Diabetes-related factors and atherosclerosis regression

7

Summary & Discussion

The main goal of this thesis was to investigate two different aspects of the 2 to 4-fold increased CVD risk in individuals with diabetes. Diabetes is an independent CVD risk factor, even after lipid-lowering therapy and further, impairs regression of atherosclerosis. A study by Nicholls *et al* in 2008 showed that lowering LDL-C to desired levels of <80mg/dL in individuals with no diabetes promotes atherosclerosis regression in almost 25% of them, measured as reduction in lesion size by intravascular ultrasound (Figure 1).[1] In individuals with diabetes, lowering LDL-C levels to <80mg/dL did only promote regression in ~17% individuals. Strikingly, the number of individuals with regression in non-diabetics with elevated plasma LDL-C and diabetics with desired LDL-C is similar. Our lab has been able to replicate findings consistent with these in mice, showing that diabetic mice have about 50% the regression of non-diabetic mice.[2]

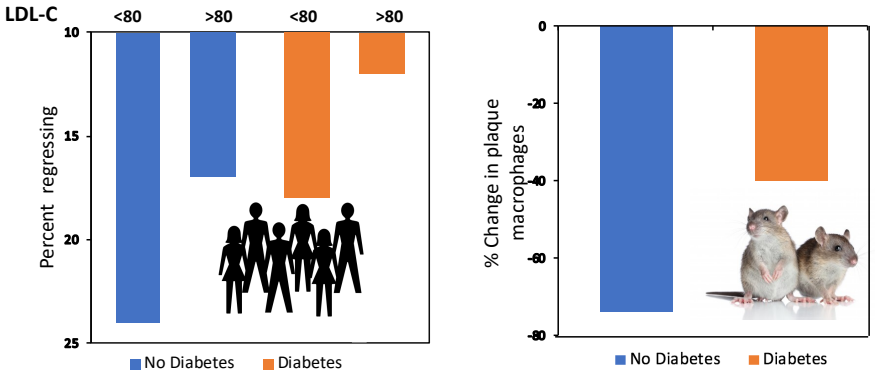

Figure 1. Comparison of atherosclerosis regression with and without diabetes in humans and mice. Human data (*left*) are based on the results published by Nicholls *et al.*[1] Mouse data (*right*) are based on our published study by Parathath *et al.*[2]

In this thesis, two probable causes for residual CVD risk, which are involved in high risk populations such as patients with diabetes, have been discussed:

- Effect of elevated plasma TG levels and low HDL (**Chapters 2-4**)
- Effect of hyperglycemia-associated circulating neutrophils and NET formation (**Chapters 5 & 6**)

Triglycerides and their role in atherosclerosis

Main findings

Chapter 2 describes an inverse association of HDL characteristics, such as HDL-C, apo-AI, HDL size and HDL-P, with pre-clinical atherosclerosis and CVD/CVE in a human cohort. Surprisingly, CEC was not associated with atherosclerosis surrogates or CVD/CVE. Stratifying the population in subjects with NGM and (pre)diabetes, revealed that associations of HDL-size and HDL-P with prevalence of CVD are lost. **Chapter 3** shows that the role of LpL in macrophage polarization *in vitro* does not resemble the *in vivo* situation. While LpL regulates lipid uptake, as well as pro- and anti-inflammatory gene expression in macrophages *in vitro,* myeloid LpL deficiency *in vivo* is dispensable for lipid accumulation and macrophage polarization. Global LpL deficiency *in vivo* reduces lipid content in adipose tissue macrophages (ATM) and the number of induced peritoneal macrophages. Further, global LpL deficiency does affect the phenotype of circulating monocytes and peritoneal macrophages, but does not affect macrophages in regressing atherosclerotic plaques. **Chapter 4** reports that LpL-induced HyperTG causes reduced HDL-C and HDL-P in mice and humans, but no dysfunctional HDL measured as CEC. Despite the reduction in HDL-C and HDL-P, LpL-deficiency-induced HyperTG did not impair atherosclerosis regression, neither in aortic arches, roots or BCAs, likely because HDL function (CEC) was maintained.

Discussion

The connection between low HDL-C and elevated TG with cardiovascular risk has been shown in observational studies, but how HyperTG affects other HDL characteristics is unknown and pre-clinical trials showing benefits of TG reduction are scant. While there are limited experimental models linking HyperTG and atherosclerosis, a large series of experimental studies have documented benefits of raising HDL levels as reviewed in [3,4]. One postulated reason for these benefits is an increase in CEC or reverse cholesterol transport (RCT).[5] However, interventions in humans that increased HDL-C levels using CETP inhibition did not show clinical benefits[6,7], indicating that an increase of HDL-C does not necessarily reflect an increase in HDL function (CEC or RCT). In line, although CETP is considered pro-atherogenic due to its contribution to an atherogenic lipid phenotype, including lowering HDL-C, CETP has also been shown to increase RCT[8], which is considered anti-

atherogenic. We showed that although inducing hCETP expression in mice lowers HDL-C and HDL-P, the ABCA1-mediated efflux per HDL particle was increased. Overall these effects resulted in atherosclerosis regression not being impaired (**Chapter 4**). Instead of solely measuring HDL-C, focus in the field of research has shifted to determining HDL functions. Therefore, we assessed HDLs' considered key-antiatherogenic function, *i.e.* CEC, and other characteristics such as apoA-I, HDL-P, and HDL size (**Chapter 2 & 4**). As the metabolic milieu may affect various properties of HDL, we looked into how diabetes (**Chapter 2**) and HyperTG alone (**Chapter 4**) impact HDL. To further determine how changes in HDL affect CVD, we associated HDL characteristics with pre-clinical atherosclerosis (endothelial dysfunction; EnD) and with CVD/CVE and were the first ones to do so in (pre)diabetic individuals (**Chapter 2**). Of note, we also associated HDL characteristics with another atherosclerosis surrogate – cIMT – but due to lack of associations, likely due to sensibility of the cIMT measurement itself, these results are not discussed here, but in **chapter 2**.

The (pre)diabetic individuals of the CODAM Cohort displayed typical features of diabetic dyslipidemia, *i.e.* reduced HDL-C and increased TG levels. As expected, HDL-C was inversely associated with EnD and CVE in the total cohort and in individuals with (pre)diabetes. Other HDL characteristics, such as apoA-I, HDL size and HDL-P were also inversely associated, with associations of HDL size and HDL-P being lost in individuals with (pre)diabetes, but not in healthy subjects. Notably, while previous studies have reported HDL-P and HDL-size to decrease in diabetes[9,10], we report an increase of both. With HDL-P and size being increased in (pre)diabetics, but lack of its association with atherosclerosis, this suggests that in individuals with NGM, the protective effect of a certain number of HDL particles against development of atherosclerosis is better than for that same number of HDL particles in (pre)diabetes. To determine if observed associations of HDL characteristics with atherosclerosis and CVE in (pre)diabetics, or the lack thereof, were dependent on TG levels, we performed TG adjusted analyses. While all HDL characteristics were still inversely correlated with atherosclerosis in healthy subjects after adjusting for TG, the association of HDL-C with atherosclerosis was lost specifically in (pre)diabetic patients. This suggests that the inverse relationship of HDL-C with atherosclerosis in (pre)diabetes is dependent on TG thereby confirming the intimate relationship of HDL-C and TG.

To allow a closer comparison of human data from the CODAM study with human and mouse data from **chapter 4**, we stratified the CODAM cohort into TG<150mg/dL (N=363; control) and >150mg/dL (N=167; HyperTG). Thereby, 66% of HyperTG patients were (pre)diabetic and had Hb1Ac levels of 6 ± 0.9%. Next to reduced HDL-C, HyperTG individuals from the CODAM Cohort also displayed decreased HDL-P, but not change in CEC, compared to control (Figure 2). This is in line with results from **chapter 4**, showing that despite reduction of HyperTG-induced HDL-C and HDL-P in mice and human, overall CEC is not impaired. Interestingly, individuals with (pre)diabetes have increased HDL-P, but also no change in CEC (**Chapter 2**). This suggests that HDL functionality can be preserved despite changes in HDL-P number. Thus, composition of HDL is of particular interest regarding its atheroprotective properties. Nonetheless, if preserved CEC is the reason for not observing impaired regression in HyperTG mice despite a reduction in HDL-C and HDL-P (**Chapter 4**), we would expect to see an association of CEC with atherosclerosis and CVD/CVE in the CODAM study (**Chapter 2**) as it has been shown in other studies.[11-14] So far, CEC measurements are not standardized and although we employed a well validated CEC assay, different protocols could have led to discrepancies between studies. Another limitation is the cross-sectional approach of our study, which hampers a direct causal interpretation of the observed associations. A better comparison of **chapter 2** and **chapter 4** would have been measuring associations of HDL-characteristics with the change in cIMT over the study period (ΔcIMT) as surrogate for atherosclerosis regression. However, as mentioned above, we did not find any significant associations of HDL characteristics with cIMT.

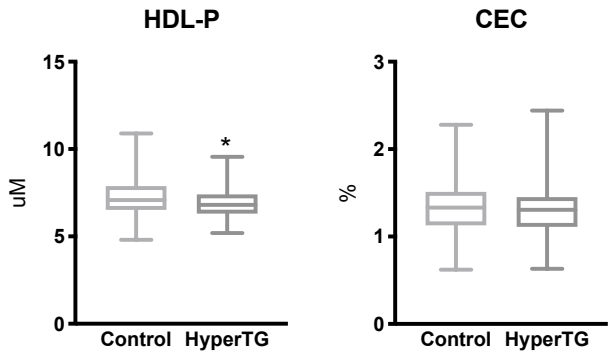

Figure 2. Comparison of HDL-P (*left*) and CEC (*right*) between Control (TG<150mg/dL, N=363) and HyperTG (TG>150mg/dL, N=167) in the CODAM study.

HyperTG has been shown to be associated with CVD in a number of studies and is not in line with our results from **chapter 4**. However, next to CEC, HDL has other functions as well as including antithrombotic functions.[15] Interestingly, proteomic analysis of HDL revealed an upregulation of proteins that are associated with platelet activation, signaling and degranulation in mice and in humans (data now shown), suggesting a role of HyperTG-reduced HDL in atherothrombosis. Current publications have reported that acute HyperTG induces platelet hyperactivity[16] and contributes to resistance of antiplatelet therapy such as aspirin and clopidogrel.[17,18] Additionally, platelet activity was also increased by TG-rich lipoproteins from T2DM patients.[19] Meta-regression analyses of 64 randomized control trials showed that TG levels were associated with stroke risk, but not cIMT measures. However, TG lowering did only result in a trend towards reduced stroke incidence.[20] Therefore, the association of HyperTG with increased CVD risk could be explained by loss of HDLs antithrombotic function, but future studies are needed.

Another aspect of the atherogenic potential of HyperTG is the lipolysis of TGs. LpL hydrolyzes TGs either at the vessel wall or within the atherosclerotic plaque, resulting in remnants and other lipolysis products (FFA, mono- diglyceride and lysolecithin). TG lipolysis at the vessel wall leads to atherogenic remnants that can enter the arterial wall and deposit lipids, while lipolysis products (FFA, mono- diglyceride and lysolecithin) can result in increased inflammation, monocyte adhesion and coagulation. In contrast, we did not find impaired vascular regression despite vascular lipolysis (**Chapter 4**). As macrophage LpL has been implicated in foam cell formation[21-23], not observing HyperTG-impaired atherosclerosis regression could also be due to counterbalancing effects of Lpl deficiency in macrophages. Thus, macrophage LpL deficiency could prevent lipoprotein uptake by macrophages, masking possible atherogenic effects of HyperTG. Macrophages are the major source of LpL in atherosclerotic plaques[24] and whether LpL in atherosclerotic plaques acts pro- or antiatherogenic is still debated. Interestingly, macrophage specific LpL knockout *in vivo* reduced atherosclerosis.[21,22] *In vitro*, macrophage LpL appears to have atherogenic functions, because it causes accumulation of atherogenic lipoproteins and their rapid uptake by macrophages[25], which we recapitulated in **chapter 3**. It is therefore feasible that LpL deficiency in macrophages overcomes the detrimental

effects of HyperTG in LpL-deficient mice. However, whether or not LpL was present in macrophages, we did not observe differences in atherosclerosis regression in mice (**Chapter 4**). The potential antiatherogenic effects of LpL are thought to rely on the fatty acids provided by lipolysis, which were reported to convert macrophages to a more anti-inflammatory phenotype via peroxisome proliferator-activated receptors (PPARs).[23,26,27] We showed that LpL *in vitro* changes both, pro- and anti-inflammatory markers, despite a decrease in FFA and increase in glycolysis suggestive of an increase in pro- and decrease of anti-inflammatory markers (**Chapter 3**). Further, transcriptomic profiling of macrophages in atherosclerosis regressing plaques *in vivo* did not reveal differences between macrophages expressing LpL and LpL deficient macrophages (**Chapters 3 & 4).**

In conclusion, in a human cohort we have shown that HDL-C, but not CEC, is associated with atherosclerosis and CVE, also in patients with diabetes. The found association of atherosclerosis with HDL-P is, however, no longer observed in diabetes patients. Experimentally reducing HDL-C and HDL-P by induction of HyperTG did not impair atherosclerosis regression, likely because CEC was unchanged. Further, lipolysis of circulating TG-enriched lipoproteins at the vessel wall or at atherosclerotic macrophages does not impair regression either. HDL proteins that changed with HyperTG were associated with lipoprotein remodeling (e.g. reduced ApoA-I) and platelet activation, signaling and degranulation. Overall, our results indicate that HyperTG does not impair atherosclerosis regression, but might be of importance for atherothrombosis via decreased antithrombotic functions of HDL and should be addressed in future studies.

Neutrophils and their role in atherosclerosis

Main Findings

Chapter 5 shows that NETs lead to inflammasome activation and inflammatory macrophages in atherosclerotic plaques. Our results illustrate that NETs can resolve during atherosclerosis regression. In a hyperglycemic environment however, NETs persisted thereby exacerbating macrophage inflammation and impairing atherosclerosis resolution. DNase1 treatment reduced plaque NETs content and macrophage inflammation, promoting atherosclerosis resolution after lipid-lowering in diabetic mice. **Chapter 6** describes a pilot study about the potential role of two different

endonucleases – DNase1 and DNase1L3 – in atherosclerosis. The data show that double-deficiency tends to increase NET formation compared to single-deficient mice and control with no change in NET content during regression. Further, NET content did not associate with atherosclerosis severity.

Discussion

The possible implication of neutrophils in the pathophysiology of CVD has long been overshadowed by other leukocyte subtypes, such as monocytes and macrophages, partly due to neutrophil's short life span in the circulation of <24h in mice[28] and human[29-31]. However, the life span of neutrophils within different tissues as well as under inflammatory conditions is not well defined. Therefore, the discovery of neutrophils ability to form NETs[32] and the presence of both in human[33-37] and mouse[38-44] atherosclerotic plaques has sparked new interest in neutrophils. It has been shown that inflammation can increase longevity of neutrophils, in part via regulation of anti-apoptotic factors, such as Mcl-1.[45,46] While survival of neutrophils could be beneficial in acute inflammation, it might be detrimental in a chronic inflammatory state due to increased NETs release as senescent neutrophils have a higher potential for NET formation.[47] NETs' proinflammatory role and contribution to chronic inflammatory disease has been postulated to trigger crosstalk of neutrophils with other cells.[48,49] Warnatsch et al. suggested that the interaction of neutrophils and macrophages leads to inflammasome activation in plaque macrophages and enhanced atherosclerosis progression.[38] In agreement, using transcriptomic profiling of atherosclerotic macrophages, we prove that macrophages in a NET+ area possess indeed a enhanced proinflammatory phenotype compared to macrophages outside of a NETs area (**Chapter 5**). Since CVD as well as diabetes are associated with neutrophilia in mice[50,51] and human[52] and hyperglycemia has been shown to prime neutrophils for NET production[53], diabetics are one clinical population in which NETs may be particularly harmful in with respect to cardiovascular risk. Further, plasma NETs markers are not only correlated with severity of CAD, but also with severity of T2DM as judged by correlation of nucleosomes (NETosis marker) with HemoglobinA1c levels (average blood glucose levels over a period of time) in humans.[54] Studying atherosclerosis regression in diabetic mice, we found impaired atherosclerosis

regression (Chapter 5), as we have shown before[2,55,56]. Thereby, macrophage content was positively associated with NET content (**Chapter 5**).

While current studies of NETs focused on atherosclerosis progression, we are the first ones reporting on atherosclerosis regression. Our novel results showed that NETs can spontaneously resolve in a non-hyperlipidemic environment, but not in hyperglycemic conditions, even with non-hyperlipidemia. Thus, presence of NETs could be one reason for the increased CVD risk in diabetic patients, even when they have desired LDL-C levels. One way to clear NETs is treatment with DNase1, as NETs are thought to be mainly degraded by endonucleases[57] and have been reported to be cleared by DNase1 in atherosclerosis in mice [38,58]. Given published studies and existent clinical use of DNase1 (e.g. cystic fibrosis[59]), we treated mice with DNase1. As expected, DNase1 treatment led to reduced NET content and excitingly, promoted atherosclerosis regression despite ongoing hyperglycemia by reducing plaque inflammation (**Chapter 5**). The pro-inflammatory nature of NETs are thought to increase monocyte and neutrophil recruitment, further enhancing plaque inflammation[38,60], leading to the hypothesis that DNase1 treatment lowers leukocyte recruitment. We do find increased monocytosis (data not shown) as well as neutrophilia in diabetic mice (**Chapter 5**), as previously reported by our lab.[50,51] In line, we also found increased plaque neutrophils in diabetic mice and a trend towards decreased circulating and plaque neutrophil levels upon DNase1 treatment. This is in agreement with a study by Wong et al. showing no difference in neutrophil recruitment in diabetic *Padi4*$^{-/-}$ (NETs abrogated) mice compared to diabetic WT mice[53], but in contrast to other studies showing that plaque neutrophil content is reduced upon the ablation of the inflammasome[39] or NETs abrogation[38]. Notably, not all neutrophils detected in plaques do undergo NETosis, which has been shown before in other *in vitro* and *in vivo* studies.[61] Thus, our results indicate that activation of neutrophils rather than neutrophil levels per se are of relevance for NET formation and its effect on plaque composition.

We have seen that restoring normal circulating cholesterol levels after a period of elevated cholesterol levels, led to spontaneous resolution of NETs without additional DNase1 treatment (**Chapter 5**), indicating that physiological amounts of endonucleases are sufficient for NETs clearance in healthy conditions. Therefore, we

were interested to know if *DNase1*$^{-/-}$ mice have higher NET content in atherosclerotic plaques and if atherosclerosis progression was worse or atherosclerosis regression impaired with DNase1 deficiency. Since it has been shown before that serum from DNase1 KO mice show some residual KO activity that displayed characteristics of DNase1L3[62], we decided to include DNase1L3 deficient and double-deficient mice. We found that double-deficient mice showed a trend towards increased NET content, which is in line with a study by Jimenez-Alcazar et al., showing that especially the double-deficient mice have more NETs than single-deficient mice leading to occlusion of vascular vessels.[63] In contrast to our previous results (**Chapter 5**), the trend towards increased NET content in double-deficient mice did not result in changes in atherosclerosis progression or regression and further, NETs content did not decrease with regression (**Chapter 6**). However, we did not observe significant differences in NET content between groups. Further, NET content in progressing mice on WT background was about 4-fold lower compared to NET content we observed in the LDLR-deficient mice and is likely due to circulating plasma cholesterol levels. In our study in **chapter 5** using *Ldlr*$^{-/-}$ fed a WD, we reached cholesterol levels of ~1000mg/dL. In our study in **chapter 6**, we switched from *Ldlr*$^{-/-}$ to WT mice injected with PCSK9 fed a WD to avoid crossbreeding of DNaseKO mice onto *Ldlr*$^{-/-}$ background. However, WT mice injected with PCSK9 only reached levels of about ~60% from those of *Ldlr*$^{-/-}$ mice. We did observe higher cholesterol levels (~1200mg/dL) using PCSK9 before[64] and the rather low cholesterol levels could be the results of a change in animal facility and staff. In line with lower cholesterol levels, we also observed lower atherosclerotic plaque sizes. Hence, low NET content could be due to lack of stimulus. If NET formation requires these high levels of cholesterol and can resolve spontaneously by lowering cholesterol levels, clinical relevance of NETs in hyperlipidemia is questionable, since patients with cholesterol levels of >200mg/dL are already put on lipid-lowering therapy. However, NETs do not resolve under hyperglycemic conditions, not even after lipid-lowering. Hence, population to benefit from DNase1 treatment are patients suffering from diabetes, especially those with not well-controlled blood glucose levels. Clinical use of DNase1 (Pulmozyme®) has been approved by the FDA (Federal Drug Administration) in 1993 and since then is on the market in >65 countries with no signs of major toxicity or detrimental long-term effects. Pulmozyme® has been shown to be effective in treatment of cystic fibrosis by

increasing lung function and reducing exacerbation of cystic fibrosis. This beneficial effect has been proposed to be partly due to NETs degradation by DNase1.[65] Thus, DNase1 treatment to lower plaque inflammation and to induce atherosclerosis resolution would be a novel therapeutic approach to overcome increased CVD risk in diabetic patients. We (**Chapter 5**) and others[38,53,58] have shown that NETs degradation via DNase1 is beneficial in the context of CVD in mice. Notably, there is one contradictory study showing no effect of DNase1 treatment on atherosclerosis [66], including the thought that DNase1 has little effect on other NET components, such as neutrophil elastase, that might continue to cause inflammation at the vessel wall.[67] NETs from isolated human neutrophils can also be degraded by DNase1.[68] Interestingly, it has been shown that an increase in circulating NETs is counterbalanced by an increase in DNase activity in healthy individuals.[69] Further, mutations in DNAse1 gene has been associated with disease states such as myocardial infarction[70] or SLE[71,72]. Thus, DNase1 activity might be impaired in individuals suffering from diabetes. In contrast, one study shows that DNase1 activity is actually increased in diabetes[73] and further research is needed for clarification.
So far, there are no reports on CVD risk in individuals treated with Pulmozyme®, however whether DNase1 reaches atherosclerotic plaques by delivery via a nebulizer is questionable. DNase1 administration in current mouse atherosclerosis studies is done via intraperitoneal injections, but in humans this may compromise immunoprotective functions of NETs e.g. antimicrobial activity. A better way to administrate DNase1 might be DNase1-coated nanoparticles, which were shown to reduce size of lung metastases in mice[74], but this way of administration needs further exploration in atherosclerosis. Although evidence for the detrimental effects of NETs in several diseases such as diabetes and atherosclerosis is raising, current research is still in its infancies. Further research is needed to deepen the knowledge about NETs in CVD and the exact mechanisms of DNase1 treatment to translate those findings into a novel therapy strategy.

Future Directions

Several questions have been raised by the results of this thesis and need to be addressed in future studies.

The effect modifications by the insulin resistant state in the CODAM study are highly interesting and are worth to further investigate in a larger cohort with sufficient power for more extensive subgroup analyses.

Interestingly, HDL proteomics revealed increased platelet associated markers on TG-enriched HDL and future experiments should focus on the role of HyperTG in thrombosis. Therefore, platelet aggregation assays using platelet-rich plasma of HyperTG patients and LpL deficient patients should be tested against control plasma to see if published results of HyperTG inducing platelet hyperactivity can be replicated. If that is the case, aggregation assays should be repeated using incubation of isolated HDL from healthy and HyperTG (LpL deficient) subjects. To determine the effect on atherothrombosis, experimental studies should be performed using a mouse model of thrombosis such as ligation of the inferior vena cava.[75] Aggregation assays should also be done using platelet-rich plasma from LpL deficient and control mice and further, flow cytometry assays using platelet markers (e.g. P-selectin, JON/A) can give additional insights on platelet activation.

Our novel results showed the therapeutic potential of NET reduction in individuals with diabetes, especially in those whose diabetes is not well controlled. Future studies should focus on understanding the mechanisms by which DNase1 leads to improved atherosclerosis regression in a hyperglycemic condition. We have shown that DNase1 treatment reduced inflammation in atherosclerotic plaques, thereby improving atherosclerosis regression. Additional inflammatory markers in the circulation are of interest. Since we were not able to detect IL1β in the circulation using ELISA and CBA assays, other markers, such as IL-17, IL-18, IL-6, can be tested. Decreased inflammation and improved atherosclerosis regression also lead to the hypothesis that fewer monocytes were recruited, macrophage retention is improved and/or proliferation is reduced. To test this, monocyte and macrophage trafficking studies should be performed. Preliminary results showed reduced circulating Ly6C^{high} monocytes with DNase1 treatment, but no differences in monocyte trafficking (data not shown) and completed study results need to be waited for to draw final conclusions.

Another aspect to consider is the possible effect of hypercholesterolemia and/or hyperglycemia on DNase1 activity. NETs have been shown to be involved in the pathogenesis of systemic lupus erythematosus (SLE)[68], partly due to reduced DNase1

activity[71,76]. This raises the question if hypercholesterolemia/hyperglycemia may have similar effects, hence the increased NET formation and inflammation. In contrast, one study reported increased DNase1 activity in serum of individuals with diabetes and of STZ-injected rats.[73] For clarification, DNase activity of hypercholesterolemic and hyperglycemic mice should be compared to control and DNase1 treated mice.

As physiological amounts of endonucleases are sufficient in clearing NETs and results of DNase deficient mouse models were modest under hypercholesterolemic conditions, future studies should focus on hyperglycemia. Given that we see positive effects on DNase1 treatment in diabetic mice and increased NET formation with the double-deficient mouse model, the impact of double-deficient diabetic mice on atherosclerosis is of interest.

Main conclusion & clinical recommendation

HyperTG and hyperglycemia have similar effects on HDL, but while HyperTG alone did not impair regression, hyperglycemia does. We observed reduced HDL-C (HyperTG and (pre)diabetics) and HDL-P (HyperTG only), but no differences in CEC (HyperTG and (pre)diabetes) in mice and humans. Experimental studies showed no impairment of atherosclerosis regression with HyperTG, but with hyperglycemia. Thereby, circulating neutrophil levels were increased along with NET content in atherosclerotic plaques and macrophage inflammation. Of note, we did not observe NETs in HyperTG mice (data not shown). Excitingly, clearing NETs using DNase1 lowers plaque inflammation and can overcome diabetes-impaired regression, despite ongoing hyperglycemia.

These results suggest that treating hyperglycemia-associated inflammation is prominent over treating HyperTG in the residual CVD risk in individuals with diabetes. Future studies should focus on establishing the mechanisms of DNase1 treatment and on evaluating the possible effect of HyperTG on other HDL characteristics such as its antithrombotic function.

References

1 Nicholls, S. J. *et al.* Effect of diabetes on progression of coronary atherosclerosis and arterial remodeling: a pooled analysis of 5 intravascular ultrasound trials. *J Am Coll Cardiol* **52**, 255-262, doi:10.1016/j.jacc.2008.03.051 (2008).

2 Parathath, S. *et al.* Diabetes adversely affects macrophages during atherosclerotic plaque regression in mice. *Diabetes* **60**, 1759-1769, doi:10.2337/db10-0778 (2011).

3 Feig, J. E. *et al.* HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. *Proc Natl Acad Sci U S A* **108**, 7166-7171, doi:10.1073/pnas.1016086108 (2011).

4 Hewing, B. *et al.* Effects of native and myeloperoxidase-modified apolipoprotein a-I on reverse cholesterol transport and atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* **34**, 779-789, doi:10.1161/ATVBAHA.113.303044 (2014).

5 Rohatgi, A. *et al.* in *New England Journal of Medicine* Vol. 371 2383-2393 (2014).

6 Barter, P. J. *et al.* in *New England Journal of Medicine* Vol. 357 2109-2122 (2017).

7 Group, H.-T. C. in *New England Journal of Medicine* Vol. 371 203-212 (2014).

8 Barter, P. J. *et al.* in *The Lancet* Vol. 23 160-167 (2003).

9 Mora, S. *et al.* Lipoprotein particle size and concentration by nuclear magnetic resonance and incident type 2 diabetes in women. *Diabetes* **59**, 1153-1160, doi:10.2337/db09-1114 (2010).

10 Festa, A. *et al.* Nuclear magnetic resonance lipoprotein abnormalities in prediabetic subjects in the Insulin Resistance Atherosclerosis Study. *Circulation* **111**, 3465-3472, doi:10.1161/CIRCULATIONAHA.104.512079 (2005).

11 Khera, A. V. *et al.* Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* **364**, 127-135, doi:10.1056/NEJMoa1001689 (2011).

12 Rohatgi, A. *et al.* HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med* **371**, 2383-2393, doi:10.1056/NEJMoa1409065 (2014).

13 Saleheen, D. *et al.* Association of HDL cholesterol efflux capacity with incident coronary heart disease events: a prospective case-control study. *Lancet Diabetes Endocrinol* **3**, 507-513, doi:10.1016/S2213-8587(15)00126-6 (2015).

14 Shea, S. *et al.* Cholesterol Mass Efflux Capacity, Incident Cardiovascular Disease, and Progression of Carotid Plaque. *Arterioscler Thromb Vasc Biol* **39**, 89-96, doi:10.1161/ATVBAHA.118.311366 (2019).

15 Mineo, C., Deguchi, H., Griffin, J. H. & Shaul, P. W. Endothelial and antithrombotic actions of HDL. *Circ Res* **98**, 1352-1364, doi:10.1161/01.RES.0000225982.01988.93 (2006).

16 Aye, M. M. *et al.* Acute hypertriglyceridemia induces platelet hyperactivity that is not attenuated by insulin in polycystic ovary syndrome. *J Am Heart Assoc* **3**, e000706, doi:10.1161/JAHA.113.000706 (2014).

17 Jastrzebska, M. *et al.* Variable gender-dependent platelet responses to combined antiplatelet therapy in patients with stable coronary-artery disease. *J Physiol Pharmacol* **69**, doi:10.26402/jpp.2018.4.10 (2018).

18 Karepov, V., Tolpina, G., Kuliczkowski, W. & Serebruany, V. Plasma triglycerides as predictors of platelet responsiveness to aspirin in patients after first ischemic stroke. *Cerebrovasc Dis* **26**, 272-276, doi:10.1159/000147455 (2008).

19 Boulet, M. M. *et al.* Large triglyceride-rich lipoproteins from fasting patients with type 2 diabetes activate platelets. *Diabetes Metab*, doi:10.1016/j.diabet.2019.03.002 (2019).
20 Labreuche, J., Deplanque, D., Touboul, P. J., Bruckert, E. & Amarenco, P. Association between change in plasma triglyceride levels and risk of stroke and carotid atherosclerosis: systematic review and meta-regression analysis. *Atherosclerosis* **212**, 9-15, doi:10.1016/j.atherosclerosis.2010.02.011 (2010).
21 Babaev, V. R. *et al.* Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J Clin Invest* **103**, 1697-1705, doi:10.1172/JCI6117 (1999).
22 Van Eck, M., Zimmermann, R., Groot, P. H., Zechner, R. & Van Berkel, T. J. Role of macrophage-derived lipoprotein lipase in lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol* **20**, E53-62 (2000).
23 Lindqvist, P., Ostlund-Lindqvist, A. M., Witztum, J. L., Steinberg, D. & Little, J. A. The role of lipoprotein lipase in the metabolism of triglyceride-rich lipoproteins by macrophages. *J Biol Chem* **258**, 9086-9092 (1983).
24 O'Brien, K. D., Gordon, D., Deeb, S., Ferguson, M. & Chait, A. Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *J Clin Invest* **89**, 1544-1550, doi:10.1172/JCI115747 (1992).
25 Mead, J. R., Irvine, S. A. & Ramji, D. P. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med (Berl)* **80**, 753-769, doi:10.1007/s00109-002-0384-9 (2002).
26 Huang, S. C. *et al.* Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol* **15**, 846-855, doi:10.1038/ni.2956 (2014).
27 Vats, D. *et al.* Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell Metab* **4**, 13-24, doi:10.1016/j.cmet.2006.05.011 (2006).
28 Suratt, B. T. *et al.* Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am J Physiol Lung Cell Mol Physiol* **281**, L913-921, doi:10.1152/ajplung.2001.281.4.L913 (2001).
29 Athens, J. W. *et al.* Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. *J Clin Invest* **40**, 989-995, doi:10.1172/JCI104338 (1961).
30 Basu, S., Hodgson, G., Katz, M. & Dunn, A. R. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood* **100**, 854-861, doi:10.1182/blood.v100.3.854 (2002).
31 Dancey, J. T., Deubelbeiss, K. A., Harker, L. A. & Finch, C. A. Neutrophil kinetics in man. *J Clin Invest* **58**, 705-715, doi:10.1172/JCI108517 (1976).
32 Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535, doi:10.1126/science.1092385 (2004).
33 Megens, R. T. A. *et al.* Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thrombosis and Haemostasis* **107**, 597-598, doi:10.1160/TH11-09-0650 (2012).
34 Oklu, R., Stone, J. R., Albadawi, H. & Watkins, M. T. Extracellular traps in lipid-rich lesions of carotid atherosclerotic plaques: implications for lipoprotein retention

and lesion progression. *J Vasc Interv Radiol* **25**, 631-634, doi:10.1016/j.jvir.2013.12.567 (2014).
35 Quillard, T. *et al.* TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J* **36**, 1394-1404, doi:10.1093/eurheartj/ehv044 (2015).
36 Naruko, T. *et al.* Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* **106**, 2894-2900, doi:10.1161/01.cir.0000042674.89762.20 (2002).
37 Pertiwi, K. R. *et al.* Neutrophil Extracellular Traps Participate in All Different Types of Thrombotic and Haemorrhagic Complications of Coronary Atherosclerosis. *Thromb Haemost* **118**, 1078-1087, doi:10.1055/s-0038-1641749 (2018).
38 Warnatsch, A., Ioannou, M., Wang, Q. & Papayannopoulos, V. Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* **349**, 316-320, doi:10.1126/science.aaa8064 (2015).
39 Westerterp, M. *et al.* Cholesterol Efflux Pathways Suppress Inflammasome Activation, NETosis, and Atherogenesis. *Circulation* **138**, 898-912, doi:10.1161/CIRCULATIONAHA.117.032636 (2018).
40 Liu, Y. *et al.* Myeloid-Specific Deletion of Peptidylarginine Deiminase 4 Mitigates Atherosclerosis. *Front Immunol* **9**, 1680, doi:10.3389/fimmu.2018.01680 (2018).
41 Drechsler, M., Megens, R. T., van Zandvoort, M., Weber, C. & Soehnlein, O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* **122**, 1837-1845, doi:10.1161/CIRCULATIONAHA.110.961714 (2010).
42 van Leeuwen, M. *et al.* Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. *Arterioscler Thromb Vasc Biol* **28**, 84-89, doi:10.1161/ATVBAHA.107.154807 (2008).
43 Rotzius, P. *et al.* Distinct infiltration of neutrophils in lesion shoulders in ApoE-/- mice. *Am J Pathol* **177**, 493-500, doi:10.2353/ajpath.2010.090480 (2010).
44 Zernecke, A. *et al.* Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circ Res* **102**, 209-217, doi:10.1161/CIRCRESAHA.107.160697 (2008).
45 Moulding, D. A., Quayle, J. A., Hart, C. A. & Edwards, S. W. Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood* **92**, 2495-2502 (1998).
46 Colotta, F., Re, F., Polentarutti, N., Sozzani, S. & Mantovani, A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80**, 2012-2020 (1992).
47 Zhang, D. *et al.* Neutrophil ageing is regulated by the microbiome. *Nature* **525**, 528-532, doi:10.1038/nature15367 (2015).
48 Jorch, S. K. & Kubes, P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med* **23**, 279-287, doi:10.1038/nm.4294 (2017).
49 Soehnlein, O., Steffens, S., Hidalgo, A. & Weber, C. Neutrophils as protagonists and targets in chronic inflammation. *Nat Rev Immunol* **17**, 248-261, doi:10.1038/nri.2017.10 (2017).
50 Distel, E. *et al.* miR33 inhibition overcomes deleterious effects of diabetes mellitus on atherosclerosis plaque regression in mice. *Circ Res* **115**, 759-769, doi:10.1161/CIRCRESAHA.115.304164 (2014).

51 Barrett, T. J. *et al.* Apolipoprotein AI) Promotes Atherosclerosis Regression in Diabetic Mice by Suppressing Myelopoiesis and Plaque Inflammation. *Circulation* **140**, 1170-1184, doi:10.1161/CIRCULATIONAHA.119.039476 (2019).
52 Hilgendorf, I. & Swirski, F. K. Making a difference: monocyte heterogeneity in cardiovascular disease. *Curr Atheroscler Rep* **14**, 450-459, doi:10.1007/s11883-012-0274-8 (2012).
53 Wong, S. L. *et al.* Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med* **21**, 815-819, doi:10.1038/nm.3887 (2015).
54 Menegazzo, L. *et al.* NETosis is induced by high glucose and associated with type 2 diabetes. *Acta Diabetol* **52**, 497-503, doi:10.1007/s00592-014-0676-x (2015).
55 Nagareddy, P. R. *et al.* Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis. *Cell Metab* **17**, 695-708, doi:10.1016/j.cmet.2013.04.001 (2013).
56 Yuan, C. *et al.* Human Aldose Reductase Expression Prevents Atherosclerosis Regression in Diabetic Mice. *Diabetes* **67**, 1880-1891, doi:10.2337/db18-0156 (2018).
57 Fuchs, T. A. *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231-241, doi:10.1083/jcb.200606027 (2007).
58 Yamamoto, K. *et al.* Augmented neutrophil extracellular traps formation promotes atherosclerosis development in socially defeated apoE(-/-) mice. *Biochem Biophys Res Commun* **500**, 490-496, doi:10.1016/j.bbrc.2018.04.115 (2018).
59 Mogayzel, P. J., Jr. *et al.* Cystic fibrosis pulmonary guidelines. Chronic medications for maintenance of lung health. *Am J Respir Crit Care Med* **187**, 680-689, doi:10.1164/rccm.201207-1160oe (2013).
60 Awasthi, D. *et al.* Oxidized LDL induced extracellular trap formation in human neutrophils via TLR-PKC-IRAK-MAPK and NADPH-oxidase activation. *Free Radic Biol Med* **93**, 190-203, doi:10.1016/j.freeradbiomed.2016.01.004 (2016).
61 Yipp, B. G. *et al.* Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med* **18**, 1386-1393, doi:10.1038/nm.2847 (2012).
62 Napirei, M., Wulf, S., Eulitz, D., Mannherz, H. G. & Kloeckl, T. Comparative characterization of rat deoxyribonuclease 1 (Dnase1) and murine deoxyribonuclease 1-like 3 (Dnase1l3). *Biochem J* **389**, 355-364, doi:10.1042/BJ20042124 (2005).
63 Jimenez-Alcazar, M. *et al.* Host DNases prevent vascular occlusion by neutrophil extracellular traps. *Science* **358**, 1202-1206, doi:10.1126/science.aam8897 (2017).
64 Peled, M. *et al.* A wild-type mouse-based model for the regression of inflammation in atherosclerosis. *PLoS One* **12**, e0173975, doi:10.1371/journal.pone.0173975 (2017).
65 Gray, R. D., McCullagh, B. N. & McCray, P. B. NETs and CF Lung Disease: Current Status and Future Prospects. *Antibiotics (Basel)* **4**, 62-75, doi:10.3390/antibiotics4010062 (2015).
66 Soehnlein, O., Ortega-Gomez, A., Doring, Y. & Weber, C. Neutrophil-macrophage interplay in atherosclerosis: protease-mediated cytokine processing versus NET release. *Thromb Haemost* **114**, 866-867, doi:10.1160/TH15-08-0623 (2015).

67 Kolaczkowska, E. *et al.* Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nat Commun* **6**, 6673, doi:10.1038/ncomms7673 (2015).
68 Hakkim, A. *et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* **107**, 9813-9818, doi:10.1073/pnas.0909927107 (2010).
69 Beiter, T. *et al.* Neutrophils release extracellular DNA traps in response to exercise. *J Appl Physiol (1985)* **117**, 325-333, doi:10.1152/japplphysiol.00173.2014 (2014).
70 Kumamoto, T. *et al.* in *European Heart Journal* Vol. 27 2081-2087 (2006).
71 Yasutomo, K. *et al.* Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet* **28**, 313-314, doi:10.1038/91070 (2001).
72 Bodano, A., Amarelo, J., Gonzalez, A., Gomez-Reino, J. J. & Conde, C. Novel DNASE I mutations related to systemic lupus erythematosus. *Arthritis Rheum* **50**, 4070-4071, doi:10.1002/art.20721 (2004).
73 Zhu, B. *et al.* Increased DNase I activity in diabetes might be associated with injury of pancreas. *Mol Cell Biochem* **393**, 23-32, doi:10.1007/s11010-014-2043-1 (2014).
74 Park, J. *et al.* Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci Transl Med* **8**, 361ra138, doi:10.1126/scitranslmed.aag1711 (2016).
75 Schonfelder, T., Jackel, S. & Wenzel, P. Mouse models of deep vein thrombosis. *Gefasschirurgie* **22**, 28-33, doi:10.1007/s00772-016-0227-6 (2017).
76 Chitrabamrung, S., Rubin, R. L. & Tan, E. M. Serum deoxyribonuclease I and clinical activity in systemic lupus erythematosus. *Rheumatol Int* **1**, 55-60, doi:10.1007/bf00541153 (1981).