Familial atherosclerosis and neuroimmune guidance cues
From in vitro assessments to clinical events
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Summary and Future Perspectives
SUMMARY

The aim of the studies described in this thesis was twofold: 1) to substantiate the role of Neuroimmune Guidance Cues (NGCs) in human atherosclerotic disease 2) to further improve treatment strategies of families where premature atherosclerosis is prevalent.

Part I: The Role for Neuroimmune Guidance Cues in Atherosclerosis

In Part I we focused on the identification of a role for NGCs in human atherosclerosis. These studies were largely conducted in vitro. Chapter 2 is a literature overview summarizing the function of Netrins in endothelial cell biology. Netrins originally known for the correct patterning of the nervous system are also important for the development of other systems, including the vascular system. The function of mature and quiescent endothelial cells is of great importance and requires active regulation. Netrins serve as intercellular and extracellular cues for endothelial cells to maintain their quiescent state and related functions, such as vascular tone, barrier function, wall shear stress and survival. The described data were mostly from in vitro studies as in vivo data at time of publication was scarce. Within chapter 3 and 4 we expand the in vitro insight with data about the EPH receptor B2 (EPHB2) and PlexinA4 (PLXNA4). In chapter 3 we examine the function of EPHB2 in atherosclerosis. We observed that the expression of EPHB2 by monocytes and macrophages increases as the atherosclerotic plaque progresses. We therefore reduced the expression of EPHB2 on monocytes and analyzed the monocyte function within different atherosclerotic processes. We observed that monocytes lacking EPHB2 expression could adhere less to endothelial cells, and that their ability to migrate was impaired. When these monocytes were analyzed under the microscope we observed that they were smaller and more rounded compared to cells with normal EPHB2 expression. To explain the changes in the cytoskeleton of the monocytes with reduced EPHB2 expression we evaluated the phosphorylation of focal adhesion kinases (FAK) as they are important regulators in cell remodeling. We observed a significant decrease in phosphorylation of FAK in monocytes with a diminished EPHB2 expression. The observations in this chapter suggest that EPHB2 on monocytes increases FAK phosphorylation resulting in cytoskeletal rearrangement, forming monocytes to be more prone to adhere, spread and migrate through the endothelial cell layer. This leads to monocyte influx into the sub-endothelial layer which eventually leads to atherosclerosis. This suggests an athero-prone function for EPHB2. In chapter 4, we report the ongoing work on another NGC, PLXNA4, and its role in atherosclerosis. When endothelial cells were subjected to the pro-atherogenic stimuli IL1β and TNFa, the gene translation of PLXNA4 was significantly downregulated. This suggested a role for PLXNA4 as an early disease stage, athero-regulatory factor at the level of the endothelium. Upon a knockdown of PLXNA4 in endothelial cells we observed decelerated endothelial cell proliferation and a decreased endothelial monolayer barrier function. Both are processes that contribute to an atherosclerotic phenotype. Endothelial cells with a decreased expression of PLXNA4 are more elongated and have an increase of
we study families with premature atherosclerosis. In chapter 5 we have generated \textit{ex vivo} human data to confirm that Netrin-1 plays a role in the atherosclerosis initiation and progression in humans. As atherosclerosis is a multistep disease, we analyzed Netrin-1 plasma levels within patient groups in multiple developmental stages of atherosclerosis. First we compared Netrin-1 against arterial wall inflammation and found a strong inverse correlation between the extend of arterial wall inflammation and circulating Netrin-1 in plasma. When comparing individuals without atherosclerosis to individuals with atherosclerosis, we observed a significantly lower Netrin-1 concentration when atherosclerosis was present. Lower Netrin-1 plasma levels were also negatively correlated with plaque burden. From these results our hypothesis arose that if an atherosclerotic plaque is present, the endothelium excretes less Netrin-1. As described in chapter 7 of this thesis and also demonstrated \textit{in vitro} and \textit{in vivo} by others, Netrin-1 reduces leukocyte migration and recruitment into the atherosclerotic plaque by inhibiting adhesion of monocytes to the vessel wall. In line with this research we observed the same effect on monocyte adhesion when endothelial cells were stimulated with plasma of patients with either high or low Netrin-1 concentrations. Attachment of monocytes to the endothelium stimulated with plasma containing high Netrin-1 was prevented, but when endothelial cells were exposed to plasma with low concentrations of Netrin-1, this inhibitory effect could not be observed. These data lend support for the hypothesis that Netrin-1 plays a role in atherosclerosis initiation and progression in humans.

Part I finishes with chapter 6, giving an overview of the function and potential of Netrin-1 in coronary artery disease. In this chapter the available literature at that time is reviewed and stated together with an expert opinion on the field by the authors. It is explained that the function of Netrin-1 differs considerably depending on excretion in the plasma or excretion within the atherosclerotic plaque. Netrin-1 produced and excreted by endothelial cells exert an athero-protective function. When human aortic endothelial cells were subjected to TNF-α and recombinant Netrin-1, a potently suppressing effect was demonstrated of TNF-α-induced adhesion of monocytes to the endothelial cells. When Netrin-1 plasma levels were measured in atherosclerotic mice, they appeared lower compared to healthy mice. But, when the downregulation of Netrin-1 was counteracted with aspirin, this resulted in reduced monocyte infiltration within the plaque and decreased lesion size. As it seems that Netrin-1 within the plasma has an athero-protective function, it is very interesting that Netrin-1 produced by accumulated macrophages within the atherosclerotic plaque has been demonstrated to enhance macrophage retention and survival and therefore stimulates the atherosclerotic process. Multiple studies, both \textit{in vitro} as \textit{in vivo} mouse studies, confirm that Netrin-1 blocks macrophage egression from the plaque. As a high macrophage content is associated with a vulnerable plaque, more prone to rupture, this elucidates also an athero-prone function for Netrin-1.

**Part II: Family Based Approach of Premature Atherosclerosis.**

Within Part II we study families with premature atherosclerosis. In chapter 7 we describe a small family comprising of 2 generations of whom 3 suffered from premature atherosclerosis in absence of any classical risk factors. Using whole exome sequencing we could identify a genetic variation in the Netrin-1 gene. This variation was not known in any large databases containing individuals with cardiovascular disease, and the position of this variation was in a highly conserved region. This, among other properties of the variation, made it likely to be deleterious and therefore interesting to explore its function. We purified the mutated protein (\textit{mut}Netrin-1) and assessed multiple of its functions within the atherosclerotic process compared to the wild type protein (\textit{wt}Netrin-1). We observed that monocytes adhere better to endothelial cells when the mutated Netrin-1 was present. Besides, where \textit{wt}Netrin-1 had an anti-inflammatory effect on the endothelial cells by inhibiting the NF-κB activation and subsequent the expression of adhesion molecules, this effect was not observed when endothelial cells were stimulated with \textit{mut}Netrin-1. As plaque stability is, among other things, associated with the macrophage content and the migration of smooth muscle cells (SMCs) we analyzed the macrophages and SMC migration in the presence of \textit{mut}Netrin-1. We observed that the presence of \textit{mut}Netrin-1 diminished the migration of both cells, compared to the effect of \textit{wt}Netrin-1. Lastly, we evaluated more in depth why this mutated protein influenced the chemotaxis of all these cells and we observed that the binding to known receptors for Netrin-1 was altered. With this study we provided the evidence that this Netrin-1 variation shows a phenotype that leads to an increased vascular inflammation and reduces plaque stability, thereby confirming previous findings that Netrin-1 plays a role in the initiation and progression of atherosclerosis.

In chapter 8 we broaden our outlook of premature atherosclerosis and evaluate 440 families at the same time. We evaluate the prevalence of sudden cardiac death (SCD) in families where premature atherosclerosis is present. In families with atherosclerotic disease, it is assumed that SCD cases in these families is caused by cardiac ischemia. This assumption was undermined when a large prospective study revealed that after autopsy only 32% of all SCD cases were due to cardiac ischemia. This led to our study where we observed that a cardiac arrhythmia (Brugada syndrome) had a similar prevalence in families with SCD and premature atherosclerosis compared to families with SCD alone. Besides, the SCD in families with premature atherosclerosis occurred more often and at an older age, while SCN5A mutations (a common gene mutation in Brugada syndrome) in these families were rare. This suggested that the SCD in families with premature atherosclerosis could be related to an underlying genetic predisposition of arrhythmias, with a different genetic origin.
Chapter 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Identifying new non-LDL driven factors in atherosclerosis, from mice to humans.

Despite the availability of multiple potent therapeutic treatments to lower LDL-cholesterol, residual CVD risk remains high [1]. An explanation for this large residual risk could be associated with non-LDL related factors. Evidence supporting this theory is that several new CVD associated loci are highly expressed in endothelial cells [2]. Phenotype association studies also point to the role of endothelium in the development of atherosclerosis [3, 4]. It is therefore not surprising that van Gils et al in 2013 hypothesized that Neuroimmune Guidance Cues, who are expressed by the endothelium, play a role in early atherosclerosis [5]. By comparing atherosclerotic susceptible regions of a mouse aorta with atheroprotective regions, it was shown that Netrin-1, Semaphorin3A and EphrinB2 were athero-regulatory factors that operate at the level of the endothelium. Besides, they influence leukocyte chemotaxis from the vessel lumen towards the sub-endothelial space. This became the cornerstone of NGC research in atherosclerosis, and the same group could also report mechanisms of macrophage retention and survival by Netrin-1 [6, 7]. More research was done, but this exceeded rarely the mouse model [8-11]. Until now, the findings in this thesis emphasize a role for certain NGCs in human atherosclerosis (Figure 1).

With the understanding and knowledge of the actions of NGCs on both endothelium and monocytes in mice studies, we were able to assess the expression of NGCs in human cells in a so called 'expression project'. After subjecting human cells to pro-atherogenic stimuli, we observed a differential expression of multiple NGC ligands and receptors in endothelial cells and monocytes. These findings suggested a potential role for NGCs in human atherosclerosis and additional studies were performed to unravel the pathways involved. This led to two functional papers; for EPHB2 on monocytes, and PLXNA4 on endothelial cells. However, these were not the only two interesting NGCs emerging from the 'expression project'. Netrin-4 and Semaphorin3F were differentially expressed on endothelial cells. The functionality of these proteins were also investigated in our group and results of these studies are eagerly awaited. Also PlexinA1, -C1, Semaphorin6D, and -7A, (Figure 2) were significantly differentially expressed after pro-atherogenic stimuli. Hu and colleagues established already a significant role for Semaphorin7A in atherosclerosis by mediating endothelial dysfunction in a β1 integrin-dependent manner[12], but future studies should be conducted to delineate the pathway involved in PlexinA1, -C1 and Semaphorin6D and could pave the way for new therapeutic options targeting atherosclerosis.

![Figure 1: Overview of the roles of the Neuroimmune Guidance Cues Netrin-1, EPHB2 and PLXNA4 established in this thesis.](image)

![Figure 2: Freshly isolated monocytes and human primary endothelial cells were stimulated with IL1β (20ng/ml) for 5 or 24 hours. Changes in mRNA levels of PLXNA1 (A), PLXNC1 (B), SEMA6D (C) and SEMA7A (D) were determined with RT-PCR. Data is expressed in copies per GAPDH, and displayed as mean ± SEM.](image)
The question, if there is a future for EPHB2 and/or PLXNA4 as therapeutic targets for cardiovascular disease, can only be answered with large clinical trials. And those can only be done if specific targeting agents are available. To date no clinical studies have been conducted on the potential therapeutic options of EPHB2 or PLXNA4 in atherosclerosis. EPHB2 is expressed in a large number of cell types [13] and one could wonder if a general antibody for EPHB2 exceeds the presumed benefits over the potential side effects. Cell specific targeting of EPHB2 in inflamed regions reducing sub-endothelial monocyte accumulation would in theory be very promising. But until a technique like that is perfected, EPHB2 trials are awaited. In our in vitro studies we observed a downregulation of endothelial PLXNA4 after stimulation with IL1β and TNFα. Subsequently we showed that endothelial downregulation of PLXNA4 led to a pro-atherosclerotic phenotype. Overexpression of PLXNA4 however, did not result in a different endothelial function. Our results suggest that maintaining a certain expression of PLXNA4 is necessary to protect against atherosclerosis in an early phase. A therapeutic agent stimulating PLXNA4 should therefore be targeting early atherosclerosis, when only arterial wall inflammation is present and PLXNA4 is downregulated. Stimulation of PLXNA4 in that phase of atherosclerosis could maintain the endothelial barrier and thereby delay the atherosclerotic process as primary prevention.

Where for most research concerning a role for NGCs in human atherosclerosis is still in its infancy, this is not the case for Netrin-1. After the effect on chemotaxis and inflammation was established in 2013 [7], multiple papers were published about Netrin-1 in cardiovascular disease. Relations were established between serum levels Netrin-1 and coronary artery calcification [14] and aspirin was found to increase circulating Netrin-1 levels in humans [15]. Moreover, an anti-inflammatory effect of Netrin-1 on endothelial cells was established by inhibiting the NF-kB activation and subsequent the expression of adhesion molecules [16] and there is still an active field of research on Netrin-1 and CVD. With this thesis we can now add an in vitro study, were a dysfunctional Netrin-1 is associated with accelerated atherosclerosis within a family with premature atherosclerosis. We also provided ex vivo evidence that Netrin-1 plasma levels could be correlated with in vivo imaging of atherosclerosis in humans. As the inflammation-hypothesis in CVD was validated by Mendelian Randomization [17] and as well with a randomized controlled trial [18], it was especially interesting that we could significantly correlate circulating Netrin-1 with the extent of arterial wall inflammation. As the CANTOS trial [18] showed to reduce cardiovascular events, multiple trials are currently investigating various anti-inflammatory agents [19]. Netrin-1 could be an interesting candidate to add to this list. Netrin-1 has been proven to suppress the TNFα induced NF-kB pathway, resulting in a diminished expression of endothelial adhesion molecules. One could hypothesize that suppletion of Netrin-1 in plasma, or stimulating endothelial excretion of Netrin-1 could enhance the anti-inflammatory effect and therefore be a preventive target for cardiovascular disease. In contrast, Netrin-1 within the plaque, produced and excreted by macrophages, has an athero-prone effect by blocking macrophage efflux [7]. Therefore, in later stages of atherosclerosis a different approach is needed and Netrin-1 should be blocked locally. Hypothetically, a drug-eluting stent excreting a Netrin-1 antibody could be placed during percutaneous coronary intervention. A humanized monoclonal antibody directed against Netrin-1 (NP137) already exists and is currently tested for its anti-cancer effects in a phase 1 trial (NCT02977195). For CVD, trials with Netrin-1 are awaited, but preclinical research shows promise.

Learning from families with premature atherosclerosis

Large genetic association studies might provide new non-LDL driven factors for atherosclerosis. Unfortunately, to date, all variants identified with GWAS either were located in regions of genes already established to play a role in CVD, or did not reach the clinic (yet) [20]. Although the majority of the variants found with GWAS are new, evaluating functional consequences of these variants requires extensive additional studies. In this thesis we therefore used an alternative approach, we studied families with a familial form of atherosclerosis prevalent at an extreme premature age (<40yrs) in the absence of cardiovascular risk factors. By identifying a genetic variant in Netrin-1 we were able to demonstrate a direct causal relationship between the genetic variant and an altered function of the protein and possibly discovered a new non-lipid related treatment target for atherosclerosis.

But also when we expand our view from one family to 440 families we can gain insight on how to handle families with premature atherosclerosis. A family history of premature atherosclerosis is widely accepted as independent risk factor for cardiovascular events [21, 22]. Besides, the risk increases further when relatives are affected at a younger age [23]. Nevertheless, unaffected family members are not naturally referred for additional medical screening after a premature cardiovascular event occurs within the family. And, to be fair, we cannot blame the general practitioner for this inconsistency. General practitioners are referred to the traditional risk algorithms to calculate cardiovascular risk and unfortunately they poorly predict the risk for relatives of patients with premature atherosclerosis [24]. This is mainly due to the fact that age is the largest predictor in these algorithms, and an old age is precisely what individuals with premature atherosclerosis are missing. Besides, when sudden cardiac death (SCD) occurs in families with premature atherosclerosis, this is often assumed to be caused by an acute myocardial infarction. Therefore relatives of the SCD victim within a family with premature atherosclerosis are usually not referred for cardio-genetic investigation, which is important to prevent more SCD cases in the future. By evaluating the rate of sudden cardiac death (SCD) in families with premature atherosclerosis and comparing the prevalence and genetic predisposition within families where no atherosclerosis was present, we were able to undermine the myth that SCD in those families is mostly caused by cardiac ischemia.
arrhythmia, like Brugada Syndrome, has a similar prevalence in families with SCD and premature atherosclerosis compared to families with SCD alone. Though the phenotype is a little different with an occurrence of the SCD at older age, general practitioners should consider family members to be tested for an underlying arrhythmia even when the SCD occurs at older age and in a family with an incidence of premature atherosclerosis.

Conclusion
This thesis demonstrates Neuroimmune Guidance Cues to play a role in human atherosclerosis and Ntrin-1 seems to have the most potential to become a new therapeutic target within cardiovascular disease. In addition, it is important to identity unaffected relatives at risk within families suffering from premature atherosclerosis because they most likely benefit from additional testing of cardiovascular risk.

REFERENCES