The effects of triglycerides and fatty acids on T cells: role in atherosclerosis

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CHAPTER 7

Appendix

Summary

Nederlandse samenvatting

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Summary

Atherosclerosis is the primary contributor to cardiovascular disease (CVD), which remains the leading cause of death worldwide and is fundamentally driven by the interactions between lipids, the immune system, and the vascular wall. Atherosclerosis is characterized by the accumulation of fats in the blood vessel walls, which attracts immune cells that then trigger an inflammatory response. Despite the immune system's attempts to clear these fats, the inflammation persists leading to chronic inflammation. This ongoing inflammation leads to an increasing buildup of fats and immune cells, resulting in the formation of atherosclerotic plaques. When these plaques eventually rupture, it can have serious consequences such as heart attacks or strokes.

Until recently, this disease was mainly associated with cholesterol, a lipid, and macrophages, a type of immune cell that continuously takes up cholesterol in the vessel walls until a fatty atherosclerotic plaque is created. However, recent research has highlighted the importance of other factors, such as triglycerides and T cells, in the development of atherosclerosis. Excessive triglycerides in the bloodstream can cause and/or aggravate the disease when present in excess, particularly through triglyceride-rich lipoproteins. Additionally, T cells, a key component of the adaptive immune system, are also found in large numbers within atherosclerotic plaques. However, these cells and lipids are already present and interacting in the human body before they reach the site of the atherosclerotic plaque. Thus, the aim of this thesis is to better understand how triglycerides and fatty acids can influence T cells in the context of atherosclerosis.

In the human body, each cell and lipid plays an important role. In this intricate system, triglycerides are crucial building blocks that provide energy and structure to cells. Triglycerides are composed of three fatty acid molecules attached to a glycerol backbone. These lipids serve as an energy reservoir because they can be broken down by the cell, releasing the fatty components, which can then be used by cells to fuel various cellular processes. As such, fatty acids also play a crucial role in the human system. Fatty acids can be divided into saturated, monounsaturated, and polyunsaturated fatty acids. This classification is based on the number of double bonds in the chemical structure of the fatty acid: saturated fatty acids have no double bonds, monounsaturated fatty acids have one, and polyunsaturated fatty acids have two or more. Generally, saturated fatty acids are considered unhealthy, while unsaturated fatty acids are seen as healthy.

T cells are specialized immune cells that can identify and respond to their environment, including triglycerides and fatty acids. T cells in the circulation are generally in an inactive, or non-activated, state. In this state, T cells do not proliferate, do not produce cytokines, and exhibit low metabolic activity in the form of beta-oxidation. Only when stimulated by antigen-presenting cells, such as macrophages or dendritic cells, do they become activated and initiate immune responses. Once activated, T cells will proliferate, produce cytokines, and shift their metabolism to aerobic glycolysis, fatty acid biosynthesis, and cholesterol biosynthesis. However, whether the interaction with triglycerides and various fatty acids influences T cells, and whether this can lead to adverse reactions in high lipid environments, like the atherosclerotic plaque, remains unknown.

To investigate this question, this thesis employs various *in vitro*, -omics, and *in vivo* techniques. An *in vitro* experiment studies things outside of a living organism. As such, the term "in vitro" literally means "in glass", referring to experiments conducted in controlled environments such as test tubes or Petri dishes. An -omics experiment studies large amounts of biological data, such as all genes (transcriptomics), proteins (proteomics), or metabolites (metabolomics) in an organism, to provide a comprehensive overview of biological processes. An *in vivo* experiment is conducted within a living organism, such as an animal or a human. Instead of working with laboratory glassware, tests are performed directly in the body. This helps scientists understand how a treatment or substance behaves in a real biological environment. As a result, this thesis leads to a more comprehensive understanding of the impact that fatty acids and triglyceride levels have on T cell function.

First, we systematically mapped the known effects of fatty acids on T cell function and then compared these with the known effects of the same fatty acids on the development and progression of atherosclerosis. By dividing T cell function into four categories (metabolism, activation, proliferation and differentiation) we could map out the individual effects of 14 different fatty acids on T cell function. Interestingly, the way fatty acids were found to influence T cells aligned with the impact these fatty acids have been found to have on the development of atherosclerosis. However, it is important to note that these results were all found in activated T cells, and may not necessarily reflect the interactions and influences fatty acids are having on T cells in the circulation, prior to activation.

To investigate how fatty acids influence T cell function prior to activation we developed an *in vitro* model. Here, we focused on $CD4^+$ T cells, which are generally thought to play a pro-inflammatory role in CVD. These $CD4^+$ T cells were exposed to one of the most abundant fatty acids in the circulation, oleic acid, which has also been shown to be associated with an increased risk of CVD. Using our *in vitro* exposure model we examined the expression levels of thousands of genes simultaneously, via RNA sequencing. After exposing non-activated $CD4^+$ T cells to oleic acid, this transcriptomics approach revealed strong changes in T cell metabolism towards a pro-inflammatory state, as the cells increased the expression of genes related to fatty acid and cholesterol biosynthesis. This result was further validated by activating the $CD4^+$ T cells after oleic acid exposure and measuring the development of T cell subsets. The results showed that when T cells were pre-exposed to oleic acid, they were more likely to become IL-9^+ producing T cells after activation compared to T cells that were not pre-exposed. IL-9 is the hallmark cytokine of the highly pro-inflammatory T_H9 subset, which has been associated with atherosclerosis pathogenesis. Interestingly, this finding was inhibited when the cells were also exposed to statins, powerful cholesterol biosynthesis inhibitors, along with oleic acid. These findings suggest that fatty acids can influence atherosclerosis by affecting immune cells, potentially through cellular metabolism.

Expanding upon these findings, we next explored the role of other fatty acid types on $CD4^+$ T cell function. Specifically, we focused on eicosapentaenoic acid (EPA), a polyunsaturated omega-3

fatty acid. This fatty acid was used in the REDUCE-IT study, which showed that patients with hypertriglyceridemia who received EPA had reduced triglyceride levels, risk of cardiovascular events, and cardiovascular mortality compared to a placebo. Despite these results, it remains unclear how EPA provides its benefits, and there are only limited studies on these effects. We identified a strong anti-inflammatory transcriptomic profile in $CD4^+$ T cells exposed to EPA *in vitro*. We also used ATAC sequencing to identify where transcription factors, proteins that influence gene expression, bind to DNA, helping us understand why certain genes were up or down regulated in our transcriptomics results. This analysis showed that GATA3 and PU.1, which are important for the development of T_H2 and T_H9 cells, were reduced, while REV-ERB, which inhibits the development of T_H17 cells, was increased. T_H2, T_H9, and T_H17 are all subsets of $CD4^+$ T cells that have known pro-inflammatory effects in different diseases and as such, the lower expression of these subsets should be beneficial. We additionally tested palmitic acid, a saturated fatty acid, and oleic acid, a monounsaturated fatty acid, to provide a more comprehensive understanding of how fatty acid saturation might affect $CD4^+$ T cells. These results showed that the anti-inflammatory effects were unique to EPA-exposed cells, a finding that may contribute to the unexpectedly strong beneficial effects observed in studies like REDUCE-IT.

Lastly, we aimed to translate our *in vitro* findings to an *in vivo* model. Here, we leveraged a "natural experiment" design, comparing the $CD4^+$ and $CD8^+$ T cells of individuals with elevated triglyceride levels (2.7–8.5 mmol/L) due to primary, or genetic mutations to those without such mutations. This set up mimics a controlled experimental setup where the genetic variation acts as a natural intervention. We once again used RNA sequencing to determine global changes in gene expression in $CD4^+$ and $CD8^+$ T cells of individuals with and without elevated triglyceride levels. We identified a pro-inflammatory transcriptomic profile in T cells derived from individuals with elevated triglycerides. Specifically, we observed upregulation of the *IL6R* gene in $CD4^+$ T cells, a gene that encodes a cytokine causally linked to cardiovascular disease. Notably, patients with triglyceride levels between 2.7–8.5 mmol/L have an increased risk of cardiovascular disease. However, this risk diminishes when triglyceride levels exceed 10 mmol/L, as triglycerides are then primarily stored in particles called chylomicrons, which are too large to easily cross the vessel walls. Interestingly, the pro-inflammatory gene expression profile was not observed in T cells derived from patients with these extremely high triglyceride levels. Furthermore, transcriptomic differences were reversed in patients with low triglycerides (0.1–0.3 mmol/L), or hypotriglyceridemia, a condition suspected to have a protective effect against cardiovascular disease. Lastly, the pro-inflammatory gene expression profile was found in T cells from patients with secondary hypertriglyceridemia (between 2.7–8.5 mmol/L), caused by secondary factors such as diabetes, although to a lesser extent. This research suggests that elevated triglycerides may contribute to cardiovascular disease, potentially by promoting inflammation in T cells.

Collectively, this thesis departs from previous research by examining the complex interaction between fatty acids and T cells, particularly in their early, non-activated stages. The research suggests that $CD4^+$ T cells are strongly influenced by their environment and that cellular metabolism plays a crucial role in cell function. It appears that different fatty acids can have varying effects

on T cells, ranging from pro- to anti-inflammatory, and that measuring global gene expression differences can provide profound insights into the cell's state. Finally, it is suggested that targeting triglycerides and fatty acids may have beneficial effects on atherosclerosis by leveraging the anti-inflammatory properties of T cells. This work opens new avenues for further research and enhances our understanding of the intricate relationship between fatty acids and T cells, which effects on how we understand human health and disease.