**Plaque expression levels of HDAC9 in association with plaque vulnerability traits and secondary vascular events in patients undergoing carotid endarterectomy: an analysis in the Athero-EXPRESS Biobank**

**Background**

Drug targets supported by human genetics have a higher probability of reaching phase III clinical trials and regulatory approval (Nelson et al, 2015). We and others previously demonstrated a **prominence of HDAC9** **in human atherosclerosis** by GWAS in stroke (Malik et al, 2018), CAD and myocardial infarction (Nelson et al, 2017), atherosclerotic aortic calcification (Malhotra et al, 2019), and PAD (Klarin et al, 2019). Despite these striking associations, the mechanisms linking HDAC9 to vascular pathologies and the ensuing therapeutic potential were poorly defined. We recently showed that HDAC9 is a key regulator of atherosclerotic plaque stability and IKK activation. Specifically, we studied the **effects of Hdac9 on features of atherosclerotic plaque vulnerability** using bone marrow reconstitution experiments and found an **increased plaque stability in *Hdac9*-deficient mice**. We further demonstrated that HDAC9 binds to the NF-κB activating kinases IKKα and IKKβ, resulting in their deacetylation and subsequent activation, which drives inflammatory responses in both Mφ and endothelial cells (EC). Pharmacological inhibition of HDAC9 with TMP195 attenuates early lesion formation by limiting myeloid cell recruitment. Gene expression profiling of Mφ by bulk RNA sequencing revealed that TMP195 downregulates key inflammatory pathways consistent with inhibitory effects on IKKβ. Moreover, therapeutic inhibition with **TMP195 reduces atheroprogression and confers plaque** **stability** in advanced lesions. *Ex vivo* treatment of monocytes from patients with established atherosclerosis reduced the production of inflammatory cytokines including **IL-1β** **and** **IL-6**. Thus, our current findings provide evidence for HDAC9 as a causal factor in atherosclerosis and a **promising target for interventional studies in humans** (Asare et al, 2020).

**Objectives**

Against this background, we now aim to make use of the data from Athero-Express Biobank Study to explore the associations of *HDAC9* expression levels in the atherosclerotic plaques from patients undergoing carotid endarterectomy with phenotypes of plaque vulnerability and secondary vascular events over a follow-up of three years.

**Study population**

We will include data from patients with available plaque *HDAC9* expression levels in Athero-Express Biobank Study (n=1201 individuals).

**Exposure**

*HDAC9* expression in the plaque, as assessed by bulk and single-cell RNA sequencing. Depending on the distribution of *HDAC9* expression levels in the plaque, they might need to be in-transformed.

**Outcomes**

We will examine **cross-sectional** associations between plaque *HDAC9* expression and the following plaque vulnerability phenotypes as well as the composite plaque vulnerability index:

1. Percentage of macrophages (continuous trait)
2. Percentage of SMCs (continuous trait)
3. Number of intraplaque microvessels per 3-4 hotspots (continuous trait)
4. Presence of moderate/heavy calcifications (binary trait)
5. Presence of moderate/heavy collagen content (binary trait)
6. Presence of lipid core no/<10% vs. >10% (binary trait)
7. Presence of intraplaque hemorrhage (binary trait)

Furthermore, we will examine in **longitudinal analyses** the associations between *HDAC9* expression levels and secondary cardiovascular events over a three-year follow-up period. The primary outcome will be a composite of fatal or non-fatal myocardial infarction, fatal or non-fatal stroke, ruptured aortic aneurysm, fatal cardiac failure, coronary or peripheral interventions, leg amputation due to vascular causes, and cardiovascular death. The secondary outcomes will be incidence of fatal or non-fatal stroke, incidence of acute coronary events (fatal or non-fatal myocardial infarction, coronary interventions), and cardiovascular death.

**Additional variables**

1. Age (continuous in 1-year increment)
2. Sex (male vs. female)
3. Presence of hypertension at baseline (defined either as history of hypertension, SBP ≥140 mm Hg, DBP ≥90 mm Hg, or prescription of antihypertensive medications)
4. Presence of diabetes mellitus at baseline (defined either as a history of diabetes, administration of glucose lowering medication, HbA1c ≥6.5%, fasting glucose ≥126 mg/dl, or random glucose levels ≥200 mg/dl).
5. Smoking (current, ex-, never)
6. LDL-C levels (continuous)
7. Use of lipid-lowering drugs
8. Use of antiplatelet drugs
9. eGFR (continuous)
10. BMI (continuous)
11. History of cardiovascular disease (stroke, coronary artery disease, peripheral artery disease)
12. Level of stenosis (50-70% vs. 70-99%)
13. Presenting symptoms (asymptomatic, ocular, TIA, or stroke)

**Statistical analysis**

We will first compare plaque expression levels of *HDAC9* across categories of the variables presented in the list above. For continuous traits, we will compare the levels across the following categories: age (years), <55, 55-64, 65-74, 75-84, 85+; LDL-C (mg/dl), <100, 100-129, 130-159, 160-189, 190+; eGFR (ml/min/1.73m2), <30, 30-59, 60-89, 90+; BMI (kg/m2), <18.5, 18.5-24.9, 25-29.9, 30-34.9, 35+

Comparisons will be done with the t-test for binary traits and with ANOVA for traits with >2 categories.

Then we will explore cross-sectional associations between *HDAC9* expression levels and the plaque vulnerability characteristics. Linear regression models will be used for continuous traits and logistic regression models for binary traits. *HDAC9* expression levels in the plaque will be added as a continuous variable (per 1-SD increment) and in a secondary approach as a categorical variable in 4 quartiles, with baseline as reference, to test for potential non-linearity in the association. We will have four additive models:

**Model 1**: adjusted for age and sex

**Model 2**: adjusted for age, sex, hypertension, diabetes, smoking, LDL-C levels, lipid-lowering drugs, antiplatelet drugs, eGFR, BMI, history of CVD, level of stenosis

Next, we will explore in logistic regression models associations between *HDAC9* expression levels in the plaque with symptomatic vs. asymptomatic plaque. As a symptomatic plaque, we will define plaques associated with an ipsilateral ischemic stroke, transient ischemic attach or central retinal artery occlusion.

To explore the mechanisms underlying these associations we will further examine whether plaque *HDAC9* expression levels are associated with plaque inflammation and matrix turnover. We will thus examine the age- and sex-adjusted associations of plaque *HDAC9* expression levels with multiple cytokines and with metalloproteinase activity in carotid plaques.

Finally, we will explore associations between plaque *HDAC9* expression levels and secondary cardiovascular endpoints over follow-up (primary and secondary outcomes, as described in the section ‘outcomes’). We will use Cox proportional hazard models for HDAC9 as continuous (per 1SD increment) and categorical trait (in quartiles) taking into account the time between surgery and the occurrence of the events, death, loss to follow-up or completion of follow-up. The same four multivariable models will be used as in the above-mentioned analyses.

We can present a Kaplan-Meier figure for the primary composite endpoints across the four HDAC9 quartiles and the results from the multivariable analyses in a Table.

For all analyses, the statistical significance threshold is set at a two-sided p<0.05.

**References**

Asare Y,…(24 coauthors)…,Dichgans M (2020). Histone Deacetylase 9 Activates IKK to Regulate Atherosclerotic Plaque Vulnerability. Circ Res. 127(6):811-823. PMID: 32546048

Klarin D et al. (2019). VA Million Veteran Program. Genome-wide association study of peripheral artery disease in the Million Veteran Program. Nat Med. 25:1274–1279. PMID: 31285632

Kundaje et al. (2015). Integrative analysis of 111 reference human epigenomes. Nature 518(7539):317-30. PMID: 25693563

Lapierre et al. (2016). Histone deacetylase 9 regulates breast cancer cell proliferation and the response to histone deacetylase inhibitors. Oncotarget 7(15):19693-708. PMID: 26930713

Magupalli VG et al. (2020). HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation. Science. 369(6510):eaas8995. PMID: 32943500

Malhotra R et al. (2019) HDAC9 is implicated in atherosclerotic aortic calcification and affects vascular smooth muscle cell phenotype. Nat Genet. 51:1580–1587. PMID: 31659325

Malik R,…(168 coauthors)…, Dichgans M (2018). Multi-ancestry genome-wide association study of 520,000 subjects identifies 32 loci associated with stroke and stroke subtypes. Nat Genet 50(4):524-537.

Meng H, Bartholomew B (2018). Emerging roles of transcriptional enhancers in chromatin looping and promoter-proximal pausing of RNA polymerase II. J Biol Chem. 293:13786–13794. PMID: 29187597

Nelson CP et al. (2017). Association analyses based on false discovery rate implicate new loci for coronary artery disease. Nat Genet 49(9):1385-1391. PMID: 28714975

Nelson MR et al. (2015). The support of human genetic evidence for approved drug indications. Nat Genet. 47:856–860. PMID: 26121088