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Diabetes-related factors and atherosclerosis regression

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Valorisation

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Type1 & Type2 Diabetes Mellitus (T1DM, T2DM) are common diseases with increasing prevalence worldwide.[1] Globally, 425 million people are diagnosed with diabetes with an estimated cost of ~$727 billion.[2] Global incidence for diabetes is projected to rise to $629 million by 2045. In the United States alone, with >30 million diagnosed patients, diabetes is the most expensive chronic disease, reaching an economic burden of $327 billion.[3] In the Netherlands, about 6.5% of the Dutch population has been diagnosed with diabetes[4] with projection to rise to 8% by 2025[5] and yearly costs of €6.8 billion[4]. These costs not only include direct costs of treating the disease, e.g. medication, but also costs of treating its complications, such as cardiovascular diseases (CVDs). Subjects suffering from diabetes have a significantly increased risk of atherosclerosis and associated CV events.[6] In the US, cost of treatment of CV-related in diabetics is estimated at $37.3 billion.[7] In the Netherlands, about 30% of the people with diabetes are suffering from CVD, and the treatment of CVD in those diabetes patients makes up ~ €1 billion of the total economic burden of diabetes. CVD is , after end-stage renal disease, the most costly complications of diabetics in the Netherlands.[4]

CVD is the deadliest disease worldwide. Despite a steady decline in death from CVDs due to advances in scientific discoveries, clinical cardiology, as well as public health, it is still responsible for 30% of deaths.[8] The global cost is estimated to be $863 billion and is predicted to increase to $1044 billion until 2030[9] due to a rising and aging population and the high incidence of secondary complications after survival of a primary CVD incident. The increased risk of patients with diabetes to suffer from CVD is due to the fact that metabolic risk factors for atherosclerotic CVD are commonly found in subjects with diabetes. Hyperglycemia is an independent risk factor for CVD, next to genetic predisposition, hypertension and diabetic dyslipidemia. The adjusted relative risk for T2DM compared to non-T2DM patients is 1.7 for cardiovascular death, 1.8 for myocardial infarction, and 1.5 for stroke.[10]

Strikingly, individuals suffering from diabetes die mostly from CVD complications rather than diabetes itself. In the US, a total of 277,000 individuals with diabetes died in 2017 of which for 111,000 CVD was the primary cause of death.[7] In the Netherlands, mortality rates of CVD have halved over the past two decades and with 26%, it holds

second place of the most non-communicable diseases, right after cancer (32%).[11,12] Interestingly, diabetes takes with 2% the 4th place, and although CVD mortality has declined, the increase in diabetes has reversed positive trends and represents a major challenge to further prevent CVD.

The underlying mechanisms of increased CVD risk in individuals with diabetes is still unclear and current treatment focuses on treating hyperglycemia in diabetic patients, followed by statins in case of elevated LDL cholesterol (LDL-C) levels. Nonetheless, even if both are treated, diabetes patients still have a ~2 to 4-fold increased CVD risk compared to non-diabetics. Other components that potentially lead to the increased risk, such as elevated triglycerides (TG) levels and low HDL cholesterol (HDL-C) levels, are not being treated due to lack of evidence. While experimental studies of raising HDL documented benefits[13,14], clinical trials raising HDL-C have mostly failed to show beneficial CVD outcome[15,16] and other HDL functions are currently explored. Experimental studies focusing on hypertriglyceridemia (HyperTG) and atherosclerosis are limited, and clinical trials of TG-lowering drugs and CVD risk are currently ongoing.

Our research has focused on the two comorbidities of diabetes, *i.e.* elevated plasma TG levels accompanied by low HDL-C and hyperglycemia-related neutrophilia/Neutrophil extracellular traps (NET) formation. The aim was to elucidate the importance of both in atherosclerosis regression to shed light into possible new treatment options and thereby lower the residual CVD risk in diabetics.

Valorisation of key findings of this thesis

The results from our pre-clinical models showed that elevated TG levels do not lead to impaired atherosclerosis regression after LDL-C lowering, despite significantly reduced HDL-C and HDL particles (HDL-Ps). This might be due maintained cholesterol efflux capacity (CEC) - the considered key-antiatherogenic HDL function.

Further, we found that clearance of NETs using DNase1 can overcome diabetes-impaired regression despite ongoing hyperglycemia, due to reduction in inflammation. This suggested that NETs contribute to the increased CVD risk in this population. Further, given that DNase1 (Pulmozyme®) is already clinically used for patients with cystic fibrosis[17], these results also suggest a potential treatment for NET reduction.

Potential of TG lowering drugs to reduce residual CVD risk of diabetics

Effective TG-lowering drugs are available and are used in patients with TG levels >500 mg/dl to reduce the risk of pancreatitis.[18-21] However, guidelines on using TG-lowering drugs to reduce CVD risk are not clearly defined due to lack of evidence. European Guidelines (ESC/EAS guidelines for the management of dyslipidemias[22] and European guidelines on CVD prevention in clinical practice[23]) recommend that individuals with TG levels >1.7 mmol/l (>150 mg/dl) should be considered at increased risk of CVD, but did not include risk estimation of TG or recommended target values. Earlier guidelines from the adult treatment panel are in line with the European guidelines[24], but more recent guidelines from the ACC/AHA on the assessment of CVD risk do not mention TGs at all.[25] There is evidence suggesting that reducing TG could be beneficial for CVD, especially in individuals with preexisting coronary heart disease and diabetes.[24,26-28] But one of the key problems is that clinical trials that appear to confirm CVD benefits of TG-lowering therapy rely on subgroup analyses, and trials designed to focus on subjects with hyperTG only were missing. Thus, these results are non-conclusive.

The REDUCE-IT trial was the first published intervention trial to determine the relationship between TG and atherosclerosis in a population with elevated plasma TG levels. Patients at high risk for CVD and TG levels ≥ 135 mg/dL and < 500 mg/dL were prescribed icosapent-ethyl (a highly purified eicosapentaenoic acid; EPA) over a median time period of 4.9 years and showed reduced CVD risk including first, subsequent, and total ischemic events.[29,30] However, it is still unclear if the reduction in ischemic events was due to lowering TG or due to a combination of effects involving plaque instability reduction and inflammation. Notably, results were consistent among diabetic patients. Another trial looking at the effect of icosapent ethyl on progression of coronary atherosclerosis in patients with elevated TG levels on Statin therapy – EVAPORATE – might help to explain the cardiovascular benefit noted in the REDUCE-IT trial, and the 9 months interim results show reduction in total plaque volume measured as coronary CTA (computed tomography angiogram), but no reduction in low attenuation plaque volume.[31,32] Further, intense research is being done on the mechanisms of action for omega-3 fatty acids (reviewed in [33]).

Less exciting was the STRENGTH Trial, which studied the effect of *Epanova* (omega-3 carboxylic acids) on CVD risk reduction in ~13.000 patients with mixed

dyslipidemia (increased TG, low HDL-C) and increased risk of CVD.[34] The Trial has been discontinued earlier this year (January 2020) due to the unlikelihood of demonstrating a benefit to patients.[35]

There are currently further clinical trials ongoing that are looking at the effect of TG-lowering therapy in high-risk CVD subjects, including the PROMINENT Trial (Pemafibrate to reduce CV outcomes by reducing TG in patients with diabetes), with results being expected over the next 1-2 years (reviewed in[36]).

Potential of targeting NETs

Two possible therapeutic targets to reduce NETs in atherosclerosis have been studied in mice – 1. PAD4 inhibition and 2. DNase1 treatment.[37] In our studies, we focused on DNase1, because it is clinically already used to treat cystic fibrosis. DNase1 (Pulmozyme®) has been approved by the FDA (Federal Drug Administration) >25 years ago, is on the market in >65 countries and did not lead to any signs of major toxicity or detrimental long-term effects. Excitingly, the positive effects of DNase1 in treating cystic fibrosis has been proposed to be partly due to NETs degradation.[38] Our, as well as other studies[39-41] showed that NETs degradation via additional intraperitoneal (i.p.) DNase1 administration is beneficial in the context of CVD in mice. In humans, however, i.p. administrations of DNase1 solely may compromise immunoprotective functions of NETs, e.g. antimicrobial activity. Cystic fibrosis patients take DNase1 via a nebulizer, but whether an effective dose of DNase1 would also reach atherosclerotic plaques, is questionable and rather unlikely. A better way to administrate DNase1 without compromising immunoprotective functions might be the use of DNase1-coated nanoparticles, which were shown to reduce size of lung metastases in mice via NETs degradation.[42] Nano-based delivery systems have been proven to be safer and more effective than delivering a drug solely, with targeted delivery to site of disease, increased efficacy per drug dose and avoidance of off-target effects[43]. Nanodrugs are clinically already used and are administered orally or intravenously[44] to e.g. treat cancer, and have been shown to be beneficial for CVD in preclinical models (reviewed in [45]). Various types of nanoparticles are available (e.g. type IV collagen, cyclodextrin-based polysaccharides, lipid nanoparticles) and the correct therapeutic strategy with DNase1 as therapeutic agent needs to be tested in atherosclerotic mouse models.

Despite the exciting potential of DNase1 treatment in (diabetic) atherosclerosis, NET research is still in its infancies and we are lacking enough evidence as well mechanistic details before therapeutically targeting NETs to lower CVD risk is clinically transferable.

Translational capacity of the animal models

To study the effect of elevated TG levels on atherosclerosis, we used an inducible lipoprotein lipase deficient mouse model (*iLpL*$^{-/-}$). Our mouse model had mild to moderate hyperTG with TG levels of ~500mg/dL, which is significantly lower than that of LpL deficient patients whose TG levels reach > 1000 mg/dL. While our mouse model also has TG levels >1000mg/dL when fed a high-fat diet, we chose to use a western diet resulting in mild-to-moderate hyperTG (~500mg/dL). These levels reflect LpL deficient patients on treatment and restricted diet as well as diabetics suffering from diabetic dyslipidemia. Further, subjects with genetic LpL deficiency are rare and not likely to impact genetic analysis of overall CVD risk associated with plasma TG levels. One limitation of our mouse model is the lack of lipolysis, which has been postulated together with hyperTG to be the cause for CVD risk by creating high concentrations of toxic lipids and remnant lipoproteins. To overcome this limitation, we have included mice with LpL expression in macrophages or endothelial cells. These studies led to the same results, showing that hyperTG does not impair atherosclerosis regression after LDL-C lowering.

Another limitation of our mouse model is the lack of cholesterol ester transfer protein (CETP) expression in mice. To humanize the lipoprotein metabolism in mice, we have introduced hCETP and again, confirmed that hyperTG + hCETP does not impair atherosclerosis regression after lipid lowering. Overall, we think our mouse model can be translated to the human condition, which we underline with the human data.

To study the effect of hyperglycemia-induced neutrophilia and NET formation, we used *Ldlr*$^{-/-}$ mice injected with streptazotocin (STZ). STZ is a glucosamine-nitrosourea compound (glucose analogue) that is preferentially toxic towards insulin-producing beta cells.[46] We used a low dose (0.05 mg/g BW) over five consecutive days leading to cytotoxic effects in the pancreas resulting in pancreatic islet inflammation, insulin deficiency and hypergylcemia.[46,47] Thereby, this method mimics human T1DM, while

the majority of individuals with diabetes are diagnosed with T2DM[48]. Further, compared to severe systemic inflammation in mice, human leukocytosis is almost always mild. Therefore, translation from the animal model to human situation has to be done with caution.

To further study the role of endonucleases in atherosclerotic plaques, we used different DNase1 knock-out (KO) mouse models - DNase1 and DNase1L3 single KO, and DNase1/1L3 double KO mice. DNase1 and DNase1L3 single KO mouse models are commonly used to study systemic lupus erythematosus (SLE). For *DNase1*$^{-/-}$ mice, it takes 9 months to develop the SLE phenotype (increase in circulating Anti-Nuclear Antibodies (ANA), as well as antibodies against double-stranded DNA, histones, and Smith proteins).[49] At 12 month of age, these mice develop severe nephritis and kidney damage. *DNase1L3*$^{-/-}$ can develop SLE as early as 5 weeks of age.[50] The double KO mice were created by our collaborators and not much is published about their phenotype. Therefore, *DNase1*$^{-/-}$ mice are suitable for non-SLE experiments shorter than 9 months, while results from *DNase1L3*$^{-/-}$ and the double KO mice might be confounded. In our performed 20 to 24 weeks long atherosclerotic study, we did not observe significant differences between our mouse models regarding NET formation, atherosclerosis progression and regression. Further, we did not detect any physical or behavioral abnormalities as well as no signs for major inflammation or tissue damage. Notably, we did not test for SLE markers.

Using administration of additional DNase1 results in beneficial effects on NET formation and atherosclerosis regression in especially diabetic mice and it is of interest to determine if the opposite effect can be observed using the various DNase KO mouse models. However, a study reported that double KO mice die within days after induction of severe neutrophilia.[51] Thus, using the double KO mice in a diabetic atherosclerosis study (shown to induce neutrophilia) might not be feasible. In conclusion, the translation of results from the various atherosclerotic DNase KO mouse models to humans is challenging.

Conclusion

Overall, our results show that treatment of hyperglycemia-associated inflammation seems to be superior over treating hyperTG, especially in individuals whose diabetes is not well controlled.

Although effective TG-reducing treatments are available, our results do not suggest benefits in CVD prevention. In agreement, the results of two recent interventional trials – REDUCE-IT & STRENGTH, studying the potential benefits of TG-lowering therapy in high CVD-risk patients, were rather disappointing. With research on the mechanisms behind EPA treatment as well as results from ongoing TG-lowering trials being on their way, we will hopefully get an answer to decide if lowering elevated TGs should be included in the CVDs treatment guidelines.

While relative lack of evidence and knowledge does not allow clinical translation of NET targeting therapeutics in CVD as of yet, rapidly evolving research holds promise for their clinical translation in the near future.

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