	Thermo Fisher Scientific

table to be continued on next page

Heraeus Multifuge X3R	41497240	Thermo Fisher Scientific
Thermo Scientific TX-750 (75003617)		Thermo Fisher Scientific
Herasafe KS12 (51022515)	41463308	Thermo Fisher Scientific
Pipet Filler S1	195942	Thermo Fisher Scientific
Heratherm Oven (51028123)		Thermo Fisher Scientific
VWR Mixer Mini Vortex	130401016	VWR International
VWR Mini Star Centrifuge	12130527	VWR International
inoLab pH7110	13171734	Wissenschaftlich Technische Werkstätten GmbH

Table 3.5: **Laboratory Equipment**

3.6 Consumables

Product (art.Nr.)	**Lot Number**	**Supplier**
MicroAmp® Optical 96Well Reaction Plate (N8010560)	I19J9Q524	Applied Biosystems by Thermo Fisher Scientific
Falcon 96Well Plate (353072)		Corning Inc.
Safe-Lock Tubes 1,5ml (0030120.086)	J190742L	Eppendorf
Cellstar Serological Pipet 2ml (710180)	F1607384	Greiner bio-one GmbH
Cellstar Serological Pipet 5ml (606180)	F1609369	Greiner bio-one GmbH
Cellstar Serological Pipet 10ml (607180)	F2005363	Greiner bio-one GmbH
Cellstar Serological Pipet 25ml (760180)	F18134C	Greiner bio-one GmbH
Cellstar Tubes 50ml (227261)	E181236J	Greiner bio-one GmbH
Cellstar Tubes 15ml (188271)	140415G	Greiner bio-one GmbH
Petri Dish (633181)	F200337Q	Greiner bio-one GmbH
MACS® LS Column (130-042-401)	5190927110	Miltenyi Biotec GmbH
C-Chip Neubauer Improved (DHC-N01)	2N16203	NanoEntek
Biosphere® Filter Tips 0,1-2,5µl (70.1130.212)	9054811	Sarstedt AG & Co.

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Biosphere® Filter Tips 0,1-10µl (70.1130.210)	0051721	Sarstedt AG & Co.
Biosphere® Filter Tips 0,5-20µl (70.1116.215)	9051211	Sarstedt AG & Co.
Biosphere® Filter Tips 2-20µl (70.760.213)	9052111	Sarstedt AG & Co.
Biosphere® Filter Tips 2-100µl (70.760.212)	9053211	Sarstedt AG & Co.
Biosphere® Filter Tips 10-100µl (70.762.211)	9052311	Sarstedt AG & Co.
Transferpipet 3,5ml (86.1171)	8053912	Sarstedt AG & Co.
Microcentrifuge Tubes 2ml (T3531-200EA)	3110	Sigma-Aldrich GmbH
Menzel Gläser Superfrost Microscope Slides (AAAA00008032E)	0789	Thermo Fisher Scientific
Menzel Gläser Superfrost PLUS (J1800AMNZ)	1476	Thermo Fisher Scientific

Table 3.6: **Consumables**

3.7 Buffers

Tris-hydroxymethyl-aminomethan 10x Buffer (TRIS)
The TRIS 10x buffer used in all steps of the immunohistology stainings was freshly made in amounts of one litre and stored at room temperature up until two weeks in case of excess buffer. 60,5 g TRIS Base were dissolved in 700 ml of distilled water before reaching a pH value of 7,6 by titrating the solution with hydrochloric acid (HCl). The buffer was completed by adding 90 g of sodium chloride (NaCl) and further distilled water up to the amount of one litre, resulting in a 1x dilution.

Citrate buffer
The citrate buffer was always freshly prepared in advance of every immunostaining. 4,2 g cytric acid monohydrate were dissolved in 2 l of distilled water. The buffer was then brought to a pH value of 6,0 with sodium hydroxide (NaOH).

Miltenyi Monocyte Buffer
The Miltenyi Monocyte Buffer was freshly prepared in required portions before isolating monocytes from human blood samples with relations of PBS + 2 mM EDTA + 0,5 % BSA.

4 Methods

If not stated otherwise in the following sections, all experiments were performed in the institutional laboratories of the AG Maegdefessel in the Department of Vascular Biology, Technical University of Munich (TUM), München, Germany.

4.1 Biobank

With biobanking experts stating the uprising importance of the collection of human atherosclerotic biomaterial retrieved from surgeries in the cardiovascular field to deepen the knowledge about the pathogenesis of such atherosclerotic lesions [Pelisek et al., 2019] the 'Munich Vascular Biobank' was installed.
Surgeries, of which biomaterial was retrieved from, were performed by the Department of Vascular and Endovascular Surgery of the Klinikum recht der Isar following the current guidelines [Eckstein, 2018]. Ethical permission for such actions had been given in advance by the universities ethics committee of the medical faculty (TU München). The biobank nowadays contains not only human tissue samples from 1.567 patients with operated atherosclerotic lesions in carotid arteries but also from patients with AAA who had to undergo surgery (n = 481) and from surgically treated PAD (n = 703). In addition, blood serum from over 4400 patients being treated for atherosclerotic lesions in carotid arteries, AAA and PAD was withdrawn and stored. Since the surgical act of kidney transplants leaves parts of the healthy abdominal aorta left behind on the explant, control tissue (n = 102) was acquired. As for healthy carotid artery tissue, 15 healthy samples were provided by the Department of Forensic Medicine. All Patients from whom biomaterial and clinical data for scientific research was acquired had previously given their informed consent. The human tissue samples for this study were entirely taken from this biobank.

4.2 Processing and Conserving Human Tissue Samples

Ensuring the biological integrity of the retrieved human tissue samples, the biospecimen were processed further between one and four hours after surgical removal.
As seen in Figure 4.1, the entire extracted atherosclerotic lesion of the carotid artery was divided into evenly split up segments (3-5 mm). Chosen segments were then directly frozen in liquid nitrogen and stored at -80 °C until needed for molecular analysis, while the other segments were fixed in 4 % formaldehyde for at least 24 h. The fixed biomaterial was then decalcified with EDTA for at least another day and a maximum of one week (depending on the level of calcification). Following decalcification, the conservation

was completed by embedding the segments in paraffin and storing the plaques at room temperature. After staining the formalin-fixed paraffin-embedded specimens (FFPE) as described in 4.5.1 to 4.5.3 the histological labeling according to the tissue morphology was performed. Therefore, individual histological characteristics such as the quality of the staining, plaque stability, counted cells, concentration of elastic fibers, extracellular matrix, neovascularization, level of calcification, infiltration of immune cells and status of rupture were rated semiquantitatively by two independent members of the institute experienced in histological pathology and unaware of the patients' clinical data. This semiquantitative score ranged from absent (-) to strongly present (+++). The histolog-

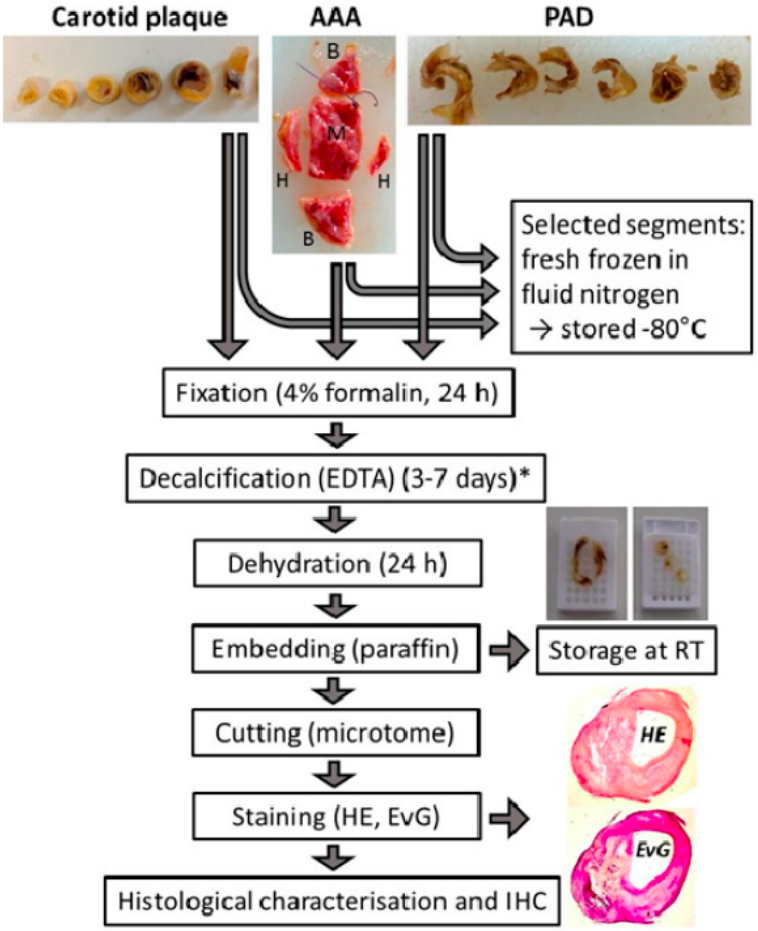

Figure 4.1: **Schematic chart of the processing of vascular tissue after surgical excision** Carotid plaque: atherosclerotic lesions from patients with high-grade carotid artery stenosis; AAA: aortic wall from patients with abdominal aortic aneurysm; PAD: atherosclerotic tissue from patients with peripheral artery disease; RT: room temperature; IHC: immunohistochemistry. HE: hematoxylin-eosin staining; EvG: Elastica van Gieson staining; * the time depends on the extent of calcification. [Pelisek et al., 2019]

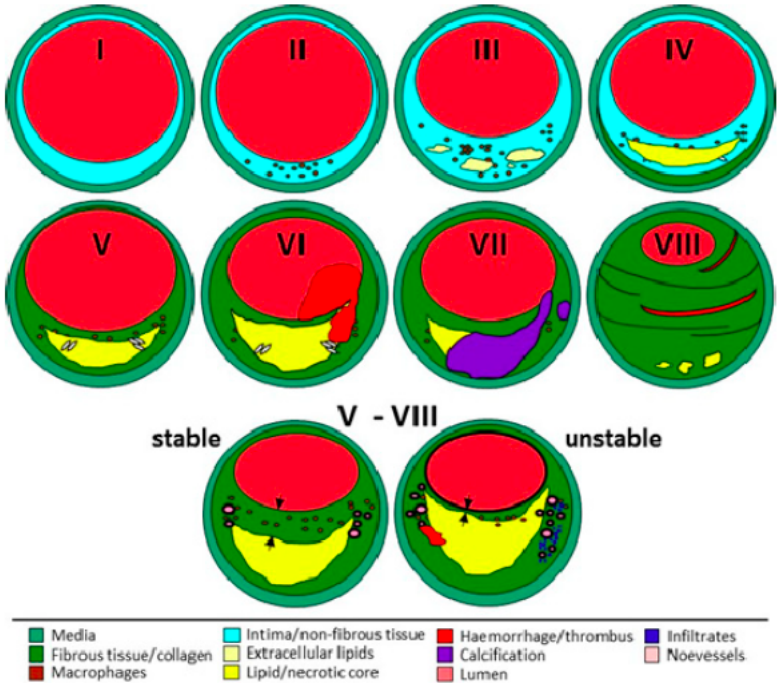

Figure 4.2: **Classification of atherosclerotic lesions according to the American Heart Association (AHA)** Type I: initial lesion with isolated macrophages and macrophage-derived foam cells; type II: fatty streaks, increased number of foam cells, intracellular lipid accumulation; type III: further accumulation of inflammatory cells and intracellular lipids, isolated extracellular lipid deposits; type IV: atheroma, formation of confluent lipid core without perceptible fibrous cap; type V: fibroatheroma, formation of fibrous layer over the lipid/necrotic core; type VI: as V but with thrombus and/or intraplaque hemorrhage; type VII: as V with calcified nodules, calcification predominates; type VIII: fibrous tissue predominates, lumen mainly small, lipid deposits minimal or absent. [...] Unstable/vulnerable plaque can develop from each plaque type of type V–VIII. [Pelisek et al., 2019]

ical morphology of the FFPE specimens was determined. The updated American Heart Association (AHA) classification of coronary atherosclerotic lesions was used for classifying under the microscope in the Hematoxylin Eosin and Elastica van Gieson staining as shown in 4.2 [Stary, 2000, Stary et al., 1995, Stary et al., 1994, Stary et al., 1992]. The progress of atherosclerotic lesions was distinguished into eight different levels. This process was performed by two independent observers experienced in vascular pathology. The segments of the FFPE specimens were hereby allocated to their level of atherosclerosis.

4.3 Serum Proteome Analysis

The serum proteome analysis of blood sera withdrawn from patients undergoing therapeutical CEA in our clinic's vascular and endovascular surgery department and stored in our Munich Vascular Biobank was outsourced to the Max Plank Institut of Biochemistry, Munich. The 96-plex PEA was used from OLink, Sweden, for the expression analysis.

4.3.1 Proximity Extension Essay

The previously described principle of the PEA technology (Section 1.5.3) was used in the 96-plex modality and the design is to be described in the following as it is in *Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability* by Assarssons et al.:
'One µL sample (buffer (PBS with 0.1% BSA), antigen-spiked buffer, or biological sample) was mixed with 0.3 µL of each proximity probe mix (A and B), 0.3 µL Incubation Stabilizer (Olink Bioscience, Uppsala, Sweden) and 2.1 µL Incubation Solution (Olink Bioscience) and incubated overnight at 4°C (Figure 1A). A combined extension and preamplification mix (96 µL) containing 10 µL MUX PEA Solution (Olink Bioscience), 0.5 units Pwo (DNA Gdansk, Poland), 1 µM forward + reverse universal preamplification primers, and 1 unit hot-start DNA polymerase was added to each reaction at RT. After mixing and a total 5-min incubation, the plate was transferred to a thermocycler (Applied Biosystem 2720) running an initial extension step to unite the two oligonucleotides (50°C, 20 min), immediately followed by a hot-start step (95°C, 5 min) and 17 cycles of amplification (95°C, 30 s; 54°C, 1 min; 60°C, 1 min) (Figure 1B). Amplification was performed with universal flanking primers to amplify all 96 sequences in parallel (Figure 1C). Finally, 2.8 µL of the preamplification products were mixed with 7.2 µL buffer containing 5 µL MUX Detection Solution (Olink Bioscience), 0.071 units Uracil-DNA glycosylase (DNA Gdansk) used to digest the DNA templates and remaining universal primers (Figure 1D), and 0.14 units hot-start polymerase. Five µL from the sample mix above was transferred to the sample inlet wells of a microfluidic real-time PCR chip (96.96 Dynamic Array IFC, Fluidigm Biomark). Five µL from the respective well of an Assay Plate (Olink Bioscience) containing 9 µM sequence-specific internal detection primers, 2.5 µM molecular beacon in 1x DA Assay Loading Reagent (Fluidigm) were transferred to the assay inlet wells (Figure 1E). The chip was run in a Biomark instrument with the following program: Thermal mix (50°C, 2 min; 70°C, 30 min; 25°C; 10 min), Hot-start (95°C, 5 min), PCR Cycle 40 cycles (95°C, 15 s; 60°C, 1 min).' [Assarsson et al., 2014]

4.3.2 Panels

Four multiplexed 96x96 PEA panels were utilized for protein expression assessment, including *Cardiovascular II* (CVDII), *Cardiovascular III* (CVDIII), *Inflammation* (IFL) and *Organ Damage* (OD). Ninety-two protein biomarkers were measured per panel, distinct in their biological impact and chosen in relation to the panel description, respectively. Within each of the panels, the biomarkers were furtherly distinguished into certain

biological processes (apoptotic processes, cell adhesion, chemotaxis, inflammatory processes, etc.), disease areas (e.g., cancer, cardiovascular, digestive, etc.), protein classes (membrane proteins, transporter, secretion proteins, etc.) and tissue expression (smooth muscle, lymph node, liver, etc.). Between CVDII, CVDIII and IFL, 10 protein biomarkers were measured in more than one panel. Each panel assessment was run using the internal controls for the PEA, including two incubation controls plus extension and detection controls. An interplate control was utilized for normalization and compensation of interplate variation for sample control in each plate. Further details, including panel performance characteristics like detection limit and distribution of analytical measuring range of every biomarker being detected, alongside additional detailed information on the biomarkers, may be found on OLink's official website (https://www.olink.com/resources-support/document-download-center/).

4.4 Assessment of Gene Expression

4.4.1 RNA Extraction

The procedure of total RNA extraction from fresh frozen tissue samples was performed with the miRNeasy® Mini Kit, Qiagen. The fresh frozen tissue samples of both stable and unstable carotid artery plaques had each been broken up and completely homogenized in 700 µl of QIAzol before they were stored in 1,5 ml Eppendorf tubes at -80 °C until further processing.
In advance of the further process of RNA isolation, the entire laboratory desk, including all necessary instruments and chemicals, was sprayed with RNAse Zap, while the homogenated tissue was incubated at room temperature for 5 min. After adding 140 µl chloroform to the tube and vortexing it for 15 s, the homogenate was incubated at room temperature for an additional 3 min. In the following step, a cooled down to 4 °C centrifuge was run for 15 min at 12.000 G to separate the RNA containing the upper aqueous phase from the lower phase and its containing cell fragments. The upper aqueous phase was then transferred into a new 1,5 ml Eppendorf tube and mixed. At the same time, 100 % EtOH was added in a ratio of 3:2. Of the homogenate, 700 µl were then transferred in the RNeasy MinElute Spin Columns containing a special membrane binding RNA and thus, enabling it to isolate RNA. After another run in the Centrifuge at 8.000 G for 30 s and discarding the flow-through, 700 µl of buffer RWT were added and the tube was once again centrifuged at 8.000 G for 30 s, including the discard of the flow-through afterward. This step was repeated twice with 500 µl of the RPE Buffer before the isolation process was completed with 1 min in the centrifuge at full speed to dry the membrane in the column. The isolated RNA was then eluted with 30 µl of RNAse-free water into a new 1,5 ml Eppendorf tube, incubated at room temperature for 1 min and then centrifuged for 1 min at 8.000G before being put directly on ice. RNA concentrations [ng/µl] were measured via spectrophotometer (Nanodrop) and the isolated RNA samples were then stored at -80 °C until further processing.

4.4.2 cDNA Synthesis

The High-Capacity RNA-to-cDNA Kit by Thermo Fisher was used for the process of cDNA synthesis out of the formerly isolated RNA. The whole experiment was conducted on ice, whereas the entire working space had been sprayed with RNAse Zap beforehand. With the knowledge of the previously measured RNA concentration, the RNA was diluted to 400 ng in 9 µl. Then, 10 µl of the kit's own BufferMix, and 1 µl of reverse transcriptase were added. The homogenate was put in the Thermo Cycler by Thermo Fisher for the 'cDNA program' via the protocol. The workflow of reverse transcription reaction - the 'cDNA program' - is built up by three steps. Step 1 incubates the homogenate for 60 min at 37 °C, followed by 5 min at 95 °C in Step 2. Completing the reaction rhythm comes with Step 3, the preservation, at 4 °C an unlimited time. After adding 60 µl, the cDNA was stored at -20 °C until further processing.

4.4.3 quantitative Real-Time Polymerase Chain Reaction

The assessment of the gene expression of AIFM1 was proceeded further with RT-qPCR in which the Taq Man Gene expression Master Mix by Thermo Fisher was used.
The Master Mix for each TaqMan™ Assay (=primer) was pipetted in advance, containing 10 µl TaqMan™ Geneexpression Master Mix, 7 µl distilled water and 1 µl primer per one well of a 96-well plate. In combination with 2 µl of the formerly synthesized cDNA, 18 µl of the Master Mix were pipetted in the proper well of the 96-well plate. As for the negative control, distilled water was taken, while the housekeeping genes RPLPO and ACTB were used. The plate was then sealed with an optical adhesive foil and shortly centrifuged, ensuring the homogenization on the bottom of each well before the RT-qPCR was run with the Quant Studio™3 Real-Time PCR Instrument by Thermo Fisher Scientific via the protocol. This protocol was built up by Denaturation-Step (one cycle; 20 sec. at 95 °C), Annealing-Step (1 sec. at 95 °C) and Elongation-Step (max. 40 cycles; 20 sec at 60 °C). Thus, cycle threshold values of the following genes were determined and normalized with the housekeeping genes.

4.5 Histology Stainings

4.5.1 Slides and Samples

The microtome Leica RM2255 by Leica Biosystems was used to obtain 3 µm thin slices from previously processed tissue samples. Slices for Hematoxylin and Eosin staining, as well as slices for Elastica van Gieson staining, were brought up on Superfrost slides by Thermo Fisher. In order to secure adequate adhesion, immunohistology stainings Superfrost PLUS slides by Thermo Fisher had been silanized for 5 min with 10 % Poly-L-Lysin. They dried at 56 °C for 1 h before slices were brought up on them. All slides were then dried at 56 °C overnight.

4.5.2 Hematoxylin Eosin Staining

The slides had been dried and fixated at 56 °C overnight before the staining. Starting off the staining, the slides were dewaxed by following the standardized procedure of 2 x 10 min xylol, 2 x 5 min isopropanol, 2 x 5 min 96% ethanol and 2 x 5 min 70% ethanol. Put in distilled water for 1 min to wash off the alcohol beforehand, the slides were then brought into Hämalaunlösung Sauer nach Mayer for 10 min to color the primarily anionic cell components. Afterward, they were washed for 5 min in a bath at room temperature with gently running tap water generating a clearer development of colour. Following these steps, the staining was then completed with the colourization of the primarily cationic cell components with 1 % eosin and the dehydration via 30 sec 96 % ethanol, 2 x 30-sec isopropanol and 2 x 2 min xylol. The slides were then mounted with Eukitt®.
Due to the electrostatic interactions, primarily anionic cell components such as nuclei and other mainly DNA and RNA-containing structures were then observed to be coloured rather blue by the cationic Hämalaun under the microscope. Primarily cationic cell structures, such as collagen fibers and mainly cytoplasmatic proteins, were seen as rather red, given their electrostatic interaction with Eosin.

4.5.3 Elastica van Gieson Staining

Before the staining, the slides had been dried and fixated at 56 °C overnight. For the beginning of the staining, the slides were dewaxed by following the therefore standardized procedure of 2 x 10 min xylol, 2 x 5 min isopropanol, 2 x 5 min 96% ethanol, and 2 x 5 min 70% ethanol. Put in distilled water von 1 min and thus cleared of excess alcohol, the slides were then taken into Resorcin-Fuchsin nach Weigert for 25 min. Afterward, the slides were then washed twice with 96% ethanol for 90 sec before being brought into HTX-Weigert (Weigert'sche Lsg. I + II; 1 : 1) for 15 min. Followed by 5 min in a water bath with gently running tap water at room temperature the slides were then put in Pikrofuchsinlösug for 2 min. The staining was then finalized with the dehydration and mountaining via protocol seen in 4.5.2.
Under the microscope, elastic fibers' expected special purple-black colouring within the tissue samples was observed. Thus, the inner and outer elastic membrane could be detected and in addition to the HE staining the classification of the atherosclerotic lesions was therefore simplified.

4.5.4 Immunohistochemistry

All immunostainings were performed with the Dako REAL™ Detection System Kit by Dako Denmark A/S and Avidin/Biotin Blocking Kit by Vector Laboratories Inc., both stored at 4 °C.

Establishing sufficient Blocking

The complete pureness of the negative control was ensured prior to establishing the primary antibody concentration in the immunostaining described later on in 4.5.4. As FFPE human heart muscle tissue was predetermined by the AIFM1 antibody supplier for the positive control, the same tissue was used, instituting sufficient blocking. Blocking with 10 % serum matching the species of the secondary plus 1 % BSA for 2 h at room temperature was recommended by the antibody supplier. As a standardized procedure for blocking FFPE atherosclerotic lesions of the carotid arteries, the combination of 15 min peroxidase blocking with 30 min of 5 % milk powder blocking had been formerly instituted within the AG Maegdefessel. Both variants were run solo and combined in test trials and also altered at the time of blocking. In Addition, blocking with avidin (15 min) and biotin (15 min) was also run in combination with those blocking trials. For sufficient blocking, the combination of 15 min peroxidase blocking with 1 h each of 5 % milk powder and 10 % goat serum plus 1 % BSA followed by avidin (15 min) and biotin (15 min) was established.

Immunostaining

Before the staining was begun, the slides had been put in the oven at 56 °C overnight. In the first steps, the slides were dewaxed via a bath in 2 x 10 min xylol, 2 x 5 min isopropanol, 1 x 5 min 96% ethanol, 1 x 5 min 70% ethanol ending in distilled water. This was followed by 7 min in a pressure cooker with the preheated citrate buffer described in 3.7. The slides were washed off for 2 min with the TRIS buffer (3.7) before being blocked with 1,5 ml 30 % hydrogen peroxide diluted in 50 ml of distilled water for 15 min. After 3 x 2min being washed with TRIS, the blocking was then continued with 100 µl of 5 % milk powder diluted in DAKO antibody diluent per slide for 1 h and after a quick wash off in TRIS with another hour of 100 µl of 10 % goat serum plus 1 % BSA (diluted in distilled water) per slide. Blocking was then completed by adding avidin and biotin with 100 µl per slide for 15 min each and a quick wash-off with TRIS in between. Following the blocking, the slides were then incubated with 100 µl each of the primary antibodies (diluted in DAKO antibody diluent) for 1 h and washed 3 x 2 min with TRIS. The staining was then continued via the incubation with 2-3 drops of the secondary biotinylated antibody (Dako Kit A) for 25 min and after another 3 x 2 min wash with TRIS with 2-3 drops of streptavidin peroxidase (Dako Kit B). The slides were then washed 3 x 2 min with TRIS once more. Out of the Dako REAL™ Detection Kit, the DAB and HRP buffers (Dako Kit C & D) were then pipetted (100 µl in ratio 1 : 50) on each slide. In between 1-5 min, the brown colour of the successful immunostaining was observed. After the last 3 x 2 min wash-off, the nuclei were then coloured briefly with 20-sec incubation in Hämalaun. Ensuring a purer development of colour, a gentle rinse-off with warm tap water was then performed. The staining was completed with the dehydration of the slides via 3 min ethanol 70 %, 3 min ethanol 96 %, 3 min isopropanol and 3 min xylol before they were mounted with Eukitt®.

Immunostainings with the following primary antibodies in the enlisted concentrations were performed:

Primary Antibody	Dilution
AIFM1	1 : 100
CD3	1 : 500
CD45	1 : 500
CD68	1 : 1000
Galectin 3	1 : 100
SMMHC I & II	1 . 4000
SMA	1 : 200

Table 4.1: **Primrary Antibody Dilutions**

The semiquantitative analysis was then performed with the Zeiss Primo Vert microscope by Carl Zeiss Microscopy GmbH.

4.6 Cellular Experiments

4.6.1 Isolating Monocytes from Human Blood Samples

The process of PBMC isolation from human blood samples was conducted with a solution of high molecular weighed succhrose and epichlorohydrin interacting with the sedimented blood compounds. The Solution was brought to room temperature prior to the experiment. Performing monocyte isolation, the Pan Monocyte Isolation Kit (Human) by Miltenyi Biotec was used. For isolating the monocytes, solutions were pre-cooled and kept at 2-8 °C.
Initially, 3 x 7,5 ml heparinized blood samples were drawn from healthy donors. The blood (15 ml) was diluted 1 : 1 with 15 ml PBS plus 1 mM EDTA. Of the reagent, 25-30 ml were then very carefully layered on top of 15 ml of Ficoll-Plaque™ PLUS in a 50 ml tube. The tube was centrifuged with 400 G at room temperature for 30 min without braking. The upper plasma phase was removed as much as possible without disturbing the intermediate leukocytic phase containing the PBMC. The leukocytic layer was then transferred into another 50 ml tube and filled to 50 ml with PBS-EDTA (1mM). The reagent was centrifuged at 400 G at room temperature for 10 min plus using the brake at the end. The supernatant was aspirated and discharged and the bottom layered pellet was resuspended with PBS-EDTA (1mM) up to 50 ml before being centrifuged for 300 G at room temperature for 10 min using the brake. This step was performed once more with only 200 G for 15 min using the braking function. The pellet was then resuspended in Miltenyi Monocyte Buffer and the cells were counted.
The counted cells were kept at about 2-8 °C the entire time, performing the isolation of monocytes. Resuspended in 40 µl per 10^7 cells, 10 µl per 10^7 cells of FcR Blocking Reagent and Biotin-Antibody Cocktail were each added to the previously isolated PBMC.

The reagent was then incubated at 2-8 °C for 5 min. Afterward, 30 µl of buffer and 20 µl of Anti-Biotin MicroBeads were added per 10^7 counted cells before the reagent was once more incubated at 2-8 °C for 10 min. For the subsequent manual cell separation, a MACS™ LS Column was placed in the Quadro MACS™ by Miletnyi Biotech. In the following steps, it was always waited until the column reservoir was entirely run through before continuing with the next step. The column was first washed with 3 ml of the kit's buffer. Secondly, the cell suspension was given into the column, and the flow through with the isolated monocytes was collected. In the last step, the column was rinsed with 3 x 3 ml of buffer and the flow-throughs were always added to the initial one. The monocytes were then counted, 10 µl RNA were extracted as described in 4.4.1 and brought into culture with RPMI 1640 (with glutamine), 10 % FBS, 1% PEST and 1 % HEPES incubating at 37 °C.

4.6.2 Developing Monocytes into Macrophages

Starting the process of developing isolated monocytes into macrophages, the cells were counted, and RNA was extracted from cell portions (via protocol found in 4.4.1). Following, cells were seeded in different wells and plates in densities of 100.000 cells/cm^2 (= 1 Mio. cells per well in a six-well plate plus 2 ml of medium) and 50 ng/ml M-CSF was added. After three days, the medium was changed, and the cells were kept in culture for three more days before being harvested. The procedure was then completed by extracting RNA once again, and the gene expression of AIFM1 was assessed, as well as markers showing the different stages in the development of the immune cells (IC), shown in the following:

IC Development Stage	**Genetic Markers**
Classical Monocytes (90-95%)	CD14, CD64
Intermediate Monocytes	CD14, CD16
Non-classical Monocytes	CD16
„M1“ Macrophages	CD68, TNF
„M2“ Macrophages	CD163, IL10

Table 4.2: **Immune Cell Biomarkers**

4.7 Software

To visualize RT-qPCR data Prism 9.0 by GraphPad Software, San Diego, USA, was used.

5 Results

5.1 Patient Collective

For this study, the human tissue samples were taken from patients treated for stable (n = 6) and unstable (n = 8) atherosclerotic lesions in the carotid artery. Both groups were built up in equal parts from female and male subjects. Further information and characteristics, as shown in table 5.1, were taken from the Munich Vascular Biobank. Clinical events, such as neurological symptoms caused by the stenosis, were not recorded for any patients before the surgery. As seen in the table, all subjects dealt with at least one of the various risk factors causing atherosclerosis. On top of that, every single patient was also being treated with either anticoagulants, statin therapy, or both medical treatments. Other atherosclerosis-related diseases, e.g., peripheral artery disease or coronary heart disease, were not confirmed for any of the patients.

	Stable (n = 6)	**Unstable** (n = 8)
Medium Age in years [range]	68,67 [59;83]	64,25 [55;77]
Medium Level of Stenosis in % [range]	88 [80;90]	85 [70;90]
Diabetes	2	4
Nicotin Abuse	0	5
Hypertonus	6	7
Hyperlipedemia	3	4
Antikoagulant Therapy	5	8
Statine Therapy	4	7

Table 5.1: **Subject Characteristics**

The whole blood samples for the experiments previously described in 4.6 were withdrawn from female (n = 2) and male (n = 2) donors with neither active vascular diseases nor cardiovascular risk factors.

5.2 Carotid Artery Plaque Classification

With the morphology observed under the microscope in the Hematoxylin Eosin and Elastica van Gieson staining, the segments of the FFPE specimens were allocated to their level of atherosclerosis by two independent observers experienced in vascular pathology. Based on the updated American Heart Association (AHA) classification of coronary atherosclerotic lesions the progress of atherosclerotic lesions was distinguished into eight different

levels.[Stary, 2000, Stary et al., 1995, Stary et al., 1994, Stary et al., 1992] All of the FFPE specimens included in this study showed characteristics such as prominent collagenous fibrotic tissue, allocating them in the group of the advanced atherosclerotic lesions (type V-VIII according to figure 4.2).

Four of the stable (n = 3) and unstable (n = 1) examined carotid artery plaque showing reparative collagenous tissue manteling one or more extracellular lipid cores in the intima layer of the artery were labeled as *fibroatheroma* (V). In four different plaques (n = 1 stable; n = 3 unstable), additional obstructions of the lumen caused by surfacing thrombi, tears in the fibrotic cap, or intralesional hematoma were observed (VI). With the calcification being regarded as the predominant component, a different group of four plaques (n = 1 stable; n = 3 unstable) was labeled *calcific lesion* (VII) independently of the existence of lipid cores. As for atherosclerotic plaque lesions containing not only hemorrhagic infiltration disrupting lesions architecture but also calcified areas, an intermediate *complex type* (VI/VII) was established. Two of the unstable and one stable plaques showed such characteristics. In accordance with *The Oxford Plaque Study*, atherosclerotic lesion stability was assessed. Thus, with their minimum fibrous cap thickness <200 µm or their respective fibrous cap thickness <500 µm, eight plaques were found to be unstable [Redgrave et al., 2008], whereas six further plaques proved stable. None of the carotid artery plaques examined in this study showed continuous communication between the necrotic core and overlying thrombus through a disrupted fibrous cap. Thus, in agreement with [Virmani et al., 2000a], no plaque was labeled to be ruptured.

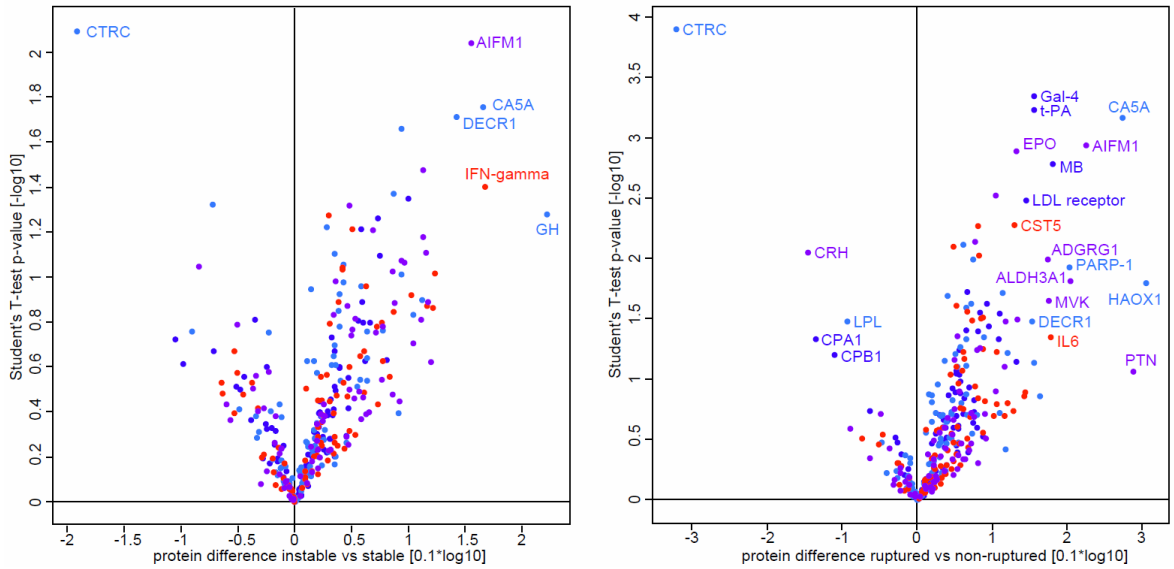

Figure 5.1: **Blood Serum Proteomics determined by Wierer/Mann, Max Planck Institut of Biochemistry, Munich:** OLink Pannels: Cardiovascular II (light blue); Cardiovascular III (blue); Inflammation (red); Organ Damage (purple); n = 106.

5.3 Proteomic Blood Serum Analysis

With the help of the Max Plank Institute of Biochemistry, proteomic analysis of blood serum from 106 patients treated for carotid artery disease was performed. The biomarkers being detected with the panels described in 4.3.2 by OLink, Sweden, were brought into perspective with the 53 stable carotid segments compared against the 53 unstable carotid plaque segments (Figure 5.1). There, statistically significant, chymotrypsin C (CTRC) showed the greatest downregulation in serum levels from patients with unstable versus patients with stable carotid plaque lesions. Looking at upregulated biomarkers in the serum of patients with unstable lesions, the mitochondria-associated apoptosis-inducing factor AIFM1 (p=0.0091) was significantly determined as such alongside mitochondrial Carbonic Anhydrase 5A (CA5A), 2,4-Dienoyl-CoA Reductase 1 (DECR1) and Interferon γ (IFN-gamma). The protein difference between blood sera from patients with ruptured versus non-ruptured plaques in perspective AIFM1, CA5A and Hydrocxyacid Oxidase 1 (HAOX1) levels showed statistically significant serum level upregulation. In this comparison, CTRC was significantly downregulated in serum levels from ruptured plaques.

5.4 AIFM1 and its Expression in Human Carotid Artery Plaques

The mitochondria-associated apoptosis-inducing factor (AIFM1) is a flavoprotein located in the inner membrane of the mitochondria in a healthy cellular environment. With its FAD-dependent NADH oxidoreductase activity, AIFM1 contributes to the functionality of the respiratory chain within the mitochondria under stable cellular circumstances [Hermann and Riemer, 2021, Miramar et al., 2001]. Located on the X chromosome at Xq26.1, in its mutated variations, AIFM1 is associated with gonosomal recessive sensorimotor neuropathy, deafness and cognitive impairment, altogether known as the Charcot-Marie-Tooth-4 disease Cowchock syndrome [Cowchock et al., 1985, Rinaldi et al., 2012]. Additionally, prior studies have linked AIFM1 mutations with further phenotypes, i.e. severe infantile motor neuron disease and fatal encephalomyopathy [Diodato et al., 2016, Morton et al., 2017]. Named after its initial identification as an apoptosis-inducing protein, AIFM1 takes part in the process of programmed cell death, inevitably designating it as potential tissue homeostasis and morphology reconstruction marker. In response to cell death stimuli, AIFM1 is released into the cytoplasm and translocates into the nucleus alongside other apoptosis regulating factors, such as cytochrome C (CYCS) and caspase-9 (CASP9), whose mitochondrial release it additionally promotes [Susin et al., 1999]. However, evidence for apoptotic pathways completely caspase-independent of AIFM1 and its requirement for rather specific cell death pathways was provided according to preceding studies [Joza et al., 2009, Joza et al., 2001]. Translocated in the nucleus, AIFM1 is partly responsible for DNA fragmentation regulation, subsequently enabling chromatin condensation and consequently proper cell disassembly. The cellular location of cytoplasmatic AIFM1 can be regulated by heat shock 70-kDa protein (HSP70) and cyclophilin A (CypA)

via an antagonistic and redox-controlled manner as prior research has confirmed [Bano and Prehn, 2018]. Respectively, HSP70 benefits cytoplasmatic retention of AIFM1, consequently preventing apoptotic formations containing AIFM1 in the nucleus [Ravagnan et al., 2001]. At the same time, CypA furthers DNA degradation by forming a trimolecular complex with AIFM1 and the degradation substrate, thus, hindering AIFM1s release from the nucleus [Candé et al., 2004]. Taking this into account, possible gene expression patterns in human carotid artery plaques were examined.
Gene expression of AIFM1, CASP9 and CYCS was assessed and compared between unstable (n = 3) and stable (n = 5) atherosclerotic CAP lesions from the study cohort via RT-qPCR as described in 4.4.1 and following. Cycle treshold (Ct) values were put in perspektive with the mean Ct-value from RPLPO and ACTB - acting as housekeeping

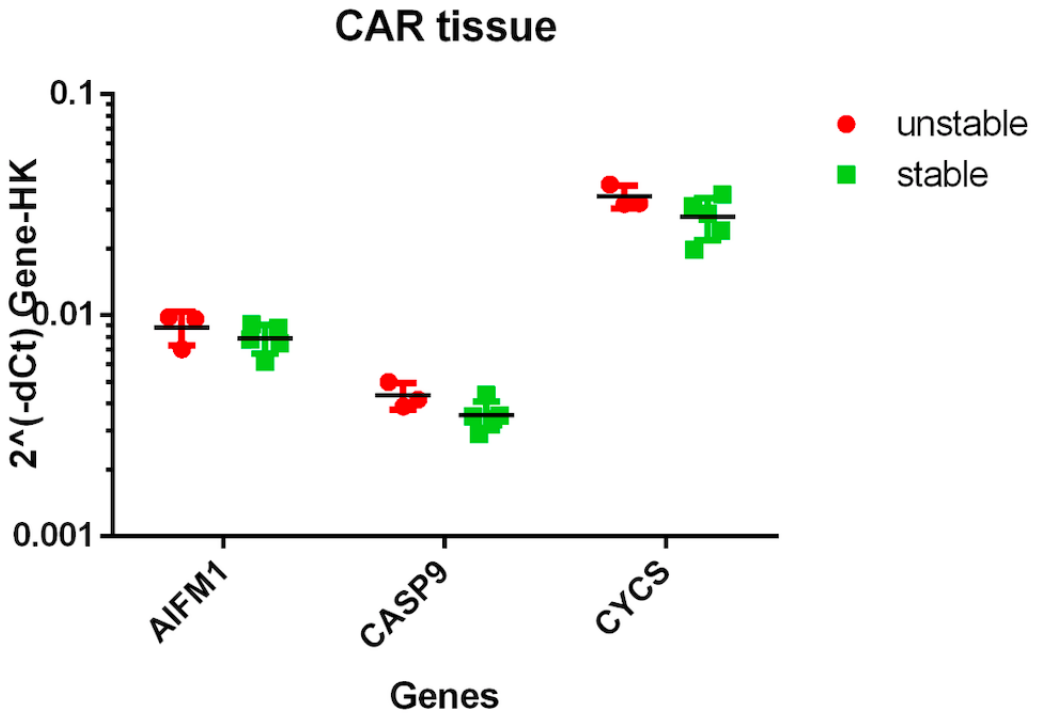

Figure 5.2: **Comparison of AIFM1 (p=0,34) expression in unstable (n=3) versus stable (n=5) atherosclerotic lesions from human carotid artery tissue** alongside CASP9 (p=0,09) and CYCS (p=0,15)

genes - being subtracted from them (delta Ct, dCt). For AIFM1, the median dCt-value in unstable atherosclerotic lesions was 6.70, with an interquartile range from 6.68 to 6.92. With the median dCt-value in stable atherosclerotic lesions being 7.01, including the interquartile range from 6.83 to 7.06, no significant result was determined. The same outcome applied to assessing gene expression of CASP9 and CYCS. However, an assumption of higher expression in unstable atherosclerotic lesions within all three biomarkers was made (Figure 5.2). No caspase-independency within AIFM1 expression was seen.

5.5 Immunohistological Examination of AIFM1 Expression in Human Carotid Artery Plaques

Observing the expression of AIFM1 in human carotid artery plaques on a histological level, the FFPE specimens were processed accordingly (Section 4.5.4). Histological expression of AIFM1 was assessed semiquantitatively in unstable and stable human CAPs, putting it in perspective with human heart muscle tissue as the positive control. Furthermore, unstable (n = 2) and stable (n = 2) specimens were cut consecutively, stained with additional antibodies (Table 4.1) and analyzed semiquantitatively. Thus, locations within the atherosclerotic lesions predominantly expressing AIFM1 could be linked to more specific cell types. The antibody binding was successfully tested with human heart muscle tissue. Amongst the tried-out primary antibody dilutions, a concentration of 1 : 100 resulted in the semiquantitatively measured most detailed stainings (Figure 5.3).

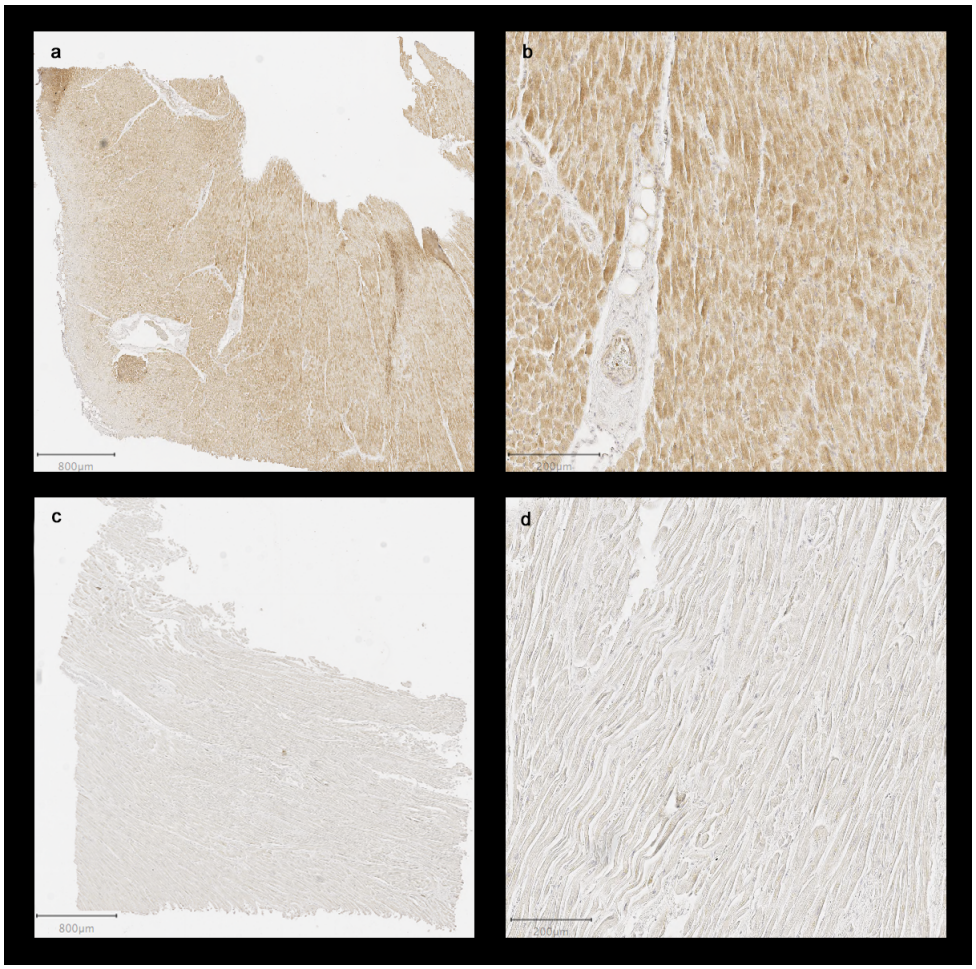

Figure 5.3: **Anti-AIFM1 establishment in human heart muscle tissue** (a+b: Anti-AIFM1 1:100; c+d: negative control; scale bar (sb): left column = 800 µm; right column = 200 µm)

With the semiquantitative analysis, no statistically significant differences in AIFM1 expression between unstable and stable human CAP were examined. Nevertheless, a clear trend of stronger AIFM1 expression in unstable human CAP was observed (Figure 5.4). As part of the mitochondrial chain and a frequent participant in the process of apoptosis, AIFM1 expression was seen slightly scattered in the necrotic core within stable human CAP. In unstable human CAP, however, AIFM1 was detected to be stronger expressed in and around the necrotic core of the atherosclerotic lesion, not only as unspecific background but also in supposedly immune cell patterns (Figure 5.4 and 5.5a). Within the inner plaque shoulder region, AIFM1 staining was also observed in cells hinting to be of the immune cell type (Figure 5.5b). As seen in figure 5.5c, no AIFM1 stained cells were detected within the media layer of the CAP, independent from its stability. The fibrous cap displayed slightly scattered AIFM1 staining (Figure 5.5d).

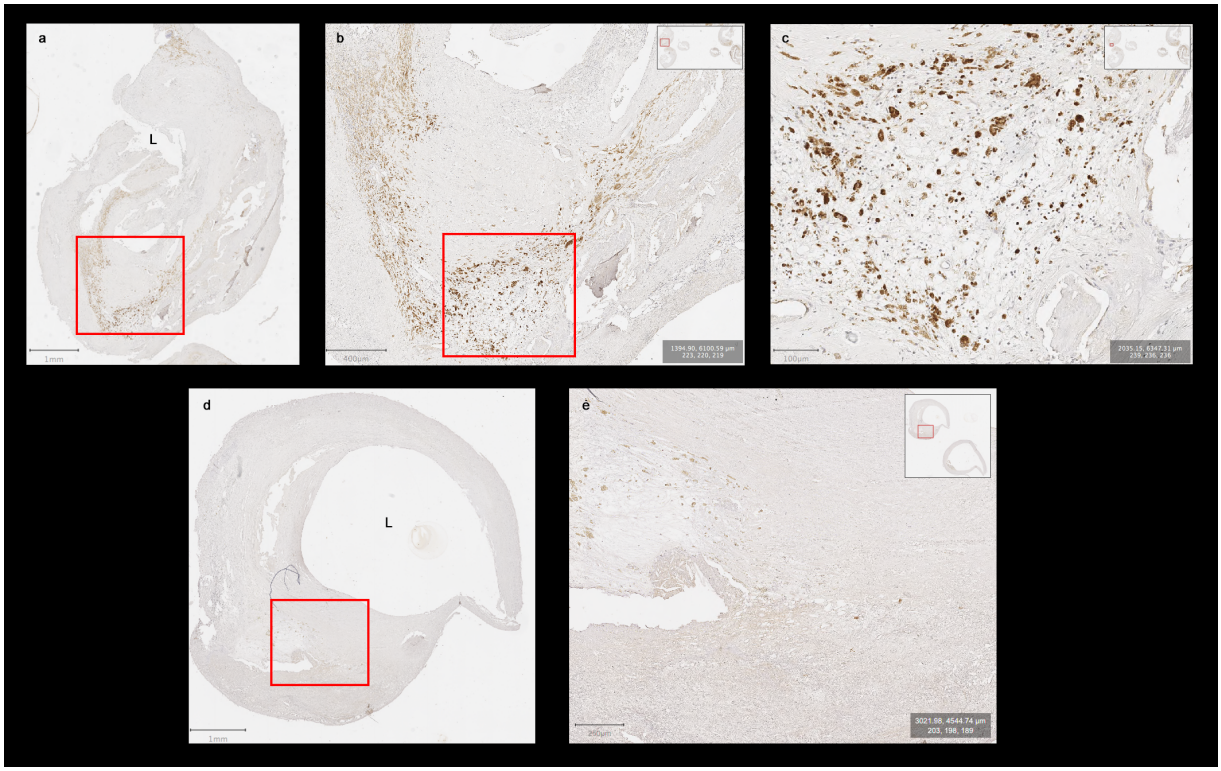

Figure 5.4: **Immunohistochemistry comparison of AIFM1 expression in unstable (a-c) vs. stable (d, e) CAP** (Anti-AIFM1 staining 1:100; sb: a+d = 1 mm, b = 400 µm, c = 100 µm, e = 250 µm)

Further, immunohistochemistry stainings highlighting the expression of CD68, CD45, CD3, LGALS3, SMA and SMMHC I & II were performed and portrayed in figure 5.6 as described in the following. The transmembranous glycoprotein CD68 is predominantly expressed in mononuclear phagocytes, especially M1 macrophages. In addition, vascular smooth muscle cells (VSMC) undergoing transdifferentiation in the process of atherogen-

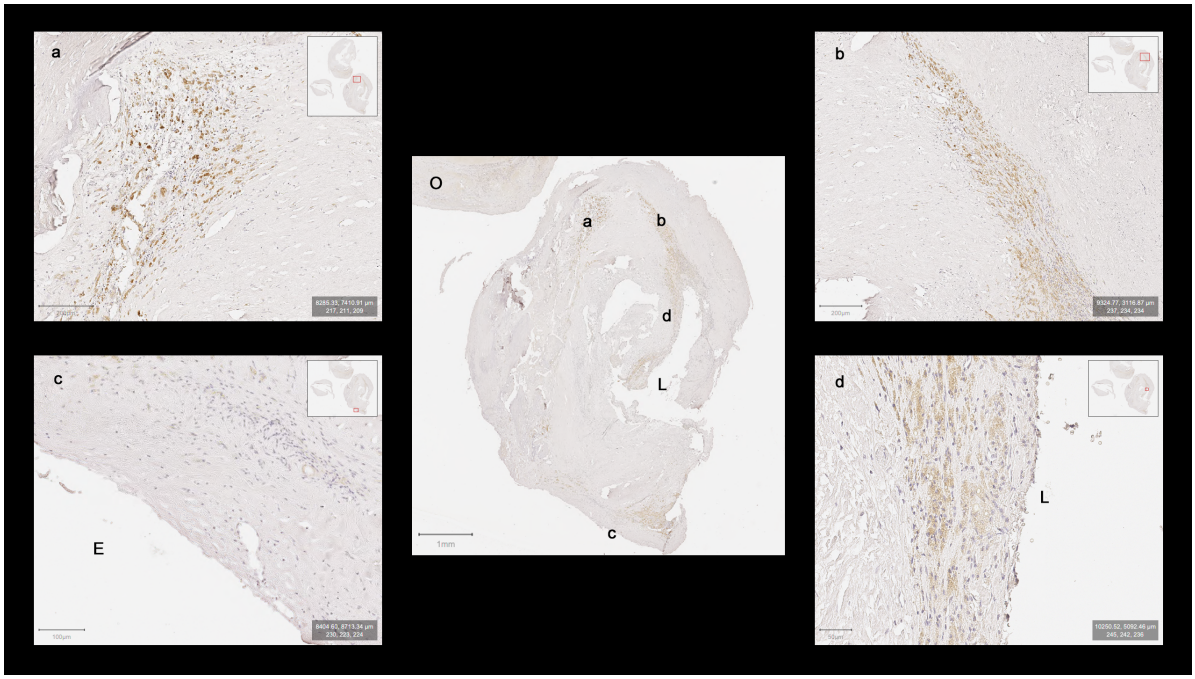

Figure 5.5: **Regional comparison of AIFM1 expression within unstable CAP** (Anti-AIFM1 staining 1:100; O = unstable CAP, a = necrotic core, b = shoulder region, c = media, d = fibrous cap; sb: a-e = 100 µm, O = 1 mm)

esis may also express CD68 due to their acquired macrophage-like phenotype [Albarrán-Juárez et al., 2016, Feil et al., 2014]. Throughout the immunohistochemistry staining, CD68+ cells were identified in the necrotic core, the fibrous cap and the shoulder region of the plaque (Figure 5.6d). With the beta-galactoside-binding protein Galectin-3 (LGALS3) regulating responses in inflammatory processes, prior studies have confirmed its expression in immune cells, mainly macrophages [Paclik et al., 2011, Hara et al., 2020]. Further, studies have identified Galectin-3 as a biomarker within atherosclerotic lesions correlating with lesion stability [Blanda et al., 2020]. The performed immunohistochemistry highlighted LGALS3+ cells in inflammatory active regions of the plaque, mainly the necrotic core with its bordering shoulder region (Figure 5.6f). Staining additional inflammatory markers, such as CD45 highlighting leucocytes and CD3 predominantly marking T-lymphocytes, did not display a significant abundance throughout the human CAP. However, singular CD45+ and CD3+ cells were accentuated in the neointima and necrotic core of the plaque (Figure 5.6b and c). None of the inflammatory markers mentioned above highlighted positively stained cells in the media vascular layer. Anti-alphaSMA (smooth muscle actin) and anti-SMMHC I & II (smooth muscle myosin heavy chain I & II) stainings were performed in order to distinguish mesodermal originated cells. AlphaSMA is expressed by myofibroblasts and VSMC, whereas SMMHC I & II stains in contractile VSMC. Since the transdifferentiation of monocyte-derived macrophages into myofibroblasts in vascular stenotic processes is known from prior studies [Haider et al., 2019, Stewart et al., 2009] alpha-SMA stained cells were also considered to eventually

be of immune cell origin. AlphaSMA expression was mainly detected in the media and muscular layer within the intima of the human CAP. However, cells were also stained with alpha-SMA around the shoulder region and fibrous cap of the plaque (Figure 5.6h). Staining with SMMHC I & II highlighted a slight abundance of cells within the media and fibrous cap of the plaque (Figure 5.6i). By putting areas with predominantly AIFM1

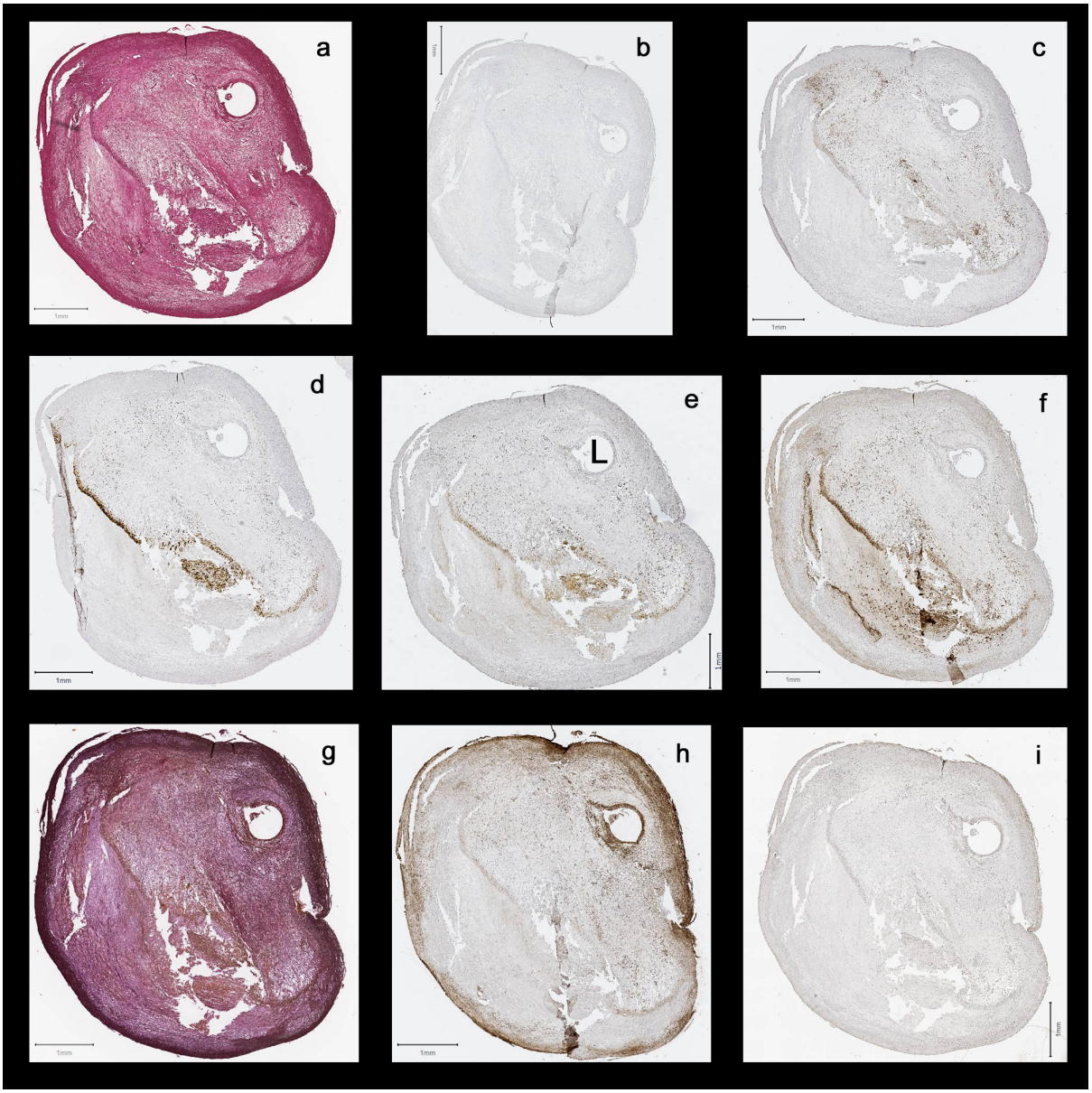

Figure 5.6: **Immunohistochemistry comparison between AIFM1 and various atherosclerotic cell markers** (One of four CAP stained with a: He, b: Anti-CD3, c: Anti-CD45, d: Anti-CD68, e: Anti-AIFM1, f: Anti-LGALS3, g: EvG, h: Anti-SMA, i: Anti-SMMHC I & II; L = Lumen, sb = 1 mm)

stained cells in perspective with the immune cell type markers, great unity with the inflammatory marker stained areas was observed semiquantitatively. Within those markers, CD68-stained cells were identified, showing the highest levels of AIFM1 expression. Analyzing areas with myofibroblastic markers, like Anti-SMA and Anti-SMMHC I & II stained cells, no congruence with AIFM1 stained cell areas was observed.

5.6 Assessing AIFM1 Expression on Cellular Level

With the semiquantitative analysis of the immunohistological examination of AIFM1 expression in human carotid artery plaques hinting a plausible strong expression in inflammatory cells, especially CD68 stained macrophages, further confirmation on the cellular level was assessed. To do so, monocytes were isolated from human whole blood samples (Section 4.6.1) and developed into macrophages using M-CSF (Section 4.6.2). Whole blood samples from a total of four healthy donors with neither active vascular diseases nor cardiovascular risk factors were withdrawn. One sample was run as pilot project (donor 1.1), followed by three further subjects (donor 1.2; 2; 3).
On the one hand, by determining genetic expression for CD14 classical monocytes may be identified semiquantitatively amongst the variously developed monocytes within the samples. These more frequent types of monocytes are mainly responsible for the phagocytosis and production of cytokines within the atherosclerotic plaque. Due to its role as an activation and differentiation marker for mononuclear cells [Naranjo-Gómez et al., 2019], CD64 expression was additionally evaluated. On the other hand, less frequent non-classical monocytes may be distinguished as expressing CD16 upholding the primary function of promoting anti-inflammatory effects and collagen deposition. [Jaipersad et al., 2014] Intermediate monocytes may be classified expressing CD14 and CD16. Investigated in earlier publications, this third subset of monocytes has shown to be involved in angiogenesis [SHANTSILA et al., 2011]. As previously stated, CD68 was used to identify M1 macrophages alongside tumor necrosis factor (TNF). Distinguishing M2 macrophages, CD163 was determined in combination with interleukin 10 (IL10), ensuring the classifications' reliability [Barros et al., 2013]. By looking at the differences within the expressed markers development of the immune cells was followed throughout the M-CSF stimulation.
Three cell cultures of macrophages were nourished from every donor-isolated monocyte sample. From the four biological monocyte sample a total amount of twelve technical monocyte specimen where hereby derived. For Comparison between macrophage and monocyte cultures the mean values from the three technical replicates per biological specimen in gene expression were determined. After the completed M-CSF stimulation, the resulting gene expression profile (Figure 5.7) did not illustrate any significant patterns between the described immune cell markers and AIFM1, CASP9, and CYCS. Following this observation, no specific immune cell type could be assigned with significantly higher AIFM1 expression. Observing the monocyte markers with the highest gene expression only led to the assumption that the macrophages initially being derived from classical monocytes. However, the markers displayed gene expression evened out and

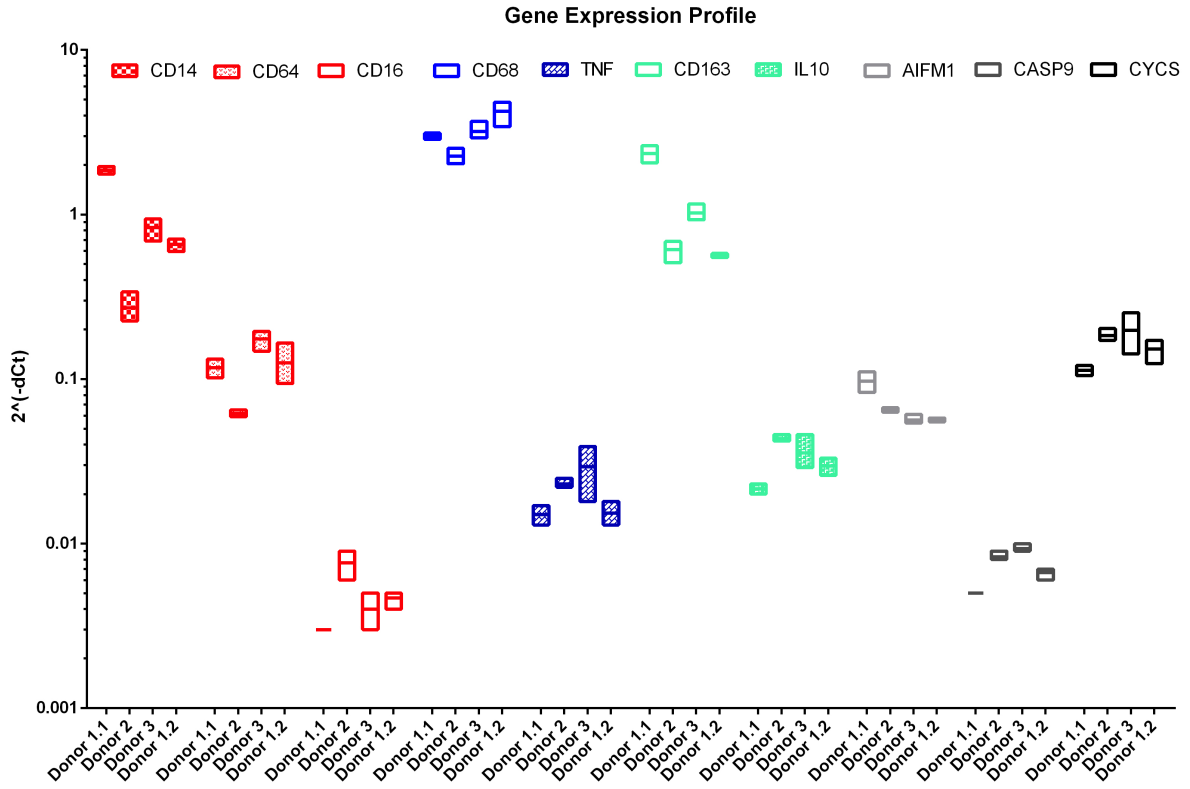

Figure 5.7: **Immune Cell Gene Expression Profile in Macrophages** (Three macrophage cultures per healthy donor isolated monocyte culture, stimulated with M-CSF for 3 days; gene expression assessed via RT-qPCR; red = monocyte-like marker, blue = M1-like markers, green = M2-like markers, uncolorful = comparing markers)

without any statistical outline within all four subjects. Subsequently, the monocyte and macrophage distribution alongside the donours was determined to be balanced enough to compare the monocyte-derived macrophages' AIFM1 expression levels to those of the initial monocytes. Hence their gene expression and origin the macrophages were rather considered to be categorized as undifferentiated M0, than M1 and M2. With the comparison between the calculated mean values of the macrophage replicates versus the four monocyte values, normalized deltaCt-values from both cell groups were evaluated to not be of normal distribution. Folowing, the monocyte-derived macrophages were therefore brought in comparison with their initial preliminary stage in a four versus four relation of sample values. The development of AIFM1 expression within one group was to be put in perspective with a nonparametric test to determine statistical significance. The performed Mann-Whitney-U test displayed a statistically significant ($p = 0{,}0286$) higher AIFM1 expression in the monocyte-derived macrophages ($n = 4$ biological replicates x 3 technical replicates) compared to the initial monocytes ($n = 4$) (Figure 5.8). These results substantiated the hypothesis of AIFM1 upregulation during monocyte differentiation

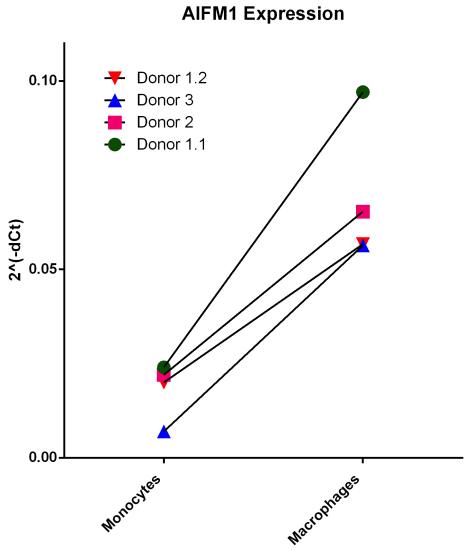

Figure 5.8: Comparison of AIFM1 expression in monocytes vs. macrophages

on a cellular level, as well as they were backing the previously corroborated hypothesis of AIFM1 being highly expressed in macrophage-dominated areas by the displayed immunohistochemistry results. Having the change of AIFM1 expression in monocyte to monocyte-derived macrophages process illustrated, AIFM1 levels from further cell types involved in the process of atherogenesis were to be examined. Therefore, endothelial cells and VSMC of different origins were withdrawn from the Munich Vascular Biobank (n = 1 per cell type) and also tested regarding their AIFM1 gene expression. Cells taken from healthy abdominal aortic and healthy carotid tissue were used as control groups. From stable and unstable carotid artery plaques VSMC samples were examined. No statistical testing was performed with case numbers of one per cell group. Hence, no statistically significant differences regarding AIFM1 expression between those cell groups were observed. Semiquantitatively examined, endothelial cells showed the lowest expression in AIFM1 expression, VSMC and monocytes displayed a rather similar level of gene expression. Macrophages in perspective showed higher levels of AIFM1 expression than all described cell types. (Figure 5.9)

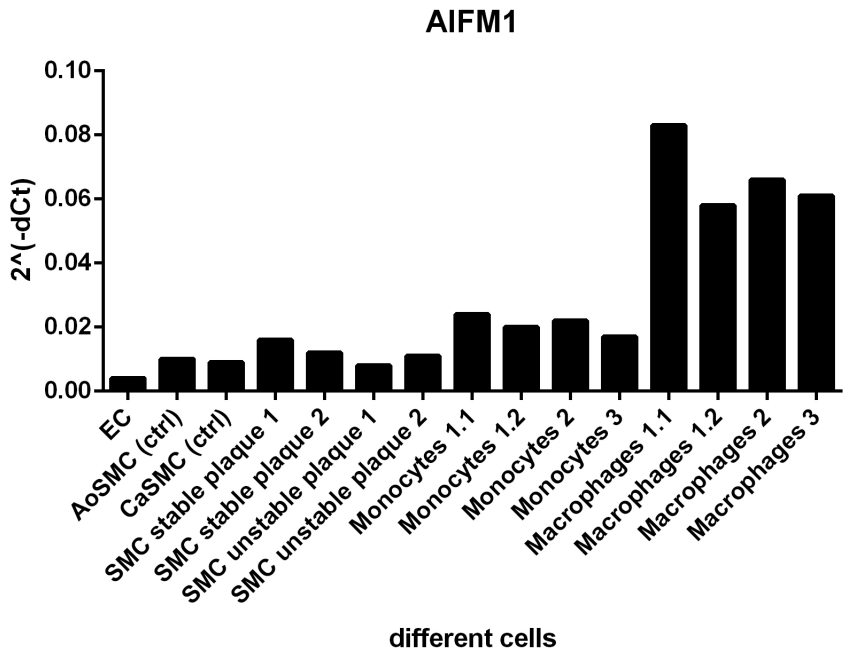

Figure 5.9: **Comparison of AIFM1 expression in human vascular tissue cells** (EC = endothelial cells, SMC = smooth muscle cells, AoSMC = Aortic smooth muscle cells, CaSMC = carotid smooth muscle cells; n = 1 per cell type)

6 Discussion

6.1 Clinical Relevance

The disastrous burden for worldwide health systems and governmental households created by ischemic stroke is beyond doubt. Carotid artery diseases' major contribution to this is also indisputable, as it accounts for up to every fifth ischemic stroke. Contemporary atherosclerosis investigations are being widely promoted to further decipher a major cause of CaAD and ischemic stroke, respectively. Atherosclerotic lesion stability has been significantly linked to fibrous cap thickness and necrotic core expansion and hence, the process of fibrous cap thinning and necrotic core development is of great present interest [Libby et al., 2019].

6.2 Methods and Limitations

Methods utilized in this study were critically examined regarding their efficiency and established by multiple test runs before performing the result-delivering trials. Single methods do have distinct limitations to be noted in the following. However, the way the study was designed and the utilized methods were tried to complement each other in their strength and weaknesses.

6.2.1 Advantages and Disadvantages of Human Biobanking

Human Biobanking as an advanced library for biological material may be seen as a critical tool for analyzing the combination between experimental research in human tissue and clinically obtained information. In accordance with experienced biobank constructors, the principal challenge for human biobanking is a low variability between different biobanks and subsequently, the ability to compare studies by them on a global level [Pelisek et al., 2019]. The analysis must result from constant and reproducible processes to enable comparison between research projects performed with resources from various biobanks. As the prominent foundation of this is the quality and integrity of the biospecimens, their proper collection, processing and storage strongly influence the research results and thus are of great interest [Malm et al., 2013, Moore et al., 2011]. A complex interplay between surgeons, clinical coordinators, technical assistants and natural scientists is required to keep the possible molecular alternation of the biospecimens to a minimum. Additionally, several quality control checkpoints have proven to be of essential value, especially in the vital period between surgical acquisition and conservation [Pelisek et al., 2019].

The Munich Vascular Biobank, consisting of FFPE, fresh frozen and serum plus isolated cells from vascular human specimen, was of great importance for this study. A standardized pathway for processing vascular tissue after surgical excision (Figure 4.1) had previously been established and was followed through in this study whenever additional biospecimens were obtained. However, the prevention of every possible error can not entirely be guaranteed. Anatomical individualities and the resulting variability of surgical preparation necessary for the prioritized successful therapeutical outcome may have led to structural alternations within the CAP specimen. This may inevitably impact the characterization on a micrological level. No significant decline of RNA integrity after surgery and in excised tissue for a considerable amount of time is to be expected in accordance with prior studies [Micke et al., 2006, Ohashi et al., 2004, Almeida et al., 2004]. On a molecular level, the integrity of the normally fragile structured RNA has been therefore assumed to be the suitable objective for biospecimen quality. In addition to advising RNA integrity evaluation before biospecimen storage, experienced biobank scientists see no disadvantage in using FFPE biospecimen as a source for RNA expression analysis [Pelisek et al., 2019].
The characterization of the differently advanced FFPE biospecimen into eight groups was standardized in accordance with the updated AHA classification (Figure 4.2) [Stary, 2000, Stary et al., 1995, Stary et al., 1994, Stary et al., 1992]. Identification of atherosclerotic lesion stability was instituted after the *Oxford Plaque Study* and rupture state after Vrimani et al. [Redgrave et al., 2008, Virmani et al., 2000a]. Lesion classification was performed by two experienced laboratory workers blinded to the clinical data and supervised by a clinical senior pathologist for each lesion. Nevertheless, minor differences in classification may have occurred. Additionally, the structure and stability within different cut sections of one FFPE specimen may vary or even portray contrasting characteristics within one another. Every specimen was overall classified once before eventual storage or further processing, but there was no standardized protocol for further subclassification. Consequently, the possibility of cells being isolated and derived from specimen parts not fulfilling the overall lesion classification characteristics must be considered. Respectively, possible histological cuts from the same FFPE specimen may portray different statuses of lesion development, stability, rupture state, or combinations of all three. To provide such possible error, a further subclassification or re-evaluation at a later point of time for the biospecimen can be seen as a potential solution but must be put in relation to the resulting additional effort.
With the utilization of patient-derived cell cultures instead of purchasable cell lines, the assessed biomarker expressions may result in greater individual meaning. Clinical patient data collected, in addition to the retrieved biospecimen, plays a key role in linking pathological experimental outcomes to actual clinical symptoms. With the detailed clinical information contained in the Munich Vascular Biobank specimen from symptomatic and asymptomatic are distinguishable and further, potential risk factors, (age, gender, etc.) may be assessed [Wendorff et al., 2016, Wendorff et al., 2015]. Even though human biobanking cannot be implied as entirely free from potential variabilities influencing experimental outcomes, the advantages of it may eventually lead to a promising foundation

for personalized medical treatment.

6.2.2 Advantages and Disadvantages of Proteome Analysis

Rapidly advancing development of proteomic technologies bears many benefits. While the performance of genome and transcriptome analysis can only deliver the generally possible protein expression, proteomics illustrates the actual protein expression pattern within the distinct cellular state at the time of measurement. In doing so, scientists have stated advanced high throughput proteomics as the least biased and hypothesis-free expression analysis technology [Aebersold and Mann, 2016]. Thus, proteomics may portray a higher predictive capacity for assessing biological functionalities and derivation heritage of certain cells than genomics and transcriptomics. With new technologies, only a minimum amount of serum sample or dissolved tissue (1 µl) is required to significantly detect biomarkers with high throughput and rapid processing; high specificity and sensitivity are being achieved [Assarsson et al., 2014]. Proteomic analysis may even be performed in isolated single cells, which enables detailed biomarker investigations on studies dealing with limited sample resources like fine-needle biopsies and small animal models [Assarsson et al., 2014, Ståhlberg et al., 2012].
Two main protein biomarker detection strategies can be identified. On the one hand, the 'triangular strategy' is being characterized by a contrasting pyramidal increase of study candidates and a decreased amount of potential biomarkers, respectively, in the three phases of biomarker detection: Discovery, validation and verification [Geyer et al., 2017, Rifai et al., 2006]. This strategy has its strengths defined in high specificity and sensitivity technologies for distinct biomarkers proven over time [Nie et al., 2017, Kim et al., 2015, Abbatiello et al., 2015, Burgess et al., 2014]. Although, with three various technologies (shotgun proteomics, targeted proteomics, and immunoassay development) utilized for all three detection phases practically achieving high numbers in significant determination of biomarkers seems mainly limited [Wu et al., 2015, Oberbach et al., 2014, Shi et al., 2013, Percy et al., 2013]. Thus, the complete and successful application of the 'triangular strategy' remains challenging. On the other hand, with recent technologies efficiently achieving sufficient proteomic depth in more time efficiently, the 'rectangular strategy' has been recently introduced [Geyer et al., 2016]. For this strategy, shotgun proteomics with maximized proteome coverage in ideally expansive study groups is performed in a discovery and a validation cohort and then analyzed in combination [Geyer et al., 2017]. An excellent study population and wide-ranging proteomics may benefit the detection of detailed biomarker patterns. The community around advancing proteomics discusses an interchange of the mainly applied biomarker detection strategy from 'triangular' to 'rectangular' due to the increasing ease and potential benefit of application with limited loss of significance described in recent analysis [Geyer et al., 2017].
This study assessed potential serum protein biomarkers for unstable atherosclerotic lesions with PEA, a dual antibody determination followed by quantification via RT-qPCR illustrating a contemporary state of the 'rectangular strategy'. However, to establish this method as clinically applicable, current difficulties need to be considered. The latest crit-

icism addresses the lack of standardized imputation of missing values in PEA technology and discusses plausible solutions for the imputation of values not missing randomly but because of the limited measurement range. [Lenz et al., 2020]

6.2.3 Limitations to Immunohistochemistry

Advanced proteomic technologies enable identifying potential serum protein biomarkers of distinct pathologies, like the PEA utilized in this study. To verify the impact of the potential biomarker on the related pathological process confirming analysis of diseased tissue is seen as a critical combining step. Thus, immunohistochemistry in this study evaluated the abundance of the detected serum biomarker, a conveniently utilized technology with low cost, robust characteristics, and ease of qualitative evaluation.
Although immunohistochemistry is a frequently implemented method, there are peculiarities to be mentioned and results require a cautious interpretation. Two main groups of potential bias have been reviewed, with reaction bias (e.g., sample fixation, antigen retrieval, detection system) on the one hand and interpretation bias (e.g., antibody selection, antibody types and clones choice) on the other hand [Yaziji and Barry, 2006]. With the consequent application of a standardized IHC protocol (Section 4.5.4), the reaction bias was tried to be minimized.

6.2.4 Limitations to Gene Expression Assessment

To find plausible genomic verification complimenting the proteomic and immunohistochemical findings in this study, gene expression of AIFM1 was compared in stable versus unstable CAP and isolated cell types from the Munich Vascular Biobank versus serum monocytes versus monocyte-derived macrophages. Detecting potential genetic predisposition targets for pathological processes was intended to lead to more efficient preventional techniques.
In this study, gene expression analysis was limited to the performance on RNA level via RT-qPCR. The utilized gene expression analysis may only provide an insufficient identification of the protein abundance corresponding to the analyzed RNA as prior studies have implied [Payne, 2015, Vogel and Marcotte, 2012]. To gain sufficient information on protein abundance, analyses like immunohistochemistry and proteomics are plausible considerations and were additionally performed in this study. Even though it is outranked in protein expression accuracy by proteomics, the transcriptome analysis brings more detailed information to the cellular state and actual presence of AIFM1 than conventional genomics. However, the transcriptome seen as a product already includes regulatory processes on the DNA level, and thus, the prediction capacity for promoting and inhibiting sequences is limited. To evaluate such sequences, genomic analysis should be taken into consideration.
Although conveniently practiced, the gene expression analysis performed on monocytes and monocyte-derived macrophages from healthy donours versus CAD patient-derived cells obtained from the Munich Vascular Biobank may portray limited comparability due

to different donour origins. A potential solution may be implied by comparing cells from physiological and pathological samples of the same donour.

6.3 Experimental Results

With the results gained from the conducted experiments within this study, a potential biomarker for the worldwide challenging impact of unstable atherosclerotic lesions was intended to be detected. With potential bias going alongside those results, being in dire need of mentioning the following is aimed to do so by bringing the results into perspective with contemporary results and thoughts from prior studies.

6.3.1 Plaque Structure and Stability

The utilized classification of extracted CAP specimen based on the updated AHA classification of coronary atherosclerotic lesions into eight (I-VIII) subclasses (Figure 4.2) may be seen as the starting point of a uniform plaque structure assessment and thus, the standardized base of the following experimental results in this study (Section 6.2.1). The CAP specimens were retrieved from patients treated after the contemporary ESVS guidelines [Eckstein, 2018, Naylor et al., 2018]. As therefore expected, none of the examined CAP specimens was classified as an initial lesion, fatty streak, or simple atheroma (I-IV), but all were characterized as portraying advanced atherosclerotic structure (V-VIII). The previously described overall classification of every single specimen in accordance with its most advanced atherosclerotic characteristics represented and the lack of further subclassification within one sample and additional follow-up classification down the road (Section 6.2.1) may also be seen as a potential cause of the determined plaque structure results. Whereas the majority of the stable specimen cohort was assigned to the group of simple *fibroatheroma* (V), nearly all but one unstable specimen were assigned to more complex *fibroatheroma* including thrombi, intraplaque hemorrhage, or scattered calcified nodules (VI & VII). Since those complexities contribute to the formation of vulnerable plaques [Bennett et al., 2016, Tabas et al., 2015, Bentzon et al., 2014], the assessed structure distribution in the study specimen may be seen as plausible.
Stability examination of CAP by ultrasound, determining echo-lucency and echogenicity, is possible in prior studies [Li et al., 2019, Topakian et al., 2011]. However, in this study, atherosclerotic lesions were labeled unstable whenever they presented a microscopically assessed minimum fibrous cap thickness <200 µm or a respective fibrous cap thickness <500 µm in accordance to *The Oxford Plaque Study* [Redgrave et al., 2008]. Thus, possible communication between the necrotic core and the overlying thrombus through a ruptured fibrous cap was counterchecked and all utilized specimens could be classified as unruptured [Virmani et al., 2000b]. Since no neurological symptoms were recorded for any of the patients included in this study (Section 5.1), this perfectly resembled the expected fibrous cap consistency in the utilized specimen.
Altogether, the atherosclerotic lesions were only characterized with AHA classification and binary stability assessment based on fibrous cap thickness. However, as scientific

research advances in deciphering the causation of vulnerable plaque formation, a more detailed classification and potential sub-classification of CAP stability might be possible. One plausible starting point might be with a more detailed analysis of metalloproteinase levels or collagen density in certain plaque regions [Hansson et al., 2015]. Additionally, with several studies showing an association of plaque morphology to age, sex and clinical data [Pelisek et al., 2016, Wendorff et al., 2015], whenever the subject cohort provides enough power, an observation of specific combinations of subgroups might be a plausible opportunity to identify more complex stability patterns and subsequently, biomarkers more detailed.

6.3.2 Protein Biomarker Variety in Proteomics

Following the 'rectangular strategy' in proteome analysis via the utilization of PEA resulted in the detection of various biomarkers potentially predicting unstable atherosclerotic plaque formation. From biomarkers presenting downregulated serum levels in patients with unstable plaque, CTRC could be identified as an outstanding hit, whereas from the variety of upregulated biomarkers in serum levels AIFM1, together with CA5A, DECR1 and IFN-gamma, portrayed significance.
The proteins with the most evident associations to atherogenesis - alongside AIFM1 - were CTRC and IFN-gamma. CTRC is widely discussed in pancreatitis pathophysiology-associated research [Zou et al., 2018, Raphael and Willingham, 2016]. However, not only a hypocalcemic activity with potential impact on atherogenesis described in prior studies [Tomomura et al., 1996], but also the hypothesis of a dysfunctional ubiquitin-proteasome system, partly presenting chymotrypsin activity, contributing to atherogenesis is being discussed in more recent studies [Wilck and Ludwig, 2014, Herrmann et al., 2010]. With Chymotrypsin C presenting downregulation in unstable plaque serum levels in this study, the importance of further proteasome-related biomarker examination in atherogenesis is highlighted. IFN-gamma has been well established as a critical player in atherogenesis for decades [Gupta et al., 1997]. Showing vital contribution to all three described stages of atherogenesis, IFN-gamma enhances the release of pro-inflammatory cytokines (e.g., TNF-α and IL-6) through macrophage activation, promoting release of metalloproteinases and oxygen radicals and supporting leukocyte recruitment through endothelial cell activation on the pro-atherogenic side [Voloshyna et al., 2014, Zhang et al., 2011, Schroecksnadel et al., 2006]. Nevertheless, preceding studies also discuss anti-atherogenic processes promoted by IFN-gamma, like the moderate stimulation of inducible nitric oxide synthase expression [Chung et al., 2011]. Altogether, IFN-gamma impacts a great variety of immune processes, which not all result in one homogenous atherogenic effect, but more in a broad and heterogenous general immunological effect, even beyond atherogenesis. Despite its vital contribution to atherogenesis, the contemporary information on IFN-gamma with a lack of specificity towards atherogenesis may hinder its establishment as an atherogenesis biomarker.
Looking at CA5A and DECR1, their association with atherogenesis may not be considered plausible enough to identify these proteins as powerful potential biomarkers yet.

While DECR1 is described as an androgen receptor possibly regulating lipid metabolism in prostate tumor cells, CA5A deficiency is being discussed in pediatric hyperammonemia screenings [Blomme et al., 2020, Nassar et al., 2020, van Karnebeek et al., 2014].

6.3.3 AIFM1 Expression and Distribution

Given its impact on atherogenesis as potential tissue homeostasis and morphology reconstruction key player [Bano and Prehn, 2018] and its significant upregulated levels in blood sera from unstable CAP patients detected in this study, AIFM1 gene expression in unstable versus stable CAP specimen was assessed via RT-qPCR and Immunohistochemistry. Additionally the Anti-AIFM1 IHC stainings were put in perspective with certain cell type marker IHC stainings in order to detect possible variation in AIFM1 expression amongst the cellular protagonists in atherosclerosis. Furthermore, the distribution of AIFM1 expression in certain plaque areas was examined.
The mitochondrial release and, consequently, the activation of the apoptosis regulator proteins CYCS and CASP9 is enhanced by AIFM1 with all three translocating in the nucleus [Susin et al., 1999]. Therefore, a positive correlation of AIFM1 gene expression levels with CYCS and CASP9 gene expression levels were assumed in apoptotic structures. Additionally, the presence of activated CASP9 marks a point of no return in apoptosis pathways [Green and Amarante-Mendes, 1998]; ongoing apoptosis is seen in direct positive correlation to defective efferocytosis in necrotic core expansion [Yurdagul et al., 2018, Tabas et al., 2015]. Subsequently, the assessment of not only AIFM1 but also CYCS and CASP9 gene expression levels was performed in unstable versus stable CAP via RT-qPCR in this study to illustrate the relation between the apoptosis regulating factors and vulnerable plaque formation. Although no significant differences could be detected, the trend towards higher gene expression in unstable CAP seen in the results may complement the hypothesis of this plausible relation.
Additionally, the hypothesis of higher AIFM1 expression fostering vulnerable plaque formation was strengthened by the utilized Anti-AIFM1 stainings in this study, in which a greater abundance of AIFM1 staining was detected semiquantitatively in unstable versus stable atherosclerotic lesions. By putting the AIFM1 stained slides in perspective with consecutively cut slides stained with EvG and HE, an overview of the AIFM1 staining distribution within the plaque could be achieved. As expected, the AIFM1 stained regions were predominantly congruent with plaque regions known for showing high apoptosis rates, like the necrotic core, shoulder region and fibrous cap. The additional slight background staining within the Anti-AIFM1 stained cells may be due to its continuous expression in mitochondria as part of the respiration chain and, thus, to its vital function in cell homeostasis [Kilbride and Prehn, 2013]. To gain more detailed information on the AIFM1 expression and distribution within unstable atherosclerotic lesions, areas with predominantly AIFM1 stained cells were put in perspective with immune and myofibroblastic cell markers in additional IHC. While no congruence was observed analyzing areas with myofibroblastic marker-stained cells, great congruence could be detected semiquantitatively with areas predominantly stained with immune cell markers. Within those

markers, CD68 shaded cell areas were identified, showing the highest levels of AIFM1 expression, semiquantitatively analyzed. Hence, amongst the tested immune cell types, M1 macrophages were substantiated to express AIFM1 the strongest.

6.3.4 Cell Plasticity in Atherosclerosis

When looking at the results of AIFM1 stainings and their comparison to immune and fibroblast cell markers, respectively, no interpretation can be made without discussing the phenotypic interchange happening in atherogenesis between immune and myofibroblast cells, predominantly macrophages and vascular smooth muscle cells.
On the one hand, prior studies have characterized CD68 as a predominant marker for the mononuclear phagocyte subset of M1 macrophages but also confirmed CD68 expression by phagocytic cells with myofibroblastic heritage via lineage tracing studies [Albarrán-Juárez et al., 2016, Feil et al., 2014]. The macrophage and potential lesion stabilization marker LGALS3 has also been detected as being expressed by VSMC-derived phagocytic cells [Shankman et al., 2015]. Furthermore, there have been scientific investigations about VSMCs expressing markers widely utilized for immune cell detection under atherogenic circumstances, created through cholesterol stimulation in vitro [Vengrenyuk et al., 2015].
On the other hand, alpha-SMA has not only been characterized and widely used as a VSMC marker but also shown to be expressed by monocyte-derived cells with myofibroblastic phenotypes within advanced atherosclerotic lesions in accordance to lineage tracing studies [Haider et al., 2019, Stewart et al., 2009]. Additionally, co-expression of alpha-SMA and CD68 in cells found in atherosclerotic lesions has been described [Basatemur et al., 2019, Allahverdian et al., 2014].
Subsequently, a hypothesis of both macrophages and VSMCs plasticity leading to a common cellular state or even a possible interchange into each other's phenotypic-like state shall be taken into consideration whenever functional cell studies based on marker expression are performed in atherogenesis research. As in this study, the unity between CD68 and AIFM1 stainings was not inevitably interpreted as AIFM1 expression being macrophage-specific but more of a marker for phagocytotic cells. However, alphaSMA-stained areas were also observed partly scattered around the AIFM1 and CD68 congruent areas. Thus, further investigations were performed in order to complement the gained immunohistochemical perceptions of higher AIFM1 expression in immune cells and not to be deceived by the cell plasticity within atherosclerotic lesions.
With the assessment of AIFM1 expression on a cellular level, even though no significance was examined, a clear trend of higher AIFM1 expression in immune cells compared to isolated VSMCs was identified. Moreover, a significantly higher AIFM1 expression in monocyte-derived macrophages versus their preliminary monocytes was observed via RT-qPCR. Thus, the limitations of functional studies based on marker expression due to cell plasticity in atherosclerotic lesions were tried to be minimized and the theorem of AIFM1 predominantly staining in immune cell regions was strengthened. Additionally, a hypothesis of AIFM1 playing an important role in myeloid cells, especially macrophage dynamics, may be formed and potentially continued to interrogate in future studies.

6.4 Outlook

Looking at the results and their discussion in this study, certain plausible ways to continue the research of AIFM1 and its role as a potential biomarker during plaque stabilization in carotid artery disease and ischemic stroke, plus the impact of AIFM1 on macrophage dynamics, may come to mind.

There may be a need to verify the specificity of upregulated AIFM1 blood serum levels resulting from the unstable CAP patients the blood was withdrawn from were being treated for. Thus, making sure the patients included in the study do not portray additional pathologies and apoptotic processes potentially leading to higher AIFM1 expression may be considered. With the cardiovascular clinical data being included in biobank data in this study, the first step following such intents has been initiated but must be put in perspective in future study designs, including further clinically acquired information. More ambitious, the process of potential AIFM1 release from the plaque may be investigated in order to complement blood serum proteomics and in combination with investigations on additional AIFM1 release generated by physiological or pathological processes.

With the high expression of AIFM1 in macrophages derived from healthy donour blood monocytes, one plausible further step could be an AIFM1 expression level development assessment in macrophages under oxidative stress to complement the hypothesis of AIFM1 expression levels increasing in apoptotic cell stages. Stimulating the monocyte-derived macrophages with oxLDL and LPS in comparison may be a plausible step to do so. Moreover, isolating macrophages out of atherosclerotic tissue from the biobank with AIFM1 expression analysis put in perspective between oxLDL-stimulated healthy macrophages and plaque-isolated macrophages may be an option. Investigating the role AIFM1 is playing in monocyte dynamics in a potential AIFM1 knockdown via special siRNA may also be taken into consideration, following a comparison of AIFM1 knockdown to AIFM1 wild-type monocytes. Using modern sequencing techniques, such as single-cell sequencing, to gain detailed information over possible morphological and, thus, homeostatic changes within the cells may show great advantages against more conservative cell culture comparison experiments. With AIFM1 being mainly located in the mitochondria in physiological cell status, mitochondria assays, including seahorse assays, also ought to be taken into consideration to further understand AIFM1 functions.

Specifically, no classic AIFM1 knock-out animal models have been established up to this day due to general embryonic lethality; however, the Harlequin mouse, intentionally characterized as a model for late-onset neurodegeneration, is expressing only 10–20% of the normal concentration of AIF due to a retroviral insertion into the AIF gene [Irwin et al., 2013]. The dreadful phenotype the Harlequin mouse is characterized by fosters ethical controversies and, once more, highlights single-cell sequencing methods as potentially better-suited experimental pathways for future inquiries in comparison. However, a cell-specific knockout with tamoxifen once the mice are fully matured would be a knock-out option to avoid dreadful phenotypes in classic knock-out mice.

7 Appendix

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7.2 Eidesstattliche Erklärung

Ich, **Lukas Bischoff**, (Vor- und Nachname) erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung **TUM Medical Graduate Center**
der TUM Graduate School zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
Apoptosis inducing factor 1 and its role during plaque stabilization in carotid artery disease and ischemic stroke
unter der Anleitung und Betreuung durch: **Univ.-Prof.Dr.med. Lars Mägdefessel**
ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 7 Abs. 6 und 7 angegebenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuer*innen für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.
Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Teile der Dissertation wurden im Zuge des Kongresses **Vascular Discovery 2021: From Genes to Medicine der American Heart Assiciation** veröffentlicht.
Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Ich habe keine Kenntnis über ein strafrechtliches Ermittlungsverfahren in Bezug auf wissenschaftsbezogene Straftaten gegen mich oder eine rechtskräftige strafrechtliche Verurteilung mit Wissenschaftsbezug.
Die öffentlich zugängliche Promotionsordnung sowie die Richtlinien zur Sicherung guter wissenschaftlicher Praxis und für den Umgang mit wissenschaftlichem Fehlverhalten der TUM sind mir bekannt, insbesondere habe ich die Bedeutung von § 27 PromO (Nichtigkeit der Promotion) und § 28 PromO (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich
○ einverstanden,
○ nicht einverstanden.

Ort, Datum, Unterschrift

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Publications

Lukas Bischoff
Munich, Germany, 5. Februar 2022

7.4 Paul Dudley White Award

The American Heart Association

Presents this

Paul Dudley White International Scholar Award

To Recognize the Authors with the Highest Ranked Abstract from ***Germany*** *at the Vascular Discovery: From Genes to Medicine Scientific Sessions 2021*

Lukas Bischoff, Jessica Pauli, Lars Maegdefessel

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Katey Rayner, PhD, FAHA
Vice-Chair, Vascular Discovery Program Committee

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